DOCTORAL THESIS

2017

Doctoral Programme of Environmental Microbiology and Biotechnology

Evaluation of the OPU approach in hypersaline environments

Merit del Rocío Mora Ruiz

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Doctor by the University of the Balearic Islands

"Reserve your right to think, for even to think wrongly is better than not to think at all"

A la persona que llegó un día pidiendo una centrífuga y se llevó mi alma

y

a los tres pilares de mi vida, esos que hacen que la peor tormenta parezca una ligera briza

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List of publications included in this Doctoral Thesis

Information of the journals of the papers related with the thesis and along the Ph. D. period.

Number	Journal	Impact Factor (publish year)	Quartile (area)	SJR
1	*Syst. Appl. Microbiol*.	3.691	Q1 (Applied Microbiology and Biotechnology)	1.282
2	*FEMS Microbiology Ecology*	3.96	Q1 (Applied Microbiology and Biotechnology)	1.687
3	*In prep*	-	-	-
4	*In prep*	-	-	-
5	*Enviro Microbiol*	5.932	Q1 (Ecology, Evolution, Behaviour and Systematics)	3.002
6	*Frontiers in Microbiology*	4.165	Q1 (Inmmunology and Microbiology)	1.970
7	*Syst. Appl. Microbiol*	3.691	Q1 (Applied Microbiology and Biotechnology)	1.282
8	*Syst. Appl. Microbiol*	3.691	Q1 (Applied Microbiology and Biotechnology)	1.282
9	*In prep*	-	-	-
10	*Malar. J*	3.079	Q1 (Inmmunology and Microbiology)	2.025
11	*Enviro Microbiol*	5.932	Q1 (Ecology, Evolution, Behaviour and Systematics)	3.002

Articles related with this thesis

1) **Mora-Ruiz,** M.D.R., F. Font-Verdera, C. Díaz-Gil, M. Urdiain, G. Rodríguez-Valdecantos, B. González, A. Orfila and R. Rosselló-Móra. 2015. Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily *Salicornioideae* (*Amaranthaceae*). *Syst. Appl. Microbiol*. 38, 406–416.

2) **Mora-Ruiz**, M. D. R., F. Font-Verdera, A. Orfila, J. Rita, and R. RosselĺLo-Ḿora. 2016. Endophytic Microbial Diversity of the Halophyte *Arthrocnemum Macrostachyum* across Plant Compartments. *FEMS Microbiology Ecology* 92(9).

3) **Mora-Ruiz**, M..R., C. Alejandre-Colomo, T. Ledger, B. González, A. Orfila and R. Rosselló-Móra. Mesophilic endophytes associated to the euhalophyte *Arthrocnemum macrostachyum* and their potential plant growth promoting activity. *In prep.*

4) **Mora-Ruiz,** M.D.R., C. Alejandre-Colomo, F. Font-Verdera, M. Ferretjans, A. Orfila and R. Rosselló-Móra Halophilic endophytic archaea in the halophyte *Arthrocnemum macrostachyum*.. *In prep.*

5) **Mora-Ruiz**, M.D.R., A. Cifuentes, F. Font-Verdera, C. Pérez-Fernández, M. E. Farías, B. González, A. Orfila and R. Rosselló-Móra. Biogeographical patterns of bacterial and archaeal communities of distant hypersaline environments. Submitted to *Enviro Microbiol*.

6) Mirete, S, **Mora-Ruiz**, M, Lamprecht-Grandío, M, Figureras, R. Rosselló-Móra and J. González-Pastor, J. 2015. Salt Resistance Genes Revealed by Functional Metagenomics from Brines and Moderate-Salinity Rhizosphere within a Hypersaline Environment. *Frontiers in Microbiology* 6(October):1–16. **Contribution**: OPUs design and diversity analysis.

7) Cortés-Lara, S, Urdiain, M, **Mora-Ruiz**, M, Prieto, L, Rosselló-Móra, R. 2015. Prokaryotic microbiota in the digestive cavity of the jellyfish *Cotylorhiza tuberculata*. *Syst. Appl. Microbiol.* 38, 494–500. **Contribution:** helping in the OPUs desing, diversity and statistical analysis.

8) Vidal, R, Ginard, D, Khorrami, S, **Mora-Ruiz**, M., Munoz, R, Hermoso, M, Díaz, S, Cifuentes, A, Orfila, A, Rosselló-Móra, R. 2015. Crohn associated microbial communities associated to colonic mucosal biopsies in patients of the western Mediterranean. *Syst. Appl. Microbiol.* 38(6), 442–452. **Contribution:** sequencing trimming, OTU clustering.

9) **Mora-Ruiz,** M.D.R., C. Díaz-Gil, J. Fullana-Leal, A. Orfila and R. Rosselló-Móra. Comparison of OPU and OTU approaches using databases of saline and hypersaline environments. *In prep*.

Other articles produced during the Ph.D. time process

10) **Mora-Ruiz**, M. D.R., P. Penilla, J. Ordoñez, A. López, F.. Solís and A. Rodríguez. 2014. Socioeconomic factors, attitudes and practices in the prevention of malaria in the coastal plain of Chiapas, Mexico. *Malar. J.* 13:157.

11) Filker S., D. Forster, L. Weinisch, M.D.R. **Mora-Ruiz** M.D.R., B. González, M, Figureras, R. Rosselló-Móra and T. Stoeckl. 2017. Transition boundaries for protistan species turnover in hypersaline waters of different biogeographic regions. *Environ Microbiol*. Jun 2. doi: 10.1111/1462-2920.13805

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Abstract

Microorganisms comprise the majority of the richness in the planet and their current analyses have dramatically expanded our understanding of biodiversity. Particularly, microbiology of extreme environments such as acidic or hypersaline habitats has been a hot scientific topic of research since it resembles the hypothesized conditions of the origin of life. Hypersaline habitats are considered extreme environments due to their extreme conditions: high salinity, UV radiation and temperature. However, life finds its way and organisms from the three domains on life: *Bacteria, Archaea* and *Eukarya* are present in high numbers in these habitats. In this thesis, we analyze the microbial communities inhabiting different environments including brines and sediments of different salterns around the world as well as associated to plants and animals using the Operational Phylogenetic Unit (OPU) as biological entity. The OPU approach is the central core of this Thesis, and therefore we have used it as a base to elucidate biodiversity, connectivity between communities and the influence of the environmental parameters on the microbial assemblage.

Throughout this thesis we have used different techniques combining culture-depending (*e.g.* the tandem MALDI-TOF/MS – 16S rRNA gene sequencing) and culture-independent (454 amplicon pyrosequencing) methods. Furthermore, we used different statistical tools such as multivariate techniques and co-occurrence networks in to unravel the spatial and temporal variations among microbial communities.

Firstly we focused on the microbial communities associated with euhalophytes due to their capacity to concentrate salt in their internal tissues. Our results confirmed that most of the epiphytic and endophytic communities were putatively moderate halophiles and few mesophiles probably because the internal compartmentalization of the plant. We also evidenced a geographical distance effect on the microbial communities and furthermore the influence of the physicochemical parameters of the rhizospheric soils. Additionally, this Thesis includes the first report of endophytic *Archaea* by 16S rRNA amplicon sequencing (principally *Halocuccus, Halorubrum,* and *Haloquadratum*), presenting also microscopy evidences and cultures.

Our results showed a strong predominance of *Euryarchaeota, Proteobacteria, Bacteroidetes* and *Firmicutes* in the different hypersaline habitats analyzed. Nonetheless, variations on specific taxa, both in *Archaea* and *Bacteria* were detected at different spatial scales (*e.g.* or the spatial differentiation of communities in distant salterns). Additionally we found novel diversity in the analyzed environments. We also used OPUs to analyze the diversity in the gastric cavity of the jellyfish *Cotylorhiza tuberculata,* finding that major key organisms were related to the genera *Spiroplasma, Thalassospira, Tenacibaculum* and *Vibrio*. Some of

these OPUs could be potential pathogens and therefore the host may serve as dispersal mechanism.

Some biotechnological applications derived from this Thesis include the identification of certain strains with plant growth promoting activity on a plant model species, which could be used as biofertilizers. The databases obtained were used in a global analysis on the suitability of OPU over traditional OTU approach. The tools used in this work produced a wide landscape of microbial diversity from mostly, but not only, hypersaline environments. Finally, the results of this Thesis are a step forward in the understanding of the diversity and the biological patterns of microbial communities based on a more detailed phylogenetic approach.

Resumen

Los microorganismos procariotas conforman la mayor parte de la riqueza biológica del planeta y su actual análisis ha incrementado considerablemente el conocimiento sobre su diversidad. En particular, el estudio de la microbiota de ambientes extremos, como pueden ser los hábitats ácidos o los hipersalinos son temas científicos prioritarios al ser utilizados como análogos de la vida primigenia. Los hábitats hipersalinos son considerados como ambientes extremos debido a su condiciones de elevada salinidad, radiación UV y temperatura. A pesar de estas condiciones extremas, en estos ambientes se puede encontrar vida en forma de organismos de los tres dominios de la vida: *Bacteria*s, *Archaea* y *Eukarya*, y que se pueden encontrar en alta abundancia en estos hábitats. En la presente Tesis se analizan las comunidades microbianas que habitan en diferentes ambientes incluyendo salmueras y sedimentos de salinas de diferentes partes del mundo así como comunidades microbianas asociadas a plantas y animales usando una aproximación basada en Unidades Filogenéticas Operacionales (OPUs, del inglés Operational Phylogenetic Units) como unidad biológica. El uso de OPUs es el eje central de esta Tesis que se ha aplicado para elucidar diversidad, conectividad entre comunidades, así como la influencia de parámetros ambientales sobre las comunidades microbianas.

A lo largo de esta Tesis se han usado diferentes técnicas combinando métodos de cultivo-dependientes (*e.g.* MALDI-TOF/MS) e independientes (pirosecuenciación por 454). Además nuestra aproximación no estuvo circunscrita a la descripción de la biodiversidad, sino que también hemos utilizado el uso de métodos estadísticos potentes tales como análisis multivariantes y otros como redes de co-ocurrencia para elucidar variaciones espaciales entre comunidades microbianas.

En primera instancia, nos centramos en las comunidades microbianas asociadas a euhalófitas. Debido a la capacidad de estas plantas para concentrar sal en sus tejidos internos, se hipotetiza la posibilidad de encontrar microorganismos halófilos asociados con este micro-ambiente hipersalino. Los resultados confirman que la mayor parte de la comunidad tanto epífita como endófita está dominada por halófilos moderados y que una menor proporción es mesófila, probablemente debido a la compartimentalización interna de la planta. También hemos evidenciado el efecto de la distancia geográfica sobre las comunidades microbianas y la influencia de los parámetros fisicoquímicos del suelo rizosférico. Adicionalmente, esta tesis incluye la primera evidencia de la existencia de arqueas endófitas (principalmente *Halococcus, Halorubrum* y *Haloquadratum*) por secuenciación, presentando evidencias de microscopía y cultivos.

Los resultados en salinas mostraron una fuerte dominancia de *Euryarchaeota, Proteobacteria, Bacteroidetes* y *Firmicutes* en los diferentes hábitats estudiados. Sin embargo,

hemos detectado variaciones en las abundancias de taxones específicos tanto en *Archaea* como en *Bacteria* a diferentes escalas espaciales (*e.g.* la diferenciación espacial de las comunidades en salinas distantes). Además hemos encontrado grupos no descritos previamente en los ambientes analizados. Asimismo, mediante OPUs se ha estudiado la microbiota que habita otro ambientes como la de la cavidad gástrica de la medusa *Cothylorhiza tuberculata*- Con la aproximación implementada, detectamos que los organismos clave estaban afiliados a los géneros *Spiroplasma, Thalassospira, Tenacibaculum y Vibrio.* Algunos de estos OPUs son considerados potencialmente patógenos y sus huéspedes (medusas) pueden ser un mecanismo de dispersión.

Algunas potenciales aplicaciones biotecnológicas generadas a partir de este trabajo incluyen la identificación de cepas con capacidad promotora de crecimiento vegetal y que pueden ser empleados como biofertilizantes. Las bases de datos generadas a lo largo de la Thesis, fueron usadas para realizar un análisis sobre la idoneidad del uso de OPUs frente el uso tradicional de OTUs. Las herramientas empleadas en este trabajo han generado una visión más precisa de la diversidad microbiana en ambientes salinos e hipersalinos. Finalmente, los resultados de esta tesis permiten ampliar nuestro conocimiento sobre la diversidad y patrones biológicos de las comunidades microbianas usando un método filogenético más detallado que el uso de OTUs.

Resum

Els microorganismes comprenen la majoria de la riquesa biològica del planeta i el seu anàlisi ha expandit considerablement el nostre coneixement sobre la biodiversitat. Particularment, la microbiologia enfocada a ambients extrems tals com hàbitats àcids o hipersalins han estat un tema d'interès actual en la ciència degut a la seva semblança a les possibles condicions de l'origen de la vida. Els hàbitats hipersalins es consideren ambients extrems degut a les seves condicions extremes: alta salinitat, radiació UV i temperatura. No obstant això, la vida és capaç de prosperar en aquests ambients i organismes dels tres dominis de la vida: *Bacteria*, *Archaea* i *Eurkarya* es poden trobar en abundància en aquests hàbitats. En la present tesi, s'analitzen les comunitats microbianes que habiten diferents parts del món, així com les associades a plantes i animals, usant la Unitat Filogenètica Operacional (OPU) com unitat biològica. L'aproximació per OPU és el nucli d'aquest treball i l'hem usat per elucidar biodiversitat, connectivitat entre comunitats i la influència de paràmetres ambientals sobre les comunitats microbianes

A través d'aquesta tesi hem fet servir diferents tècniques combinant mètodes cultiu-dependents (p.e. MALDI-TOF/MS) i cultiu-independents (454 pyrosequencing). A més a més, no només descrivim la diversitat dels esmentats entorns, sinó que hem recorregut a l'ús de mètodes estadístics com tècniques multivariants o xarxes de co-ocurrència per aclarir les variacions espacials entre comunitats microbianes.

En primera instància, ens enfoquem en les comunitats microbianes associades a euhalòfites. Degut a la capacitat d'aquestes plantes per concentrar sal en els seus teixits interns, inicialment vàrem hipotetitzar la possibilitat de trobar microorganismes halòfils associats amb aquest micro-ambient hipersalí. Els nostres resultats confirmen que la major part de la comunitat tant epífita com endòfita està dominada per halòfils moderats i que una menor proporció era non- halòfils. També hem evidenciat l'efecte de la distància geogràfica sobre les comunitats microbianes i la influència dels paràmetres fisicoquímics del sòl rizosfèric. Addicionalment, aquesta tesi inclou la primera mostra d'arqueus endòfits per seqüenciació (principalment *Halococcus, Halorubrum* i *Haloquadratium*), però també presentant evidència microscòpica i cultius.

De manera global els nostres resultats relacionats amb salines van exhibir una forta dominància *d'Euryarchaeota, Proteobacteria, Bacteroidetes* i *Firmicutes* en els diferents hàbitats analitzats. Malgrat això, hem detectat variacions en les abundàncies sobre taxes específiques a diferents escales espacials tant en *Archaea* com en *Bacteria* (*e.g.*. la diferenciació espacial de les comunitats en salines distants). A més a més, hem trobat nous grups no descrits prèviament en els ambients analitzats. Descriure la diversitat microbiana és una cerca incessant, per la qual, vàrem utilitzar OPUs per analitzar la diversitat associada a la cavitat gàstrica de la

medusa *Cothylorhiza tuberculata*, trobant com a resultats que els organismes clau estaven afiliats als generes *Spiroplasma*, *Thalassospira*, *Tenacibaculum* i *Vibrio*. Alguns d'aquests OPUs són considerats potencialment patògens i els seus hostes poden estar actuant com a mecanismes de dispersió.

A més a més, algunes de les potencials aplicacions biotecnològiques generades a partir d'aquesta tesi inclouen la identificació de soques amb activitat promotora del creixement d'un model d'espècie de planta. Les bases de dades obtingudes en aquest treball s'ha usat per a realitzar un anàlisi global de la idoneïtat del ús d'OPUs sobre l'ús tradicional d' OTUs. Les eines emprades en aquest treball produeixen una sinèrgia complementària obtenint una visió més àmplia de la diversitat microbiana en ambients salins i hipersalins. Els resultats d'aquesta tesi són un pas més enllà per a la nostra comprensió de la diversitat i patrons biològics de les comunitats microbianes mitjançant l'ús de OPUs.

Section I. General introduction

Thus we hope to find an un-ambiguous "beginning of life" or "definition of death," although nature often comes to us as irreducible continua

Stephen Jay Gould (1941-2002)

Microbiology in hypersaline environments: biodiversity and driving factors

1.1 Definition and classification of hypersaline environments

In microbiology, the "extreme" environments and their different forms of life have been considered as one of the most intriguing topics. The fascinating exploration of these environments hostile or even lethal for living beings such as plants or most animals led to consider them as models of hypothetical life outside the Earth (Rampelotto 2013). Extreme environments can be divided in different categories depending on the environmental factors, as extreme pressure (high pressure as *e.g.* abyssal zones in the sea), temperature (thermophiles as *e.g.* hot springs), pH (either acidophiles as *e.g.* Río Tinto, Spain as an acidophilic environment or alkalophiles as *e.g.* soda lakes with highly alkaline conditions), salt (halophiles as *e.g.* the ones studied in this thesis), among others (Svetlichny *et al.* 1991; Meintanis *et al.* 2006; Ventosa and Arahal 2009; Rampelotto 2013).

Hypersaline environments are those exceeding 35 psu salt concentration (~ the concentration of earth's oceans; (Grant, W.D., Gemmel, R.T., McGenity 1998). In general, hypersaline environments can be divided into thalassohaline and athalassohaline. Thalassohaline systems are those having a similar ionic composition as oceans and seas. They originated from the evaporation of seawater or by dissolution of evaporites (Ventosa 2006; Ventosa, Oren and Ma 2011). Typical examples of thalassohaline systems are solar salterns, which are located along tropical and subtropical coasts (*e.g.* Exportadora de Sal, México; The Petchaburi salterns, Thailand; Salinas de Levante and Santa Pola salterns, Spain). During evaporation there is a serial precipitation of salts due to the different coefficient of saturation (Ω) of each salt. Firstly, calcium carbonate precipitates at about 100 psu. Secondly, at 220 psu gypsum precipitates and later the precipitation of NaCl (halite), which takes place near to 340 psu. Finally, the remaining salts, that are usually hygroscopic salts of Mg^{2+} and K^{+} (Ventosa and Arahal 2009)

By contrast, athalassohaline systems have different ionic composition than seawater, and they are conditioned by the geochemical properties of rocks and substrates of their specific location which, through dissolution, tend to concentrate in endorheic lagoons (Rodríguez-Valera, Acinas and Antón 1998). Common examples of athalassohaline waters are the Dead Sea in Jordan, Israel and Palestine; Great Salt Lake in USA; cold hypersaline lakes in Antarctica, Tirez lagoon in Spain, Atacama´s lakes in South America; Lake Magadi or the lakes of Wadi Natrun (Rodríguez- Valera 1988; Javor 1989; Grant, W.D., Gemmel, R.T., McGenity 1998; Demergasso *et al.* 2004; Moreira, Rodríguez-Valera and López-García 2006; Ventosa 2006; Montoya *et al.* 2013).

Additionally, solar salterns and natural salt lakes have attracted the attention of ecologists because they are considered to be relatively simple ecosystems with apparent low richness and high abundance of microorganisms (Ventosa, Oren and Ma 2011). Therefore, these environments have been used for the study of organisms living in these extreme environments since the early twentieth century (Hof 1935) which, with a few exceptions, the microorganisms inhabiting these environments are called halophiles (Rodríguez- Valera 1988; Grant, W.D., Gemmel, R.T., McGenity 1998). These halophiles have a consistent ability to colonize natural hypersaline environments (Ventosa, Oren and Ma 2011) and therefore, saline lakes or solar salterns are not the only environments where these microorganisms can be found. There are also halophiles in other unexpected locations such as salt deposits (mines; Carpa *et al.* 2014), the interior of plants growing in saline soils (see Chapter 1, 2 and 3), seabirds nostrils (Brito-Echeverría *et al.* 2009; See Annex), and even a broad variety of salt-preserved food products such as fish, meats or vegetables (Henriet *et al.* 2014).

1.2. Halophiles the "salt-loving" microorganisms: diversity and physiological mechanisms of survival.

Hypersaline environments are considered hostile due to their extreme conditions such as high salinity, temperature and UV radiation. However, unicellular (and some pluricellular) forms from the three domains of life (*Bacteria*, *Archaea* and *Eukarya*; Figure I.1) can be found at high concentrations in these habitats (Oren 2016).

Since the Helge Larsen's diverse studies of the life in hypersaline habitats (considered the first comprehensive review written on halophilic microorganisms), the knowledge related with diversity in hypersaline environments has evolved in leaps and bounds (Ventosa, Oren and Ma 2011). Currently most of the microbiologists in this area distinguish different groups of halophiles based on the salt concentration at which they attain their optimal growth. Kushner & Kamekura (1988) defined several categories of microorganisms: *non-halophiles* are those that grow optimally in media containing less than 0.2 M NaCl (however if they can tolerate higher concentrations of salt they are called *halotolerants*); *slight-halophiles* (marine bacteria) that grow best in media from 0.2 to 0.5 M NaCl; *moderate-halophiles* that grow best in media between 0.5 to 2.5 M NaCl; and finally, *extreme-halophiles* that present their optimal growth in media containing > 2.5 M NaCl.

Briefly, the halophiles in the archaeal domain are principally represented by the known as haloarchaea, which constitute a large group of extremely halophilic mostly aerobic organisms that classified within a single family (*Halobacteriaceae*) within the phylum *Euryarchaeaota* (Grant, W.D., Gemmel, R.T., McGenity 1998; Ventosa 2006). *Halobacterium*, *Haloarcula* and *Haloquadratum* are the most representative genera. The bacterial domain is represented by a

higher diversity of lineages, in where the most common halophyles are affiliated to the phyla *Rhodothermaeota* (Munoz, Rosselló-Móra and Amann 2016), *Bacteroidetes, Proteobacteria, Firmicutes,* and *Actinobacteria*. These lineages include relevant genera such as *Alkalilimnicola, Owenweeksia, Desulfuromonas, Halovibrio* and *Salinibacter,* the latter considered as one of the most relevant members of the known halophilic microbial communities (Antón *et al.* 2002; Benlloch *et al.* 2002; Oren 2008; Rosselló-Mora *et al.* 2008a; Peña *et al.* 2010; Gomariz *et al.* 2014)

The presence of eukaryotes is minor in abundances in comparison with the other two domains and usually is represented by algae such as *Dunaliella salina* (Ventosa and Arahal 2009) and *Chlamydomonas* spp.; flagellates such as *Pleurostomum flabellatum* (De Jonckheere *et al.* 2009); fungi as *Alternaria* spp. or *Trimmatostroma salinu* (Butinar *et al.* 2005; Liu *et al.* 2014b); and the brine shrimp *Artemia salina.* It has been suggested that microbial eukaryotes have greater difficulties reproducing due the selective effect of high salinity, resulting in large decrease in the number of species as salinity increases (Benlloch *et al.* 2002). But, as most information about microbial eukaryote diversity in such environments derives from microscopy and fingerprinting approaches, the true extent of their diversity in these extreme habitats is still unknown. Recent studies evidenced that diversity of microbial eukaryotes in such environments is much higher than previously described , and also that different salt regimes harbor distinct ecosystems (Casamayor, Triadó-Margarit and Castañeda 2013; Stoeck *et al.* 2014; Filker *et al.* 2015; Filker *et al.* 2017).

Apart from the three domains of live previously described, halophilic biodiversity also embraces viruses. It was not until the 1970s that viruses in halophilic environments were first described. More recently their participation in biogeochemical cycles as well as the genetic plasticity of their hosts has been investigated (Pina *et al.* 2011). Haloviruses (viruses that infect halophiles) are also an important component in the microbial community with abundances up to 10^9 virus-like particles per mililiter (VLPs)/mL (Santos *et al.* 2012). Evidence of infection in some archaeal groups as *Haloquadratum, Halorubrum,* and *Halobacterium* by haloviruses has been shown in previous studies (Santos *et al.* 2012; Atanasova *et al.* 2015). However, new characterization of haloviruses showed that hypersaline environments contain a higher richness than previously assumed (Ventosa *et al.* 2014).

Regarding the strategies of halophiles to live in these adverse habitats, including their physiological responses to the high extracellular concentration of salt and the intense UV radiation, halophiles have found ways to thrive in these extreme environments. A few groups (*e.g.* taxa from families *Halobacteriaceae* and *Salinibacteraceae*) accumulate intracellularly salts (K^+, Cl^-) reaching molar concentrations equivalent to the environment in order to maintain the osmotic pressures. Their proteins are adapted to the ionic high concentrations to be functional (Oren 2016). Those organisms use the Na^+ pump, pushing Na^+ ions out of the cell,

while concentrating K^+ ions within the cell in order to balance osmotic pressure. This balance consists of an internal concentration of K^+ at around 5 M and an outside concentration of Na^+ at around 4 M (Grant, W.D., Gemmel, R.T., McGenity 1998; Roberts 2005). Other groups (most salt-adapted members of the *Bacteria*, halophilic algae, and fungi) accumulate organic molecules (such as glycerol, betaine, ectoine and hydroxyectoine) as compatible solutes to prevent the loss of cellular water to the environment, maintaining the osmotic balance (Roberts 2005; Oren 2016). Another survival strategy is the use of pigments for photoprotection against the intense UV radiation in such enviroments: halophilic microorganisms are usually colored with C50 carotenoid compounds in their cell membrane that are partially responsible for the typical pink-orange overall coloration in brines (Grant, W.D., Gemmel, R.T., McGenity 1998; Demergasso *et al.* 2004). However, there are exceptions of non pigmented taxa as members of the genus *Natrialba* (Ventosa and Arahal 2009). Some other species encode for different rhodopsin chromo-proteins that are light-activated pumps (Dassarma and Dassarma 2006; Ventosa 2006).

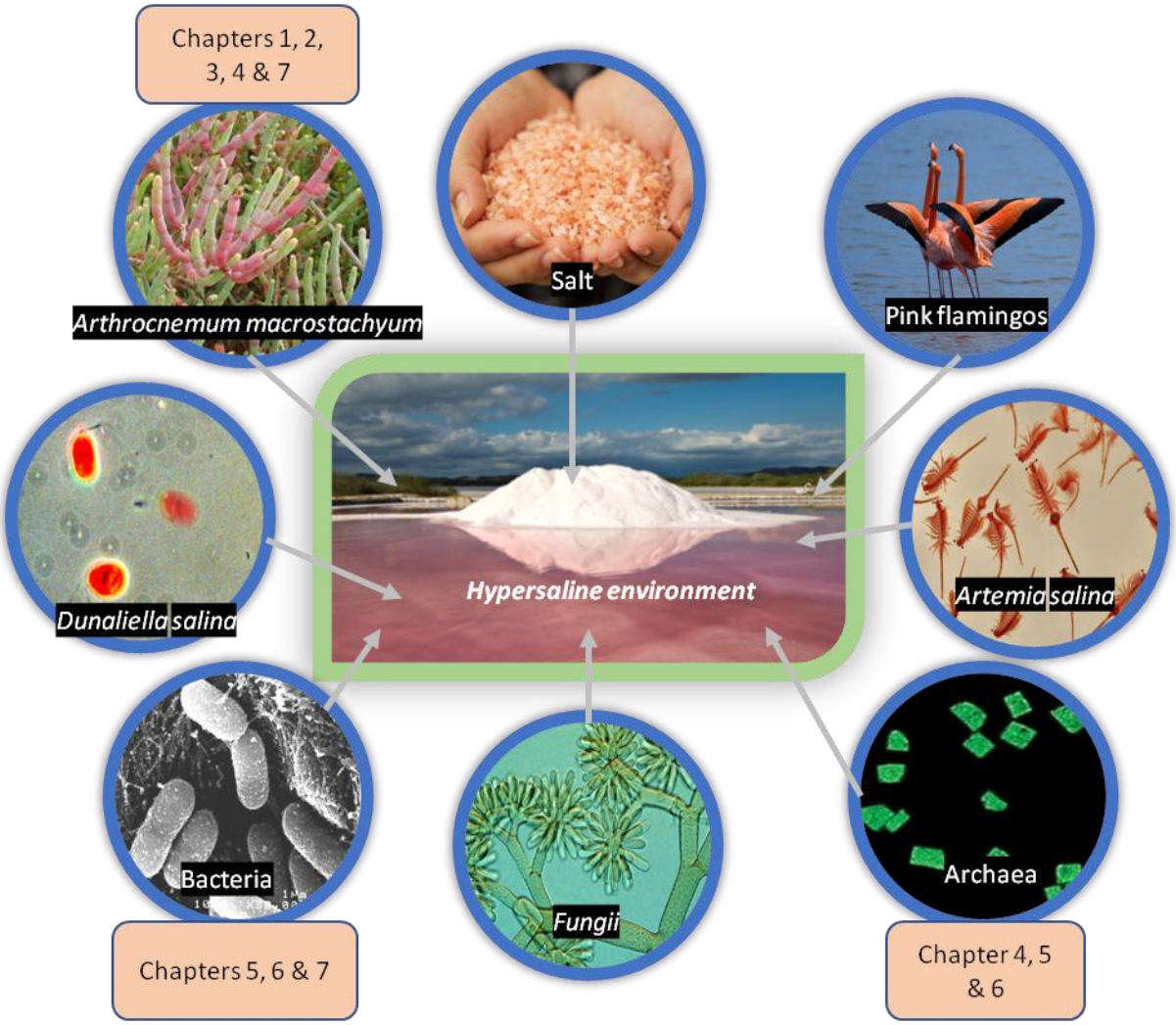

Figure I.1 Biodiversity associated to solar salterns. As seen, organisms from the three domains of life can be present. Along the different chapters of this thesis the description of some of these organisms is developed.

1.3 Driving factor of halophilic communities.

Although the study of particular halophilic taxa is of great interest for the scientific community, as for example the study of the halophilic bacteria *Salinibacter ruber* (Antón *et al.* 2002, 2013a; Peña *et al.* 2010), the knowledge on the structure of the communities, population dynamics, successions as well as interspecific interactions generates other level of awareness (Fargione, Brown and Tilman 2003; Kraft and Ackerly 2014; Kraft *et al.* 2015). For example, the variation of the microbial communities inhabiting different structures of plants (Chapter 2). Among the environmental variables acting as driving factors for microbial communities, salinity has been considered as the most important selection force (Lozupone and Knight 2007). The relevance of salinity is reflected in the energetic costs associated with osmoregulation and the requirement of some adaptive mechanisms to cope with high salt concentrations (Gunde-Cimerman, Oren and Plemenitaš 2005; Oren 2016). Nonetheless, within this thesis additional relevant variables (see

Chapter 2 and 5) as pH and even geographical distance seemed to strongly influence the structure of the communities. Other factors that may influence their biodiversity are air pressure, low nutrient availability, solar radiation or the presence of heavy metals and other toxic compounds, or even predation (Rodríguez- Valera 1988; Ventosa 2006).

Methods for analysing halophilic microbial communities: from laboratory techniques to statistical tools

Environmental microbiology is one of the branches of microbiology that is responsible for the exploration of the microbial biodiversity of different environments using a variety of laboratory techniques and statistical tools. Within this thesis, we have used a laboratory multiapproach to tackle the analysis of halophilic communities. In some chapters, we have used "classic" culture techniques (Chapters 3), molecular techniques in others (Chapters 2, 5 and 6) or a combination of both (Chapters 1, 4 and 7). Regarding to the statistical approach we have used diverse techniques from univariate (Chapter 3 and 8), to multivariate (Chapters 1, 2, 4, and 5) and even other exploration tools such as co-occurence networks (Anex; see Figure I.2 for the schema of standard microbiology workflow)

2.1 Laboratory techniques

The exploration in microbial ecology has been supported by the use of different laboratory techniques. Most of the studies have been developed under controlled laboratory conditions being cultures one of the most important approaches. Classical culture-dependent approaches include all techniques focused in recovering and analysing pure cultures from the environment. This approach has been partly relegated since the molecular studies have showed an underestimation of richness. However, recently culture-dependent techniques have been revisited (Viver *et al.* 2015; Diop *et al.* 2016) with the so called "*Culturomics"* (Lagier *et al.* 2012) and currently conform an important tool in the study of halophilic microorganisms (as further explored in Chapters 1, 3, and 7) and other areas (Greub 2012; Tandina *et al.* 2016). *Culturomics* is based on the extensive culture usually accompanied by the identification of the microorganisms using Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) mass spectrometry. Additionally, cultures are still necessary for a proper characterization of diversity, and yet for the description of a new taxa (as the deposit in biological resource centres is a requirement).

During the last century, molecular techniques have been developed, most of them with the objective of identifying the members of the communities as well as for quantifying their abundances (Figure I.2). While techniques such as Single Stranded Conformational

Polymorphism (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE) and, Restriction Fragment Length Polymorphism (RFLP) were largely used during past year to describe the microbial communities in hypersaline environments (Benlloch *et al.* 2002; Casamayor *et al.* 2002; Pedrós-Alió 2005), they are currently almost obsolete. Other techniques as Random Amplification of Polymorphic DNA (RAPD) have been used for a long time and they are still useful at the present to evaluate intraspecific clonality (Peña *et al.* 2005; Munoz *et al.* 2011; see also Chapter 1 and 3). Finally, additional laboratory techniques such as flow cytometry, pigment composition through High-Performance Liquid Chromatography (HPLC) and other molecular techniques have allowed a deeper analysis of the microbial communities (Pedrós-Alió 2005).

2.2 Next Generation Sequencing methods

During the last decades, the knowledge of microbial communities has advanced enormously thanks in part to the use of the high-throughput DNA sequencing technologies that made possible a deeper exploration of microbial communities (Barberán *et al.* 2011). Different platforms are currently available with own advantages and disadvantages. For instance, one of the most used is Illumina MiSeq, which produces greater depth and breadth of coverage (ditto for Illumina HiSeq 2000 and Illumina GAIIx), but the run time is higher than other platforms (from 27 h to 11 days) as Ion Torrent, which is unequalled for speed of sequencing, but with lower accuracy. PacBio is a more recent sequencing platform which produces long reads (average 1,500 pb), but with considerable higher error rates (12.86%). Within the highly competitive massive sequencing, new products rapidly surpass adversaries, as happened with the platform used in this thesis (Roche 454-pyrosequencing Titanium Plus), which is currently unavailable. The decision in the use of Roche 454-pyrosequencing in this work was based on the consistent longer reads (up to 900 pb) in comparison with Illumina MiSeq (2 x 250 pb). The length of the reads is very relevant in the affiliation process because a longer read implies a higher accurate richness estimations and accurate classifications (Yarza *et al.* 2014). Although the benefits of 454-pyrosequencing related with the read length, the high costs presented a considerable disadvantage which ultimately led to its removal from the market (Quail *et al.* 2012; Sharon *et al.* 2013; Frey *et al.* 2014; Yarza *et al.* 2014).

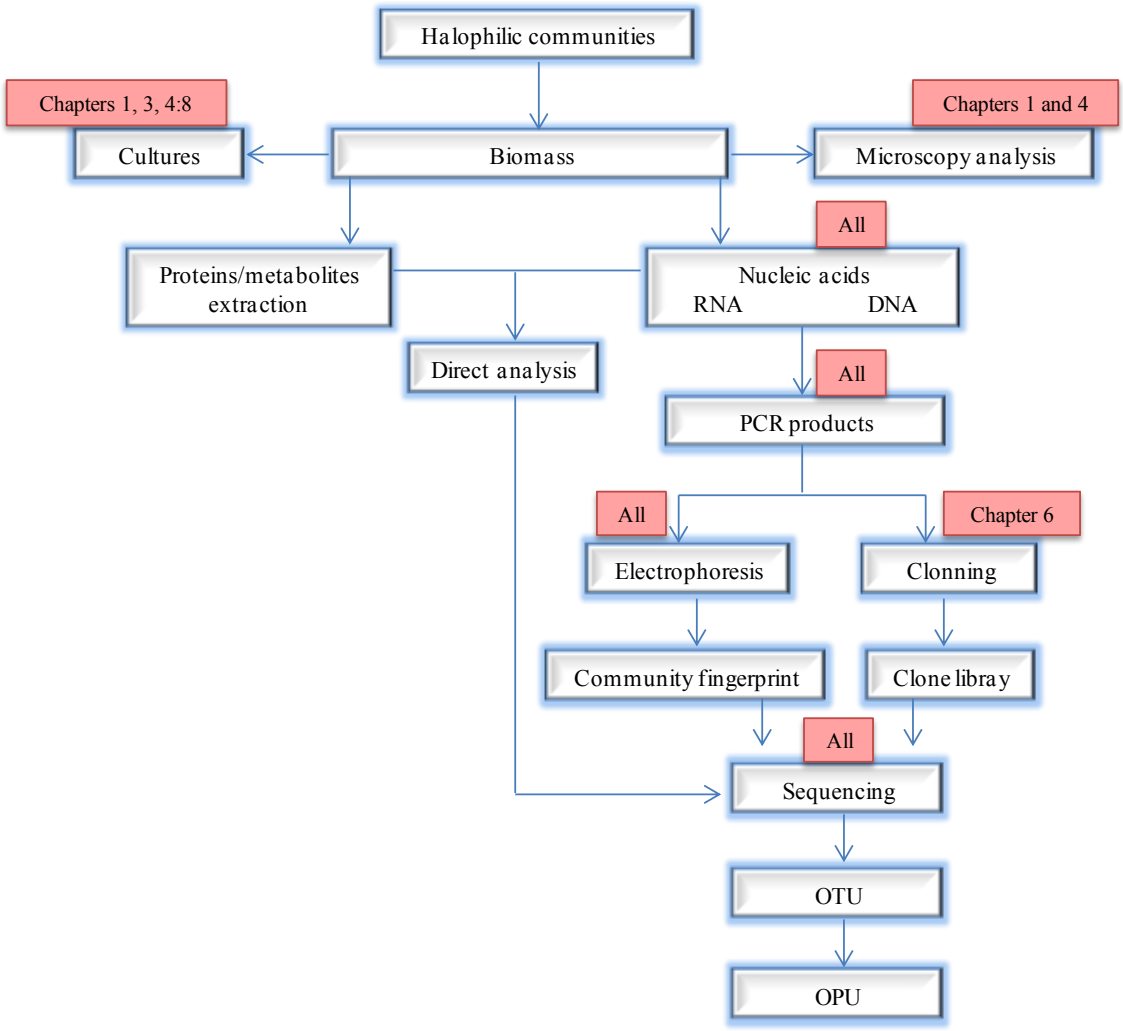

Figure I.2 Schema of approaches used in ecology of halophilic microorganism (modified of Pedrós-Alió, 2005). In red the Chapters which the different approaches were used.

Currently, the use of the –omics technologies such as genomics, transcriptomics, proteomics and metalobolomics generates massive amounts of data. The use and interaction of these different approaches produce a synergic effect allowing a deeply characterization of microbial communities obtaining information such as interaction among populations, characterization of novel diversity, or response in front environmental perturbations (Paul *et al.* 2008; Crits-Christoph *et al.* 2016; Lopatina *et al.* 2016). Direct sequencing metagenomic analyses have also been relevant in the study of communities avoiding biases of PCR (Podell *et al.* 2014; Andrade *et al.* 2015). However, unassembled or partially assembled metagenomes cannot provide information about the role of individual community members. Nevertheless, when applied to assembled and well curated genomes reconstructed from such samples, metagenomics can provide metabolic insight at the species level (Sharon *et al.* 2013). In fact, the future in the classification of uncultured microbial species (*Bacteria* and *Archaea*) could be based on the use of almost complete assembled genomes, retrieved through binning approaches,

as type material and the basis for valuable taxonomic information retrieval (Konstantinidis and Rosselló-Móra 2015).

However, high-throughput data still needs to be corroborated using other techniques as those based on the direct observation of cells by microscopy such as the fluorescence *in situ* microscopy (FISH). These techniques are constantly used not only to observe the cell morphology, but also as shown in Chapter 4 of this thesis to confirm results produced in Next Generation Sequencing (NGS) analysis related principally to the abundances or the identification of specific taxa (Jiang *et al.* 2006; Douterelo *et al.* 2014; Fernández *et al.* 2014; Deshmukh *et al.* 2016).

2.3 Phylogenetic analysis and statistical tools

In microbiology one keystone for data analysis is associated with the phylogenetic identification and it will be discussed in detail along the next section for hypersaline environments. Despite of this, it is important to briefly remark here the relevance of the phylogenetic analyses in microbiology. The most commonly used marker for microbial taxa identification is the sequence of the 16S rRNA gene. It has been widely used principally because this gene has relevant properties as 1) Universal distribution: it is present in all prokaryotic organism; 2) Structural and functional conservation: the functional constancy of this gene assures is a valid molecular chronometer, which is essential for a precise assessment of phylogenetic relatedness of organisms; 3) Their primary structures are alternating invariant, more or less conserved to highly variable regions, critical for the concurrent universal amplification. Additionally, this also permits investigation of a wide spectrum of phylogenetic lineages ranging from the domain to the species level; 4) Sufficient size: the gene is large enough to provide enough phylogenetic signal and statistically valid measurements; 5) Large databases of this gene are currently available such as RDP, Greengenes and SILVA, and therefore the sequence from an unknown strain can be compared against these databases (Ludwig and Schleifer 1994; Clarridge and III 2004; Janda and Abbott 2007; Rossi-Tamisier *et al.* 2015; Srinivasan *et al.* 2015).

The novel sequencing techniques described in the previous section produce a huge amount of information that need to be properly analyzed. Therefore, the statistical treatment of these datasets is paramount in actual microbiology. The major pitfall encountered in microbial ecology when trying to summarize and explore large datasets is related to the choice of the most adequate numerical tools to properly evaluate the data, both statistically and visually (Ramette 2007). This thesis is an example of the huge number of taxa usually obtained in a microbiology study: in the meta-analysis of this thesis some samples included almost 8,000 Operational Taxonomic Units (OTUs, see below) representing a challenge for their analyses. Some tools as the multivariate analyses are well described in the literature (Dufrene, Marc; Legendre 1997;

Oksanen *et al.* 2016), however the microbial ecologists rarely use them and most of the works are summarily reported using Principal Component Analysis (PCA) and cluster analysis (Table I.1). Among these multivariate techniques some of the less explored techniques in microbiology are Canonical Correspondence Analysis, non-Metric Multidimensional Analysis (nMDS), Linear Discriminant Analysis (LDA), Redundancy Analysis (RDA) or Mantel test, most of them used along this thesis. Other tools as co-occurrence analysis and networks have been used with the objective to elucidate the niche of different taxa within the community (Freilich *et al.* 2010; Barberán *et al.* 2011; and also in the meta-analysis). These networks, based upon natural environmental co-occurrence patterns, make possible to examine the complex interactions among microorganisms and evidence the keystone species in the systems (Freilich *et al.* 2010; Steele *et al.* 2011).

Table I.1 Usage (%) of multivariate methods in different fields (From Ramette, 2007).

	Exploratory analysis				**Hypothesis-driven analysis**						
	Cluster	**PCA**	**MDS**	**PCoA**	**CCA**	**RDA**	**MANOVA**	**Mantel**	**ANOSIM**	**CVA**	**Total number**
Bacter	48.5	38	4.5	0.4	3.2	1.8	1.3	0.4	0.9	1.1	1141
Microb	45.8	40.2	3.9	1.1	2.2	2.2	1.1	1.7	0.6	1.1	179
Plant	40.3	28.5	4.6	1.7	15.5	3.7	1.9	2.3	0.6	0.9	3335
Fungi	54	27.2	2.8	1.1	8.5	2.8	0.9	1.1	0.2	1.4	563
Fish	30.1	33.7	9.8	0.3	13.5	2.7	3.6	2.9	2.3	1.2	1464
Bird	41	20.5	5.4	0.7	21.2	3.5	2.1	4.2	0.5	0.9	429
Insect	54.3	13.7	6.1	0.8	11.5	4.4	3.5	3	1.1	1.7	637

OTUs and OPUs: Definition, use, and limitations

The rapid advances in DNA sequencing technology have allowed the study of microbial communities in greater depth than previously (He *et al.* 2015). Although the new approaches are mainly addressed by metagenomic analysis, undoubtedly, the 16S rRNA gene surveys are still widely used for characterizing microbial communities (Suau *et al.* 1999; Delbès, Moletta and Godon 2000; Mohit *et al.* 2014; Suh *et al.* 2015; Guo *et al.* 2016). As mentioned above even if sequencing microbial DNA recovered from microbial community samples has the potential to provide huge amount of information, the data should be properly managed to evaluate the underlying hypotheses (Sharon *et al.* 2013).

A common problem in the study of communities is the accurate use of a microbial species definition (Pedrós-Alió 2005), which refers to which parameters have to be used to circumscribe units in environmental samples from which we only obtained 16S rRNA sequences. Although the topic on which definition of species to take remains controversial, different approaches have been employed to calculate units taking into account the DNA bands in a DGGE gel and the different sequences in a DNA clone library. As these methods are of low resolution, the universal approach used is the consideration of the distinct measurable entities as Operational Taxonomic Units (OTUs; Pedrós-Alió, 2005). An OTU is an abstract concept of a unit that depends on the biological nature of the entities to be counted. The concept originates from the pheneticists' school who developed numerical taxonomy. The basic premise is that all OTUs represent identical kind of things (Rosselló-Mora and López-López 2008a). Although the use of OTU is previous to NGS, the analysis of massive sequencing data uses also OTUs to cluster the sequences based on sequence identity, which has facilitated the analysis of the huge number of sequences generated in NGS (Rosselló-Mora and López-López 2008a; Nguyen *et al.* 2016). In general, the identification of the environmentally occurring discrete 16S rRNA groups (often assumed to be species) has been performed using an identity threshold of 97% similarity (Turnbaugh *et al.* 2009; Pilloni *et al.* 2012; Gobet, Boetius and Ramette 2014; Birtel *et al.* 2015) or even lower (95%; Willing *et al.* 2010). The 97% threshold has been actually taken in microbial molecular ecology to circumscribe putative species based on the observation made by Stackebrandt and Goebel (1994). These authors described this value as the minimum sequence identity cutoff below which the distinction of two different species is guaranteed. Above this threshold the recognition of whether two organisms belong to the same species or not had to be evaluated using DNA-DNA hybridization techniques. However, in 2006 this value was re-evaluated and increased up to 98.7% (Stackebrandt E 2006). A more restrictive threshold would be more adequate for the environmental surveys, as it would not lead to underestimation of diversity (Yarza *et al.* 2010). Despite of such recommendations, this threshold had not been readily implemented in molecular ecology of prokaryotes.

There have been many criticisms in the use of restricted cutoffs on the percentages of sequence identities to define OTUs (Preheim *et al.* 2013; He *et al.* 2015). The main criticism is that there is no-consensus in the use of the same cutoff value among the scientific community, limiting the comparison of the different studies. Nemergut *et al.* (2011) and Youngblut *et al.* (2013) reported that the use of different identity cutoffs may change the results observed. Specifically, Nguyen et al. (2016) mentioned two basic problems derived from the use of OTUs:
1) The similarity in a non-evolutionary-based distance metric: some evidences suggest the overestimation of the evolutionary similarity between pairs of sequences.
2) The "common" 97% 16S rRNA sequence identity threshold is an "approximation" that cannot face some limitations such as the use of different hypervariable regions for the analysis. Moreover Yarza *et al.* (2014) pointed in this direction indicating that considering 97% threshold could be too conservative and would lead to underestimation of diversity as distinct species of the same genus may cluster together.

Furthermore, the problematic is not only the cutoff used to circumscribe OTUs, but also a key issue is the selection of the algorithm to cluster the sequences into OTUs. Different methods have been developed for clustering SSU rRNA gene sequences based on identity or genetic distance alone such as taxonomy-independent, taxonomy-unsupervised or *de novo* clustering (Preheim *et al.* 2013). First, sequences are aligned to create a distance matrix that will be used into the consequent clustering. There are many available algorithms for clustering sequences, most prominently hierarchical clustering algorithms (HCA) and their heuristic approximations (*i.e.* uclust), cd-hit or the ESPRIT suite of algorithms. One of the principal objectives in the development of new algorithms has been to decrease the computational cost (Preheim *et al.* 2013; Westcott and Schloss 2017). For example, in this thesis some datasets having ~500,000 sequences (see Annex) took more than one month to obtain the OTUs (method: uclust). The availability of different algorithms generates also an additional problem: it has been noted that different methods often provide different conclusion of the same dataset, both quantitatively and qualitatively (Schmidt, Matias Rodrigues and von Mering 2014; Nguyen *et al.* 2016).

However, the major pitfall of the NGS approach is that the length of the sequences is too short for taxonomic identifications at the species level (Yarza *et al.* 2014), and the almost complete sequence of the 16S rRNA gene would be desirable. Generally, 454 pyrosequencing rendered in the past sequence lengths <300 bp (*e.g.* Turnbaugh *et al.* 2009; Willing *et al.* 2010; Pilloni *et al.* 2012; França *et al.* 2014; Gobet, Boetius and Ramette 2014). However, the results obtained with such short sequences seemed to be robust enough to mirror the observations made with classical techniques (Pilloni *et al.* 2012; Gobet, Boetius and Ramette 2014). Lately, the improvements in the methodology allowing much longer reads of the amplicons (up to 800 bp and with means >550 bp; (Mora-Ruiz *et al.* 2015) led to a much robust identification power due

to the larger information content. However, due to the higher costs of 454 pyrosequencing, Illumina is being the method of choice for most of the new studies on environmental microbial diversity despite some evidences that both, the method and the different regions studied, may not produce robust results comparable to the conventional methods (Birtel *et al.* 2015). For these reasons, among the different NGS techniques, still 454 may be the best choice for amplicon analyses due to the larger stretches produced with relative low error rates (Liu *et al.* 2012).

Considering all these problems derived from the use of the OTU approach and with the aim of having a more "realistic" unit of species in sequencing analysis, our group (França *et al.*, 2014) developed the approach of identifying Operational Phylogenetic Units (OPUs). An OPU is the smallest monophyletic group of sequences containing OTU representatives together with the closest reference sequence, including the sequence of a type strain when possible (Mora-Ruiz *et al.* 2016; Figure I.3). In this way, one or more OTUs may belong to the same OPU due to their common phylogenetic signal, usually reducing the richness observed with the former method. OPU approach has been used in previous works (França *et al.* 2014; Viver *et al.* 2015).

Differently to the OTU, the OPU approach uses a phylogenetic inference for the classification of sequences. In the OPU approach, as in all phylogenetic affiliation methods, has as prerequisites for reliable phylogenetic conclusions an optimal alignment of the primary structures and a careful data selection (Ludwig and Schleifer 1994). In order to consider those points, for the OPU approach the sequences are automatically aligned using the program package ARB (Ludwig *et al.* 2004) with the SINA aligner (Pruesse, Peplies and Glöckner 2012a) that takes into account the secondary structure of the gene to refine the aligments (Yarza *et al.* 2010). For the first affiliation step, the Living Tree Project (LTP) database is used. LTP is a curated dataset, periodically updated which comprises high quality 16S rRNA gene sequences only of type strains of the hitherto classified species with a validly published name (Yarza *et al.* 2010). With such a curated dataset one avoids the use of sequences with species names that may be incorrect, misassigned accession numbers or wrong strain collection numbers, which can produce serious problems in the correct identification. However, it is not uncommon to have OTUs with no close references sequences; in such cases the high quality SILVA-ref is used to select the closest relatives of unidentified sequences with the LTP database. The whole process is performed with the ARB software (Ludwig *et al.* 2004) using the parsimony tool to insert partial sequences in a pre-existing tree. With ARB-parsimony, new sequences are successively added to existing trees according to the parsimony criterion, without modification of the original tree topologies (Ludwig *et al.* 2004). Attending to the previous reasons, we consider the OPU approach as the best and most accurate option for the classification of partial sequences obtained through NGS.

Finally, the OPU approach is the central core of this thesis to specifically test wether OPU is more efficient and reliable than grouping sequences just on the basis of a standardized cut-off values. Furthermore, we aim to demonstrate that this method is suitable to describe microbial communities in hypersaline environments, providing enough resolution to detect and identify different taxa and to infer biogeographic and evolution-time patterns in microbial communities.

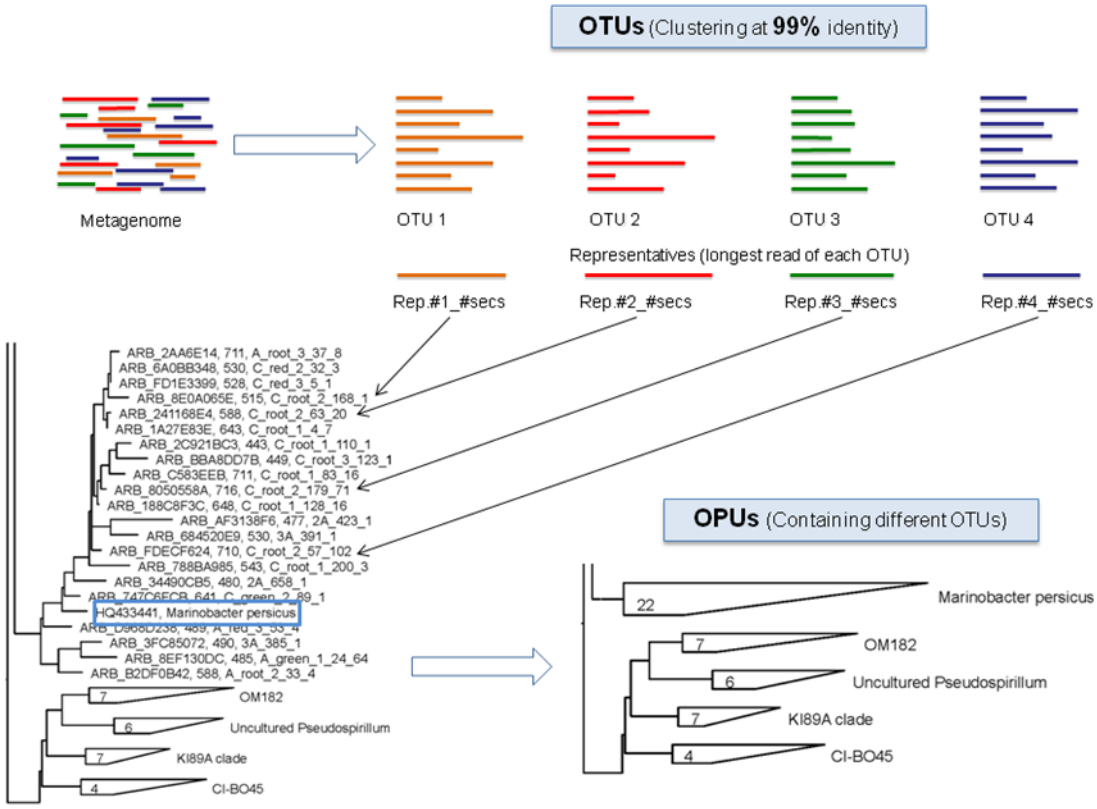

Figure I.3 Rationale to circumscribe the Operational Phylogenetic Units (OPUs) for representative sequences of OTUs. Briefly, the pyrosequencing data, after being trimmed, is clustered into OTUs at 99% identity. The longest representative of each OTU is selected for a parsimony insertion using a pre-reconstructed and optimized tree containing the representative type strain sequences and additional relevant sequences. After insertion, the tree is manually inspected and the OPUs circumscribed according to their phylogenetic uniqueness.

Section II. Objectives and structure

Along this thesis, we used the OPU approach to study different communities, especially halophilic microorganisms, trying to understand the structure of the total community as well as the environmental variables shaping them. In this research, we analyzed the microbial community inhabiting different environments including exosphere and endosphere of halophytes, solar salterns, saline lakes, hypersaline sediments and, animals (*e.g.* jellyfish) using both dependent and independent-culture approaches. The questions answered within this work provide an unprecedented view on halophilic microbial communities and their ecology using the novel OPU approach. We have divided this thesis into three subsections, which are listed according to the following structure:

Microbial diversity associated to halophytes

In this particular subsection, we describe the bacterial and archaeal communities associated to halophytes. Due to the special mechanism of osmoregulation of these plants (accumulation of salt), they provide a novel environment where halophilic communities can be explored.

Chapter 1. Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily *Salicornioideae (Amaranthaceae)*

Objective: To investigate the presence and identity of the halophilic microorganisms conforming the epiphytic and endophytic communities of halophytes.

Chapter 2. Endophytic microbial diversity of the halophyte *Arthrocnemum macrostachyum* across plant compartments

Objective: To study the bacterial community associated to three different compartments in the endosphere of *Arthrocnemum macrostachyum* as well as the influence of the microbial community and the environmental parameters of the soil.

Chapter 3. Mesophilic endophytes associated to the euhalophyte *Arthrocnemum macrostachyum* and their potential plant growth promoting activity.

Objective: To analyze the mesophilic and halotolerant bacterial community associated with the endosphere of *Arthrocnemum macrostachyum´*s phyllosphere as well as to evaluate the Plant Growth Promoting Activity (PGPA) of selected isolates on the plant model *Arabidopsis thaliana.*

Chapter 4. Halophilic endophytic archaea in the halophyte *Arthrocnemum macrostachyum*.

Objective: To explore the diversity of halophilic archaea inhabiting the endosphere of *Arthocnemum macrostachyum.*

Microbial diversity in salterns

Within this subsection, we attempt to describe the biogeography of the distribution of halophilic microorganism in salterns distributed in distant locations as well as revealing the underlying environmental variables driving the shift in the communities along time.

Chapter 5: Biogeographical patterns of bacterial and archaeal communities of distant hypersaline environments.

Objective: To know the effect of the geographical distances on the microbial diversity and to evaluate the effect of the environmental parameters on the total structure of the community as well as on specific taxa.

Alternative uses of OPU approach: evidences in other environments

We have previously mentioned we applied the OPU approach in other environments different from hypersaline, therefore we provide in this last subsection some additional examples in where the OPU approach was used.

Chapter 6. Exploring the diversity in other environments: and OPU approach

Objective: To evaluate the OPU approach as a tool in the exploration of clinical microbiology and complement in the search for salt resistance genes of microorganisms inhabiting brines of solar salterns and associated with rhizosphere of *Arthrocnemum macrostachyum*.

Chapter 7. Prokaryotic microbiota in the digestive cavity of the jellyfish *Cotylorhiza tuberculata.*

Objective: To study the microbiome *Cotylorhiza tuberculata* by dependent and indepeden-culture approaches.

Section III. Methods and techniques used and optimized in this thesis

The research performed in this thesis is the result of a synergic confluence produced by the use of different techniques. The studies of microbial communities evolve fast, developing new tools for the understanding of the microbiological composition in different environments. In this section, we outline some methods explored in this thesis. Nonetheless, each of the chapters presents a more detailed section of the methods respectively used.

1. Culture-dependent approach

* Cultures: In Chapters 1, 3, 7 large-scale culturing was performed using saline (principally SeaWater) and non-saline culture media. Additionally, Chapter 1 describes a random method for the selection of colonies and a protocol for the plant surface sterilization to isolate epiphytic and endophytic communities associated with halophytes. The sterilization method was evaluated using electron microscopy (see above). Additionally, a culture media based on plant extract was designed for isolation of "rare" taxa (Chapter 3).

*Whole Cell MALDI-TOF: This technique was used for the identification of isolates (see Chapter 1, 3 and 7). MS (Main Spectra) obtained were clustered in OTUs and representative of each OTU was selected for identification by 16S rRNA gene sequencing. Chapter 1 also includes a method to calculate the minimum sample size of colonies analyzed by WC-MALDI-TOF.

*****Random Amplification of Polymorphic DNA: In Chapter 1 and 3 RAPD screening was done to estimate the clonality of selected strains.

2. Culture-independent approach

*****DNA extraction: Along this thesis DNA extraction from plants, soils, sediments and brines samples were performed using specific protocols depending the type of sample. In Chapter 1, a protocol for the enrichment of the endophytic microbial fraction was optimized to address the drawbacks of chloroplastidial and mitochondrial DNA interferences, using differential and gradient centrifugations. For all samples, PCR for 16S rRNA gene amplification and sequencing using 454 GS-FLX+ Titanium technology were performed.

*Microscopy: This thesis includes in Chapter 1 and 4 the use of microscopy techniques such as Scanning Electron Microscopy (SEM) and Catalyzed reporter deposition Fluorescence In Situ Hybridization (CARD-FISH). The latter with the objective to quantify the number of bacteria and archaea in brine samples.

3. Phylogenetic reconstruction

* Sequencing trimming, OTUs clustering and OPU design: For all chapters, the treatment of the sequences originated from massive sequencing with 454-pyrosequencing includes firstly a trimming step using Mothur software (Schloss *et al.* 2009). Low-quality sequences were trimmed and no ambiguities and mismatches in reads with primer pairs and barcodes were allowed. Chimeras were removed with the application Chimera Uchime implemented in Mothur. Sequences were clustered into OTUs at using the UCLUST tool included in QIIME (Caporaso *et al.* 2010). Finally, the longest read of each OTU was selected as representative for the OPU design by phylogenetic inference as previously described (Franca *et al.* 2015). For the OPU approach, the longest representative of each OTU is selected and inserted by parsimony in a pre-reconstructed and optimized tree containing the representative type strain sequences and additional relevant sequences. Consecutively, the tree is manually inspected and the OPUs circumscribed according to their phylogenetic uniqueness.

*Physiochemical characterization of the environment: Environmental parameters such as salinity, water temperature, pH, oxygen were measured in each location and others additional obtained from databases. The ionic characterization was performed by ionic chromatography and also suing Total Bernard's calcimeter method by the Research Technical Services of the University of Alicante.

4. Ecological and statistical tools

The diversity was calculated using the "real" diversity Jost index with a by Monte-Carlo resampling designed in Chapter 2. Regarding to the statistical approach we used diverse techniques from uni to multivariate analyses in most chapters to analyze the structure of the communities and their relationship with the environmental variables. All the datasets were analysed using R (www.r-project.org), MATLAB© and PAST v 3.01 (Hammer, Harper and Ryan 2001) software

Section IV. Results

Results encompasses seven different chapters

Microbial diversity associated to halophytes

Thanks to the human heart by which we live,
Thanks to its tenderness, its joys, and fears,
To me the meanest flower that blows can give
Thoughts that do often lie too deep for tears

William Wordsworth (1770–1850)

Chapter 1. Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily *Salicornioideae* (*Amaranthaceae*)

Abstract

Halophytes accumulate large amounts of salt in their tissues, and thus are susceptible to the colonization by halotolerant and halophilic microorganisms that might be relevant for the growth and development of the plant. Here the study of 814 cultured strains and 14,189 sequences obtained by 454 pyrosequencing were combined to evaluate the presence, abundance and diversity of halophilic endophytic and epiphytic microorganisms in the leaf's phytosphere of members of the subfamily *Salicornioideae* of five locations in Spain and Chile. Cultures were screened by the tandem approach of MALDI-TOF/MS and 16S rRNA gene sequencing. In addition, differential centrifugation was used to enrich endophytes for further DNA isolation, 16S rRNA gene amplification and 454 pyrosequencing. Culturable and non-culturable data showed strong agreement with a predominance of *Proteobacteria*, *Firmicutes* and *Actinobacteria*. The most abundant isolates corresponded to close relatives of the species *Chromohalobacter canadensis* and *Salinicola halophilus* that made up nearly 60% of all isolates and were present in all plants. Up to 66% of the diversity retrieved by pyrosequencing could be brought into pure cultures and the community structures were highly dependent on the compartment where microorganisms thrive (plant surface or internal tissues).

Introduction

Plants are naturally associated with microorganisms, and these relations range from beneficial to pathogenic interactions (Verma 1991). In many cases, they can play an important role in the growth and development of plants (Kuklinsky-Sobral *et al.* 2004; Hardoim, van Overbeek and Elsas 2008). The beneficial functions reported are as diverse as: promotion of plant growth (Vessey 2003; Lugtenberg and Kamilova 2009), N_2-fixation (Cocking and Cocking 2003; Franche, Lindström and Elmerich 2009), protection against plant pathogens (Compant, Clément and Sessitsch 2010) and synthesis of secondary metabolites (Nagarajkumar, Bhaskaran and Velazhahan 2004). Such microorganisms can colonize both external surfaces and internal tissues. Those associated to phylloplane and rhizoplane are named epiphytes (Andrews and Harris 2000), and those invading tissues for all or part of their life cycle are called endophytes (Newman and Reynolds 2005; Reinhold-Hurek and Hurek 2011), and seem to be ubiquitous in the plant (Lodewyckx *et al.* 2002).The microbial community residing in the phylloplane (leaf's epiphytes) faces a variable environment that is characterized by fluctuating temperatures, humidity, UV radiation, wind, plant topography and quality of photosynthate (Andrews and Harris 2000; Lindow and Brandl 2003), whereas endophytes may colonize a more stable environment.

According to their salinity tolerance, plants can be divided in glycophytes and halophytes. The former include sensitive and relatively salt tolerant species (Radyukina *et al.* 2007), whereas halophytes are plants that can tolerate high salt concentrations and can complete their whole life cycle at salt concentrations in soil higher than 200 mM NaCl (Kosová *et al.* 2011; Flowers, Munns and Colmer 2014). In general, the exploration of the microbial diversity in plants has been focused on the phylloplane of glycophytes, and especially on the major commercial crop species (Ercolani 1991; Knief *et al.* 2010). *Bacteria* generally constitute the microbial fraction. On the other hand, *Archaea* had been reported just associated to the rhizosphere (Ramakrishnan *et al.* 2001; Bomberg *et al.* 2003; Ochsenreiter *et al.* 2003; Conrad *et al.* 2008) and phyllosphere (Knief *et al.* 2010), and have never been convincingly detected in the internal tissues (Reinhold-Hurek and Hurek 2011). The microbial community association with halophytes has rarely been investigated, and the few reports focused just on the rhizosphere (Ruppel, Franken and Witzel 2013), notwithstanding that the phyllosphere is generally predicted to be more diverse from evidences using cultivation-dependent (Emiliani *et al.* 2014) and independent (Bodenhausen, Horton and Bergelson 2013) approaches. Furthermore, there is an study describing the bacterial communities found on leaves of *Atriplex halimus*, a salt-excreting plant (Simon, Abeliovich and Belkin 1994), but there are no reports of endophytic microorganisms isolated from the endophyllosphere of halophytes.

Here, we investigated the presence and community structures of halophilic microorganisms colonizing halophytes by means of culture-dependent and -independent (high-throughput pyrotagging) approaches. This pilot work has been centered on the endophytic and epiphytic microbiota associated to the leaves of *Salicornioideae*, growing under natural conditions in five different locations. In addition, a protocol for the enrichment of the endophytic microbial fraction was optimized to address the drawbacks of chloroplastidial and mitochondrial DNA interferences.

Materials and methods

Collection and identification of plant material

The aerial parts, comprising stems and green leaves of five halophytes, were collected during the months of March and April 2013 in Pichidangui (PI: 32°08'21.56''S, 71°31'16.26''W, Chile), Lo Valdivia (LV: 34°41'50.16''S, 72°00'42.86''W, Chile), Alicante (AL: 38°21'03.3''N, 3°00'44.3''W, Spain), Campos (CA: 39º21'03.3'' N, 3º00'44.3'' E, Spain) and Ses Fontanelles (SF: 39°32'4.64''N, 2°43'56.41''E, Spain). Individual stems with green leafs of the plants were excised at about five centimeters above the soil level and stored in zip-lock plastic bags using sterile gloves. The five plants were identified in the Biology Department of the University of the Balearic Islands (UIB). Additionally genetic identification was performed to verify the identity of plant specimens. Plant DNA isolation was performed using DNeasy Plant Mini Kit (Qiagen). The maturase K gen (*matK)* was amplified using Master Mix (5 PRIME GmbH, Germany) following the manufacturer's instructions. The reaction was carried out in a final volume of 25 µL with the specific primers F2cariophyllales and R2cariophyllales with the previously published conditions (de Vere *et al.* 2012). A fragment of about 800 bp was visualized on 1.5% agarose gel stained with ethidium bromide (1 µg/mL) and amplicons were purified using MSB® Spin PCRapace kit (INVITEK GmbH, Berlin). Purified PCR products were sent to Secugen (Madrid, Spain) for DNA sequencing. Sequences were trimmed using the software Sequencher *v* 4.8 (Gene Codes Corporation, Michigan). The new sequences were aligned and compared with reference sequences of the GenBank database using the ClustalW aligner implemented in the ARB software package (Ludwig *et al.* 2004). The identity values between the sequences were also calculated with the ARB package.

Culture-dependent analyses: Surface sterilization and isolation of epiphytic and endophytic microorganisms

Approximately 150 g of each plant shoots of 7-10 cm were randomly selected; the damaged plant material was previously excised and removed. Shoots were carefully manipulated to avoid tissue damage (Chase and Hills 1991), and the excision areas were first hot cauterized using an

incandescent metal loop to avoid the loss of sap. Isolation and cultures of the plant-associated microbiota was performed on agar plates with five different salt concentrations (5%, 15%, 20%, 25% and 30%) using Sea Water (SW) culture media (Rodriguez-Valera *et al.* 1985) supplemented with 0.05% yeast extract. Three different fractions or conditions: epiphytic (P), endophytic (N) and sterile test (S) were taken after each manipulation step. Briefly, 25 g of plant material were placed into 50 mL tubes with 35 mL SW of each five different salt concentrations, gently vortexed for 3x 45 s, and then the plant material was removed and placed in a sterile tube. This initial suspension was considered as the P inoculum. Immediately, the plant material surface was sterilized with sequential washes: 10 min in sterile distilled water, 5 min in 0.2% Triton X-100, 10 min in sterile distilled water, 5 min in 2% bleach (NaClO), 10 min in sterile distilled water, 2 minutes in 70% ethanol and, finally, two rinses of 5 min in sterile distilled water. Between steps, plant material was dried with sterile paper towels. The sterilization test was performed by submerging and mixing the sterilized plant material in SW media, and following the same mixing process as for the isolation of epiphytes. This suspension was considered as the sterilized inoculum S. Finally, the plant material was mixed with 10 mL of PBS 1X and the mixture was gently macerated using a sterile pestle and mortar. The tissue extract was considered as the N inoculum.

The P, S and N inoculates were serially diluted (until 10^{-8}) in each of the five different salt concentration SW media and spread-plated onto the respective SW agar media supplemented with 10 $\mu g \cdot mL^{-1}$ of the fungicide itraconazol (Bexal Farmacéutica). Samples were plated in triplicates and incubated at room temperature. The colonies were randomly picked and selecting at least 10% CFUs (colony forming units) growing in plates with abundances between 10-100 colonies in each location. Colonies were replicated onto plates with the same culture conditions and to obtain a random subsample of isolates no attention to the colony morphology was paid. For storage purposes, isolates were grown on liquid SW media with the same salt concentration, and the grown suspensions were supplemented with glycerol (5%) for storage at -80°C.

A piece of a shoot of approximately 3 g was selected to verify the efficacy of the surface sterilization by scanning electron microscopy (SEM). For this purpose, a variable pressure microscope S-3400N (Hitachi, Japan) was used. Previously, samples were fixed with 2% glutaraldehyde (4°C) during 48 h. Shoots were then washed with phosphate buffer (0.1 M, pH 7.2-7.4) for 24 h. Finally, samples were submerged in consecutive concentration steps of acetone of 30-50-60-70-90 and 100% for 30 min each one. Micrographs of fifteen areas in each sample were taken using 10 kV of accelerating voltage and 40 Pa of pressure with a low pressure ESED secondary detector (Hitachi Trademark, Barcelona, Spain). Additionally, with the object to visualize endophytes, previously sterilized shoots were opened carefully using a

sterile scalpel. The opened shoots were fixed and micrographs were taken using the same protocol mentioned above.

MALDI-TOF/MS analyses, cluster identification by 16S rRNA gene sequencing

Randomly selected colonies were analyzed by MALDI-TOF/MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) as previously reported (Viver *et al.* 2015). Groups of spectra clustering in the dendrograms were considered as Operational Taxonomic Units (OTUs; see below). OTUs selected from the P and N dendrograms were defined with a cut-off of 720 and 750 distance level respectively. Several representatives of each OTU were selected for their DNA extraction, 16S rRNA gene amplification and sequencing, and phylogenetic reconstruction as previously reported (Viver *et al.* 2015). The sequences have been deposited at the EMBL repository under the accession numbers (LN651124-LN651155). OPUs (Operational Phylogenetic Units; see below) were circumscribed by manually inspecting the resulting final tree (Viver *et al.* 2015).

Clonality estimated by RAPD fingerprinting

RAPD fingerprints were generated for 193 isolates representing the different OPUs. These were randomly taken to cover at least 12% of each group. RAPD screening was performed using the Master Mix (5 PRIME GmbH, Germany) following the manufacturer's instructions with the primer RAPD1 using the same conditions previously reported (Peña *et al.* 2005). The reaction was carried out in 25 µL volume, containing 3 µL of DNA template. The amplified PCR products were visualized by electrophoresis in 2% agarose gel running in 1X TAE buffer, at 25 V for 50 min. The mass-ruler express forward DNA ladder mix (Thermo Scientific, Massachusetts, USA) was used as a molecular weight standard. Ethidium bromide stained DNA gels were photographed and analyzed with Bionumerics *v* 7.1 software (Applied Maths, Belgium).

Culture-independent analyses: Separation of plant and microbial fraction

Approximately 60 g of each plant were sterilized and macerated as described above. Twenty mL of macerated biomass were centrifuged in five subsequent steps at 200, 500, 800, 1000 and 3000 *xg* for 20 min at 4ºC, and collecting the pellets after each centrifugation step. Pellets and the final supernatant (3000 *xg*) were stored at -20°C. Finally, a sucrose density gradient centrifugation was applied to the pellet of 3000 *xg*. The density gradient was prepared by overlaying six solutions of 4 mL of sucrose of increasing concentrations (15%, 20%, 30%, 40%,

50% and 60% w/v) avoiding perturbations and mixing. The pellets of 3000 *xg* were mixed with 2 mL of sucrose solution at 2.5% w/v and 20 µL of blue toluidine (1 mg/L) and placed on the top of the gradient. Gradient was centrifuged (79,880.3 *xg*, 2:00, 4°C - Optima TLX Ultracentrifuge Rotor SW30.1 Centrifuge, Beckman Coulter, California, USA). After centrifugation, four layers were observed. Each layer was recovered using a syringe with needle. All layers were stored at -20°C.

Microbial DNA extraction, PCR amplification and pyrosequencing

Samples of each layer of higher density were used in the microbial DNA extraction as it was expected that mitochondria and chloroplast would be retained in the upper layers (between 10% and 30% w/v of sucrose solution; Galbraith *et al.* 1995). 1200 µL of extraction buffer (EB; Li *et al.* 2001) were added to 2 mL of sample, mixed by vortex (60 s) and centrifuged (7000 *xg*, 15 min, Room Temperature-RT). Pellet obtained was used to extract DNA as previously published (Li *et al.* 2001) with the following modifications: no RNAse was added to the samples, and an initial step with pre-warmed (40ºC) phenol:chloroform:isoamyl alcohol mixture (25:24:1 v/v/v) in a proportion of 1:1 (v/v) was also incorporated before subsequent treatments with chloroform:isoamyl alcohol (24:1). DNA precipitation was made using 11% (v/v) of 3 M sodium acetate and 60% (v/v) of cold (4ºC) isopropyl alcohol and the air-dried DNA pellet was resuspended in 80 µL of Milli-Q grade water. DNA was quantified using NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA).

PCR amplification was performed using bacterial primers GM3 and S (Table A 1.1) as previously reported (Viver *et al.* 2015). A second short PCR (five cycles) was performed in a final volume of 25 µL in triplicate to incorporate tags and linker into the amplicon using 1:25 dilution of the original products as templates, and also using the same conditions as the first PCR. In this case the primers GM3-PS and a variant of 907-PS (Table A 1.1) were used. The PCR products were visualized by electrophoresis in 1% agarose gel running in 1X TAE buffer, at 25 V for 50 min. Two bands were observed, the first of ~1500 bp and the second of ~960 bp. The second band was excised and the PCR product was eluted using the Zymoclean™ Gel DNA recovery Kit (Zymo Research, California, USA) following the manufacturer's instructions. The concentration of the barcoded-amplicons was measured with NanoDrop and mass-ruler express forward DNA ladder mix and finally, an equimolar mixture of the amplicons was sent to the sequencing company Macrogen Inc., Seoul, Korea. The samples were sequenced using 454 GS-FLX+ Titanium technology. The set of sequences has been deposited at the ENA sequence repository under the study accession number PRJEB7624.

Sequence trimming, OTU clustering and OPU design

Data was processed using Mothur (Schloss *et al.* 2009) software. Low-quality sequences were trimmed (<300 bp with a window size of 25 and average quality score of 25). No ambiguities and mismatches in reads with primer pairs and barcodes were allowed. Chimeras were removed with the application Chimera Uchime implemented in Mothur. Sequences were clustered into OTUs at 99% using the UCLUST tool included in QIIME (Caporaso *et al.* 2010). The longest read of each OTU was selected as representative for the OPU design by phylogenetic inference as previously described (Viver *et al.* 2015).

Ecological indexes and statistical analysis

A non-parametric Kruskal-Wallis test was performed for comparing the abundances between epiphytes and endophytes in each plant as they did not satisfy assumptions for normality and homogeneity of variances. The relation between the composition of the microbial communities, compartment (P or N) and locations was examined by a Non Metric Multidimensional Scaling (NMDS; Kruskal, M. and Wish 1978). The goodness of the NMDS was evaluated according to the stress value, which for ten samples is considered acceptable if smaller than 0.133 (Sturrochk, K. Rocha 2000). The abundances of endophytes obtained from both methods (cultured and no-cultured) were further explored using a Principal Component Analysis (PCA). All multivariate statistical analyses were performed using the package *vegan* (Oksanen *et al.* 2016) and FactorMineR (Lê, Josse and Husson 2008) in R *v* 3.1.1. (www.r-project.org). OPUs were used to calculate rarefaction curves and the Shannon-Wiener (*H´*), Chao 1, Evenness (*E*), and Dominance (*D*) indexes per sample with PAST *v* 3.01 software (Hammer, Harper and Ryan 2001).

OTU and OPU definitions in this study

In our reports (França *et al.* 2014; Viver *et al.* 2015) OTUs are defined by clustering the units according to an identity threshold. Here, the OTUs based on MALDI-TOF/MS measures correspond to each independent cluster formed by profile identity limits, which are dependent on each dataset. On the other hand, the trimmed sequences obtained by 454 pyrotagging are stringently clustered in OTUs sharing ≥99% identity. In both cases, the representative sequences of each OTU are used for a phylogenetic inference (by reconstruction for almost full sequences, or parsimony addition for partial sequences) and OPU assignment. An OPU is considered as the smallest clade containing one or more amplified sequences affiliating together with reference sequences available in the public repositories (Viver *et al.* 2015). When possible, the OPUs should include a type strain sequence, and for identity values >98.7% with type strain sequences the amplicons would be considered to belong to the same species using this conservative

threshold, as previously recommended (Stackebrandt and Ebers 2006). On the other hand, for the identity values <98.7% and >94.5% with the closest relative type strains the OPU would be considered to be the same genus (Yarza *et al.* 2010) but from a different unclassified species.

Results

Species identification of the host plants

Molecular identification results confirmed the morphological identification. The five exemplars could be identified as members of the family *Amaranthaceae,* subfamily *Salicornidoideae*. The affiliation observed with the gene *matK* confirmed the identification of two very close species: *Allenrolfea vaginata*, taken from LV, and *Arthrocnemum macrostachyum* taken from PI, AL, CA and SF (Table A 1.2).

Culturable abundances

The heterotrophic colony forming units (CFU) was enumerated after 3, 7, 15, 25 days since platting. After the day 25, CFUs were checked every 15 days for the following seven months. None of the S plates (sterile tests) exhibited growth. Accordingly, all areas observed under SEM showed the presence of colonizing epiphytic microorganisms in the untreated tissues, whereas after the sterilization of plant surface, no traces of attached microorganisms could be detected (Figure 1.1 A and B) as a confirmation that attached epiphytic microbiota could be successfully removed from the plant material. Moreover, the micrographs of sectioned sterile plant material exhibited presence of endophytic microcolonies (
Figure 1.1 C and D).

Plate counts showed abundance values of aerobic heterotrophic epiphytes with a maximum of 2.7 x 10^6 $CFUg^{-1}$ (PI), whereas the major value for endophytes was 2.7 x 10^7 $CFUg^{-1}$ (CA), both in SW 5%. No growth was detected in SW 30% after seven months of incubation with the unique exception of SF endophytes, which reached values of 1.4 x 10^7 $CFUg^{-1}$ (Table A 1.3). There was a decreasing pattern of microbial abundances with the increase of salt concentration in the culture media for both epiphytes (P) and endophytes (N) samples (Table A 1.3). The abundances of CFU's were considerably different between endophytes and epiphytes (Figure 1.2). In general, endophytes were more abundant than epiphytes and the differences were significant for all samples (Kruskal-Wallis X^2, d. f.=1, p<0.05; Table A 1.3), excepting LV that did not show significant differences (X^2, d. f.=1, p>0.05; Table A 1.3).

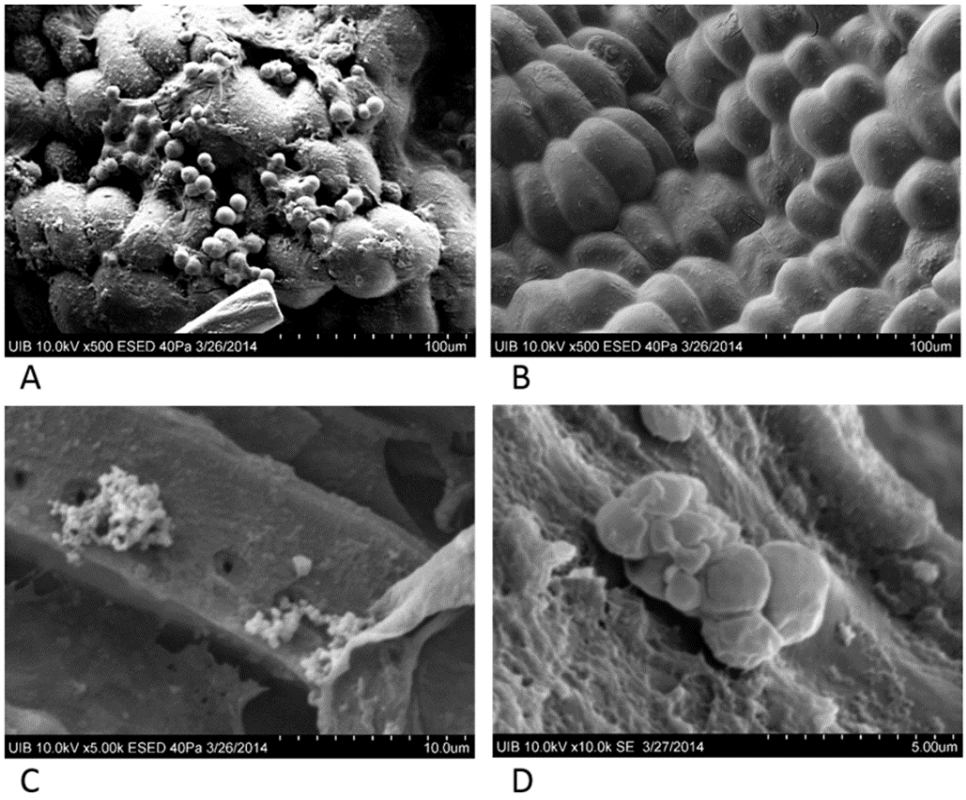

Figure 1.1 Scanning electron microscopy micrographs of the untreated phyllosphere plant surface (A) and after sterilization process (B). Endophytic microorganisms (C and D) from the phyllospheric area after sterilization of the surface. Sample corresponding to *Arthrocnemum macrostachyum* obtained from Ses Fontanelles, Spain.

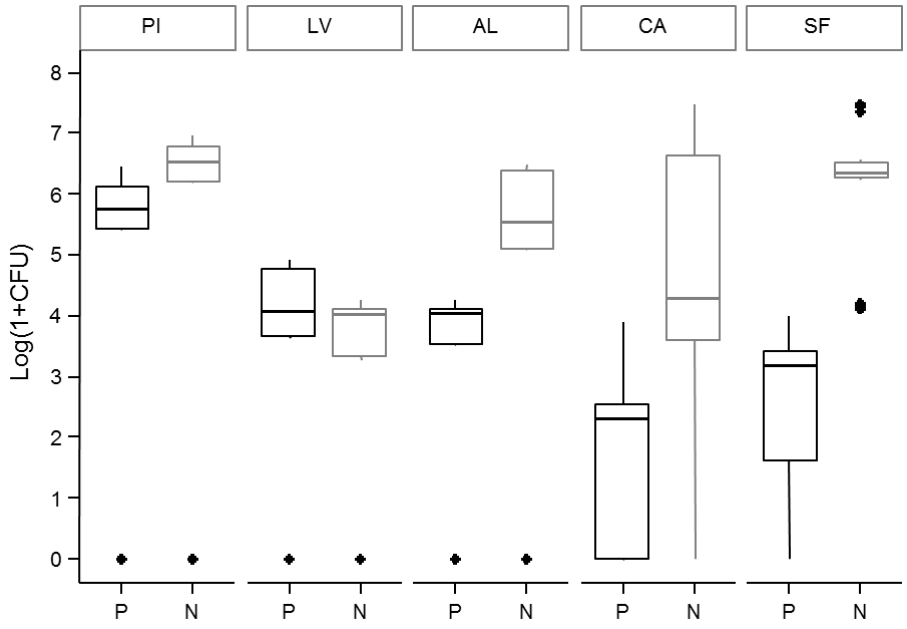

Figure 1.2 Total abundances box plot of endophytes and epiphytes in each location. PI=Pichidangui, LV=Lo Valdivia, AL=Alicante, CA=Campos, SF=Ses Fontanelles, P= epiphytes and N= endophytes.

MALDI-TOF/MS analysis of isolates and identification of OTUs by sequencing

A total of 813 colonies corresponding to 318 epiphytes and 495 endophytes of different plants: PI (n=247), LV (n=173), AL (n=170), CA (n=79) and SF (n=144) were analyzed by MALDI-TOF/MS. The dendrograms clustered the MALDI-TOF/MS profiles into 32 distinct OTUs (*i.e.* clusters based on profile similarities; Figure A 1.1, Figure A 1.2). Representative isolates of each OTU were identified by 16S rRNA gene sequence affiliation with their closest relative type strains (Table 1.1, Figure 1.3 and Table A 1.4). In all cases, the new isolates and the closest type strains was at least 98.7%. The 32 OTUs could be grouped in a total of 17 OPUs (*i.e.* unique phylogenetic clades affiliating the new sequences with reference sequences; note that the numeration of the OPUs is unique for both culturing and pyrosequencing approaches Figure 1.3 and Table 1.1). Six OPUs corresponded only to epiphytic isolates (OPUs 2, 7, 10, 11, 16 and 17), five only to endophytic (OPUs 5, 6, 9, 13 and 14), and six were common to both compartments (OPUs 1, 3, 4, 8, 12 and 15). The bacterial domain was represented by 16 OPUs that harbored 809 isolates (99.5%) while OPU 38 (an epiphyte) was the unique affiliating with the archaeal domain with 4 isolates. Most of the OPUs affiliated with *Gammaproteobacteria* (52.9%) and *Firmicutes* (17.6%), and in minor extent to *Alphaproteobacteria* (0.4%), *Actinobacteria* (4.7%), *Bacteroidetes* (0.4%) and *Euryarchaeota* (0.5%). The most abundant isolates corresponded to OPU 1 identified as *Chromohalobacter canadensis* and OPU 3 identified as *Salinicola halophilus*, with 204 and 269 strains respectively, representing 58.2% of the total isolates in this study.

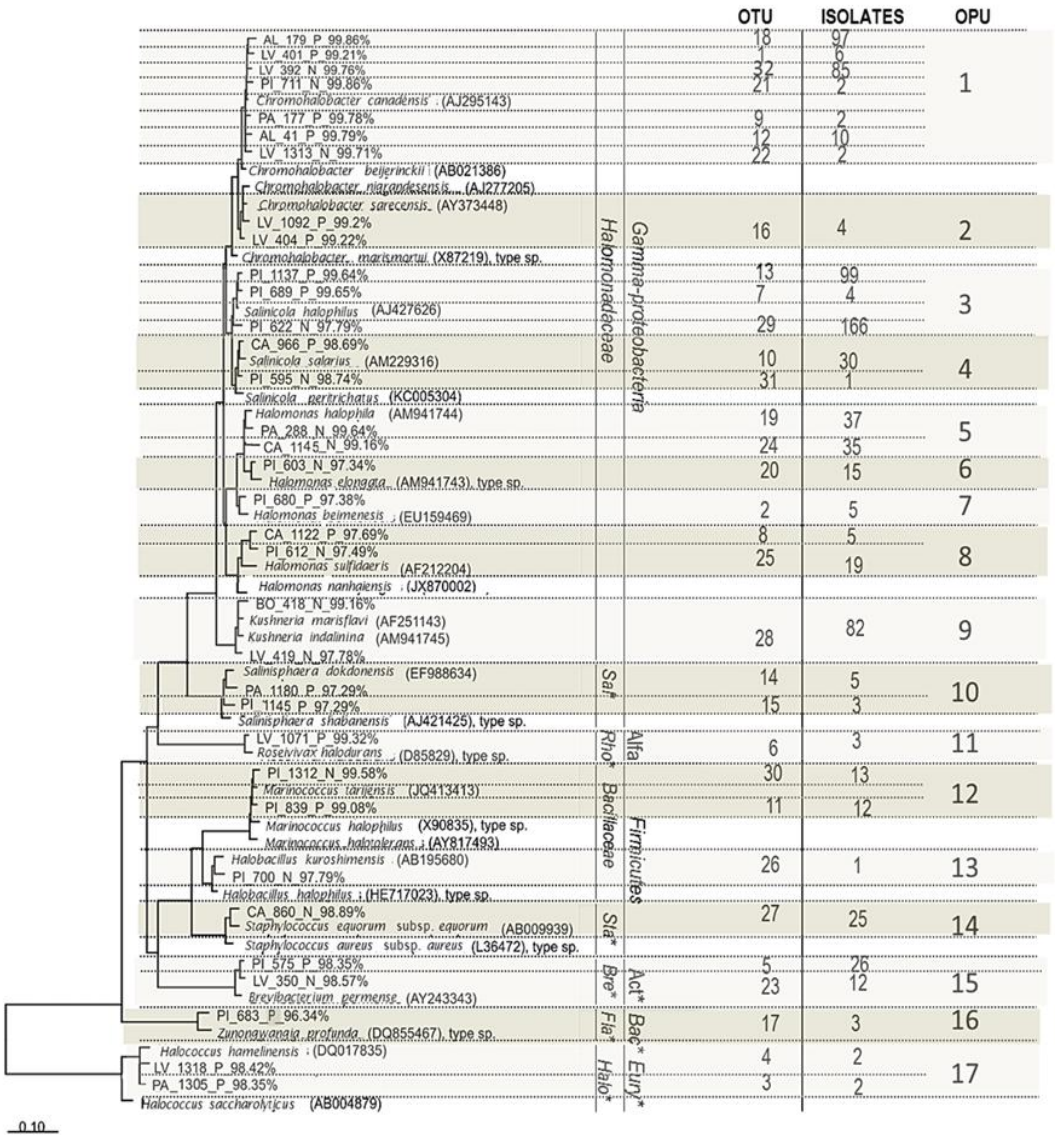

Figure 1.3 16S rRNA phylogenetic reconstruction of representative isolates, and their close relative type strains and additional reference sequences. Sequence names are formed by: origin (PI=Pichidangui, LV=Lo Valdivia, AL=Alicante, CA=Campos and SF=Ses Fontanelle Fontanelles), number of isolate, origin (N=endophyte and P=epiphyte) and identity percentage with the closest type strain. Columns indicate (from left to right): family (*Sal=*Salinisphaeraceae*, *Rho=*Rhodobacteraceae*, *Sta=*Staphylococcaceae*, *Bre=*Brevibacteriaceae*, *Fla=*Flavobacteriaceae* and *Halo=*Halobacteriaceae*); phylum; (*Alfa=*Alphaproteobacteria*)*Act=Actinobacteria, *Bac=*Bacteroidetes* and *Eury=*Euryarchaeota*); OTU number in the dendrograms; number isolates; number of OPU.

Table 1.1 Most relevant OPUs summing abundances above 91.8% of the total pyrosequencing data. The affiliation of the sequences and isolates is given with the closest relative and the identity value. (*id = identity). OPUs marked in bold include those more abundan abundant in all locations and those marked in grey are exclusively epiphytic (dark grey) or endophytic (light grey).

OPU / affiliation	# accession	% id*	ENDOPHYTES										EPIPHYTES				
			PYROSEQS %					ISOLATES %									
			PI	LV	AL	CA	SF	PI	LV	AL	CA	SF	PI	LV	AL	CA	SF
1 Chromohalobacter canadensis	**(AJ295143)**	**>98%**	**1.2**	**0.4**	**50.6**	**33.1**	**37.0**	**22.4**	**17.5**	**15.3**	**0.0**	**26.5**	**50.5**	**37.7**	**9.6**	**15.4**	**38.1**

2 *Chromohalobacter sarecensis*	(AY373448)	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
3 Salinicola halophilus	**(AJ427626)**	**>99%**	**0.3**	**2.8**	**0.7**	**1.0**	**0.6**	**32.2**	**43.9**	**63.6**	**0.0**	**16.7**	**30.9**	**21.1**	**80.8**	**0.0**	**16.7**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
4 Salinicola salarius	**(AM299316)**	**>97%**	**0.1**	**0.1**	**0.1**	**0.2**	**0.0**	**0.7**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**9.7**	**5.8**	**53.8**	**19.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
5 Halomonas halophila	**(AM941744)**	**>96%**	**0.6**	**0.8**	**20.0**	**7.8**	**10.0**	**5.9**	**0.0**	**0.0**	**37.9**	**37.3**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
6 *Halomonas meridiana*	(AJ306891)	>96%	0.1	38.1	0.1	2.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7 *Halomonas elongata*	(FN869568)	-	0.0	0.0	0.0	0.0	0.0	9.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8 *Halomonas beimenensis*	(EU159169)	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
9 Halomonas sulfidaeris	(AF212204)	>98%	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.8	22.7	0.0	0.0	0.9	0.0	30.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10 Kushneria indalinina / Kushneria marisflavi	**(AM941745) (AF251143)**	**>94%**	**0.1**	**38.4**	**0.3**	**1.0**	**0.2**	**24.3**	**17.5**	**19.5**	**1.5**	**10.8**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
11 *Salinisphaera dokdonensis*	(EF988634)	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	0.0	11.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12 *Pseudomonas seleniipraecipitans*	(FJ22810)	>95%	2.1	12.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13 *Pseudomonas alcaliphila*	(AB030583)	>96%	91.7	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14 *Pseudomonas pseudoalcaligenes*	(Z76666)	>95%	0.0	0.0	0.0	0.2	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15 *Marinobacter daepoensis*	(AY517633)	>94%	0.04	0.03	0.1	0.2	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
16 *Rudaea cellulosilytica*	(EU741687)	>95%	0.4	0.5	6.5	18.4	13.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
17 *Chelonobacter oris*	(EU331064)	>94%	0.4	0.8	5.0	8.4	2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18 *Nevskia terrae*	(GQ845011)	>98%	0.0	0.0	0.2	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
19 *Curvibacter lanceolatus*	(AB021390)	>96%	0.0	0.1	0.1	0.7	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20 *Burkholderia rhynchosiae*	(EU219865)	>97%	0.0	0.3	0.1	2.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
21 *Cupriavidus gilardii*	(AF076645)	>98%	0.1	0.4	1.5	3.7	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22 *Nautilia lithotrophica*	(AJ404370)	>80%	0.2	0.0	1.0	1.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
23 *Roseivivax halodurans*	(D85829)	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24 *Rhodobiaceae*	-	>84%	0.0	0.2	0.1	0.1	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
25 *Methylobacterium platani*	(EF426729)	>93%	0.0	0.0	0.4	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
26 *Blastochloris viridis*	(AF084495)	>94%	0.2	0.0	0.1	0.1	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
27 *Gluconacetobacter liquefaciens*	(X75617)	>94%	0.1	0.9	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
28 *Rhizobium tarimense*	(HM371420)	>98%	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
29 *Sphingomonas oligophenolica*	(AB018439)	>98%	0.04	0.6	0.3	4.7	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
30 *Marinococcus halotolerans*	(AY817493)	>99%	0.1	0.03	0.9	1.2	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
31 *Marinococcus tarijensis*	(JQ413413)	>99%	0.2	0.03	2.8	0.2	0.1	2.0	0.0	0.8	0.0	8.8	4.1	1.8	3.8	0.0	9.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
32 *Halobacillus kuroshimensis*	(AB195680)	-	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
33 *Alkalibacillus salilacus*	(AY671976)	>85%	0.2	0.8	3.4	1.4	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
34 *Staphylococcus spp.*	-	>96%	0.0	0.1	0.0	1.6	1.0	0.0	0.0	0.0	37.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
35 *Brevibacterium permense*	(AY243343)	>98%	0.2	0.3	1.1	1.4	1.0	0.0	21.1	0.0	0.0	0.0	1.0	21.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
36 *Zunongwangia spp.*	-	>96%	0.2	0.4	0.1	1.2	0.4	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
37 *Chitinophaga jiangningensis*	(KF150362)	>89%	0.0	0.0	0.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
38 *Halococcus hamelinensis*	(DQ017833)	-	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.9	0.0	0.0	4.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
TOTAL			**98.8**	**98.7**	**95.9**	**91.8**	**94.1**	**98.1**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

Comparison between culturable epiphytic and endophytic isolates in different exemplars

Among the 17 OPUs that configured the complete set of phylogenetic clades detected in culturable fraction, OPUs 1 (*C. canadensis*) and 3 (*S. halophilus*) were common to all samples and appeared also in both epiphytic and endophytic fractions, with exception of CA samples in where OPU 1 was present only in the epiphytic fraction. In addition, the samples showed other common microbiota as the OPU 31 (*Marinococcus tarijensis*), common in PI, AL and SF; OPU 4 (*Salinicola salarius*) common to all epiphytic communities, but only present as endophytic in PI; OPU 9 (*Halomonas sulfidaeris*) common as epiphytic in CA and LV, and endophytic CA, AL and PI; and OPU 35 (*Brevibacterium permense*) only present in the Chilean samples PI (only epiphytic) and LV. The remaining OPUs detected were either exclusive of the epiphytic fraction, as OPUs 2 (*Chromohalobacter sarecensis*, exclusive of LV), 8 (*Halomonas beimenensis*, exclusive of PI), 11 (*Salinisphaera dokdonensis*, present in SF and PI), 23 (*Roseivivax halodurans*, exclusive of LV), 36 (*Zunonwangia profunda*, exclusive of PI) and 38 (*Halococcus hamelinensis*, present in PI, LV and SF); or exclusive of the endophytic microbiota, as OPUs 5 (*Halomonas halophila*, present in PI and SF), 7 (*Halomonas elongata*, exclusive of PI), 32 (*Halobacillus kuroshimensis*, exclusive of PI) and 34 (*Staphylococcus equorum*, exclusive of CA). It was remarkable that OPU 10 (*Kushneria marisflavi – Kushneria indalinina*) was exclusively endophytic and common to all samples (Table 1.1).

The most relevant OPUs given the high amounts of strains isolated were OPU 1 (*C. canadensis*) with 36.2% of the epiphytic and 17.9% of the endophytic fractions; and OPU 3 (*S. halophilus*) with 32.4% and 33.5% of the respective fractions. Both were the more highly retrieved of the complete dataset making 25.1% and 33.1% of the total isolates. From the epiphytes, and after *C. canadensis* and *S. halophilus*, the more highly retrieved groups were OPU 4 (*S. salarius*) with 9.4% and OPU 35 (*B. permense*) with 8.2% (Table 1.1). The most important and exclusive endophytic OPU present in all samples was OPU 10 that affiliating with *K. marisflavi – K. indalinina* with 16.7% of the endophytes (Table 1.1).

The comparison of abundance and distribution of OPUs among all sites and compartments, visualized by NMDS analysis (Figure 1.4), revealed with a stress value of $7.9 \cdot 10^{-2}$ that the two distinct compartments (endophytic vs epiphytic) were remarkably different even in the same plant. However, the same compartments in different plants shared a common culturable microbiota with independence of their origin (and plant species for LV). For both compartments, CA samples always exhibited the most different culturable microbiota. With respect to diversity indexes, the richness estimator index (Chao 1 in Table 1.2) ranged from 3 to 10.5 among all samples, and in general was higher in the Chilean samples (PI and LV) than those of the Mediterranean area (AL, CA and, SF). On the other hand, rarefaction plots showed similar saturation trends (Figure A 1.3). The diversity measure (*H*' index) showed to be higher

in the Chilean and SF samples; PI, AL and CA showed higher values for endophytes than epiphytes, and SF and LV presented an opposite trend. Evenness (*E*) showed the lowest values for PI, and the highest values were observed for CA (0.9) and SF (0.8) in epiphytic fraction, and LV (0.9) and SF (0.9) for the endophytes. Dominance (*D*) presented a maximum of 0.7 in AL (P), and the rest exhibited values under 0.5 (Table 1.2).

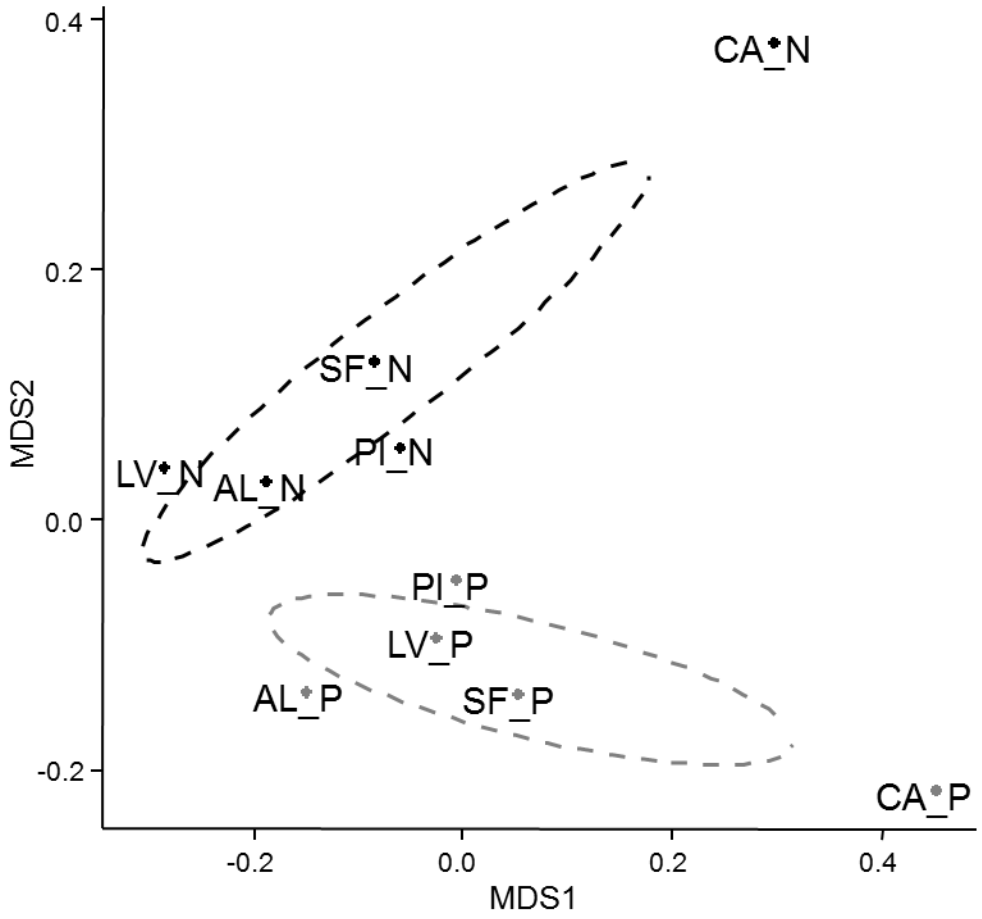

Figure 1.4 Two dimensional Non-metric multidimensional scaling (NMDS) of epiphytes (P) and endophytes (N) in all locations. Standardization by Wisconsin, euclidean distance and stress value of 0.0786.

Table 1.2 Comparison of the Operational Phylogenetic Units (OPUs) diversity (H'), richness (Chao 1), evenness (E) and dominance (D) indexes of the profiles obtained from MALDI-TOF analysis and sequences from pyrosequencing.

	Col/seq*	OTUs	OPUs	*H´*	Chao 1	*E*	*D*
Culturable data							
Epiphytes							
PI	95	12	8	1.30	10.5	0.40	0.37
LV	116	10	9	1.66	10	0.58	0.23
AL	52	5	4	0.69	4	0.50	0.66
CA	13	4	3	0.98	3	0.89	0.40
SF	42	8	6	1.60	6	0.83	0.23
Endophytes							
PI	152	11	10	1.54	10	0.58	0.23
LV	57	5	4	1.30	4	0.92	0.30
AL	118	5	5	0.97	6	0.78	0.47
CA	66	4	4	1.14	4	0.77	0.39
SF	102	6	5	1.47	5	0.87	0.26
Pyrosequencing data							
PI	2,334	57	30	0.52	53	0.04	0.84
LV	3,145	132	35	1.64	55	0.12	0.29
AL	1,747	106	44	1.85	56.4	0.12	0.31
CA	1,097	102	55	2.53	63.6	0.21	0.16
SF	1,262	94	56	2.53	68.5	0.22	0.17

*Colonies for culturable data and sequences for pyrosequencing data.

RAPD fingerprinting analysis

In order to understand whether isolates showed clonality, we performed RAPD analyses. RAPD analyses were performed at the OTU level as one OPU could harbor more than one OTU (*e.g.* OPU 3 formed by OTUs 7, 13 and 29). The profiles exhibited diverse patterns ranging from 2 to 16 bands with product lengths ranging from 250 to 4000 bp (data not shown). In all cases, each OTU showed its own profile composition, and in no case the same pattern was shared by two different OTUs. In addition, all strains of the same OTU belonging to different samples or compartments never shared an identical profile. Clonality was only observed among the strains of the same OTU and isolated in the same location. We found OTUs with monoclonal subpopulations (*e.g.* OTU 6 – *R. halodurans* and OTU 24 – *H. elongata)* or high percentage of clonality (*e. g*. OTUs 13 and 29 – *S. halophilus* for PI; Table A 1.5). However, in most cases (60%) OTUs were formed by subpopulations with no clonality (Table A 1.5).

Direct pyrosequencing, OTU affiliation and OPU distribution

For endophytic microbiota, we performed a culture-free analysis of this fraction to be compared with the cultured diversity. In order to enrich with the prokaryotic endophyte fraction we modified and improved a protocol of differential centrifugations using density gradients with sucrose (as detailed in the Materials and methods section). Pyrosequencing analysis of the amplified 16S rRNA gene generated a total of 14,189 sequences ranging between 1,472 (SF)

and 5,659 (LV). The mean of the length of the sequences was 540 bp as we removed all fragments with size <300 bp. After trimming the sequence set was reduced to ~76% of the total (Table 1.3). Sequences affiliating with mitochondria (<5%) and chloroplasts (<0.2%) were removed. A total of 491 OTUs (unique clusters of sequences embraced by a minimum threshold of 99% identity, Table 1.3) were obtained, and for each location the number ranged from a minimum of 57 (PI) and a maximum of 132 (LV). After the phylogenetic reconstruction, the diversity of pyrotags was reduced to 103 OPUs (*i.e.* unique phylogenetic clades affiliating the new sequences with their references), including 28 singletons and 14 doubletons (Table A 1.4). Among them, 31 OPUs gathered > 90% of the sequence diversity, whereas the remaining 72 just generated values < 0.8% in their corresponding sample (Table 1.1 and Table A 1.4). Most of the OPUs affiliated with *Proteobacteria* (64 OPUs comprising 91.7% of the total sequences), and among them 32 affiliated with *Gammaproteobacteria* (84.3%), 19 with *Alphaproteobacteria* (3.7%), 7 with *Betaproteobacteria* (2.8%), 5 with *Deltaproteobacteria* (0.2%) and 1 with *Epsilonproteobacteria* (0.7%). *Bacteroidetes* were represented by 7 OPUs (1.0%), *Acidobacteria* (0.2%) by 3 OPUs, *Chlamydiae* (0.01%) by 1 OPU, *Deinococcus-Thermus* (0.1%) by 1 OPU, *Firmicutes* (5.7%) by 18 OPUs, *Armatimonadetes* (0.3%) by 2 OPUs and *Actinobacteria* (1.0%) by 7 OPUs Table 1.1 and Table A 1.4).

The most representative OPUs (Table 1.1) due to their abundances and common occurrences were *Alkalibacillus salilacus* (OPU 33), *C. canadensis* (OPU 1), *Halomonas* spp. (OPUs 5, 6 and 7) and *Kushneria* spp. (OPU 10). The first one just detected by pyrosequencing, and the later three also present in the culture collection. Finally, two OPUs affiliating with *Pseudomonas* (OPUs 12 and 13) were present in high percentages in the Chilean, but nearly absent in the Mediterranean samples. Among 103 OPUs detected, only 19 (OPUs 1, 3, 5, 6, 10, 15, 16, 17, 21, 29, 30, 31, 33, 35, 36, 81, 95, 99 and 103) were detected in all locations, and occurred with a remarkably high number of sequences making up to 70.3% of total pyrosequencing sequences, and with a mean of 70.3% ± 37.1% per location (Table 1.1 and Table A 1.4). In fact, no distribution pattern (Figure A 1.4) seemed to be associated with the microbial community in relation with the location. Richness estimator index (Chao 1 in Table 1.2) ranged from 53 (PI) to 68.5 (SF), and rarefaction curves showed to be close to saturation in all but SF, which seemed to harbor higher diversity (Figure A 1.3). Accordingly, the diversity (*H'*) index and evenness (*E*) showed to be higher in SF (2.53 and 0.22) and lower in PI (0.52 and 0.04) and LV (1.64 and 0.12). Dominance (*D*) presented an inverse behavior in comparison with *H'* and *E* indexes (Table 1.2).

Table 1.3 Sequence distribution obtained by pyrosequencing including chloroplastidial and mitochondrial sequences detected (PI=Pichidangui, LV=Lo Valdivia, AL=Alicante, CA=Campos and SF=Ses Fontanelles).

	Total	Removed	% Removed	Chloropl.	Mitoch.	FINAL	OTUs	OPUs
PI	3,085	751	24.4	0	85 (5.48%)	2,334	57	30
LV	5,659	2,514	44.4	4 (0.12%)	197 (6.05%)	3,145	132	35
AL	2,035	335	16.5	5 (0.16%)	180 (5.90%)	1,747	106	44
CA	1,938	447	23.1	2 (0.07%)	129 (4.60%)	1,097	102	55
SF	1,472	210	14.2	0	53 (5.13%)	1,262	94	56
Total	14,189	4,257	-	11	644	9,585	491	104
Mean	2,837.8	851.4	24.5	2.2	128.8	1,917	98.2	44.8
SD	1,683.7	950.8	11.9	2.3	1.5	838.5	27.1	11.4

Comparison between culturable and pyrosequencing endophytic data

Endophytic microbiota could be considered as authentically autochthonous and not incidentally occurring. In this regard, among the 11 OPUs that constituted the endophytic cultivable fraction (Figure 1.3), 9 were detected by pyrosequencing (Table 1.1), only the rare OPUs 32 (singleton, affiliating with *H. kuroshimensis*) and 7 (affiliating with *H. elongata*) were not detected by the pyrotagging approach. Almost all sequences of cultured OPUs matched with high identity (above 97%) their closest relatives (at genus level) obtained by pyrosequencing. Both pyrosequencing and culture data coincided with the principal OPUs, which affiliated with *Proteobacteria* and gave similar abundances (91.7%-88.2%; pyrosequencing-culture). Also, *Firmicutes* (5.7%-6.3%; pyrosequencing-culture) and *Actinobacteria* (1.0%-4.7%; pyrosequencing-culture), showed similar occurrences (Table 1.1 and Figure 1.3). About 45.0% of the pyrosequences corresponded to the cultured taxa: *C. canadensis* (24.5%), *S. halophilus* (1.1%), *S. salarius* (0.09%), *Halomonas* spp. (7.9%), *Kushneria* spp. (8.0%), *Staphylococus* spp. (1.6%), *Marinococcus* spp. (0.8%) and *B. permense* (1.1%; Table 1.1). The PCA based on OPUs (Figure A 1.4) did not show any association between pattern and origin of the sample in both culturable and pyrosequencing fractions. In both cases, CA seemed to be slightly different in relation with other samples.

Discussion

To our knowledge, this is the first report of halophilic microorganisms inhabiting the endophyllosphere of halophytes combining culture dependent and independent approaches. Contrary to other studies that report values of 10^2 to 10^5 $CFUg^{-1}$ (Lindow and Brandl 2003; Al-Mailem *et al.* 2010; Finkel *et al.* 2011) for bulk endo- and epiphytes, and specifically 10^4 to 10^5 $CFUs\ g^{-1}$ for epiphytes (Ercolani 1991) and 10^5 $CFUg^{-1}$ for endophytes (Emiliani *et al.* 2014), we could retrieve up to two orders of magnitude higher culturable yields (10^2 to 10^6 $CFUg^{-1}$ for epiphytes and from 10^4 to 10^7 for endophytes). Conspicuously, our data resembles more the abundances obtained in rhizosphere, where ranges from 10^4 to 10^{10} $CFUg^{-1}$ have been reported for soybean (Kuklinsky-Sobral *et al.* 2004) or *Salicornia* (Mapelli *et al.* 2013). Our results

suggest that the halophytes studied here are mainly colonized by moderate halophiles (5-20% salt; 95% of total counts), and in less extent halotolerant (2-5% salt; 3.1% of total counts) or extreme halophiles (20-30% salt; 1.9% of total counts; Table A 1.6) in accordance with what has been reported by Mapelli and cols. for the *Salicornia* rhizosphere (Mapelli *et al.* 2013).

Culturable and uncultured diversity measured by means of sequence identities showed high consistency, and the paradigm considering that culture-based techniques recover only 1% or less of the true prokaryotic diversity (Amann, Ludwig and Schleifer 1995) fails. We could recover in culture a maximum of 62.7% (LV sample) of the pyrosequenced diversity. In addition, from the 38 most relevant OPUs (making > 90% of the diversity), 11 of them were recovered as endophytic culturable microbiota. Only two cultured taxa (OPU 7 and OPU 32 affiliating with *H. elongata* and *H. kuroshimensis* respectively) were not detected by pyrosequencing, thus perhaps being part of the rare biosphere (Pedrós-Alió 2006). The success in mirroring the most abundant taxa detection for the cultivable and culture-independent fractions was also reinforced by the fact that >95% of the pyrotagging sequences affiliated at the genus level (>94.5% similarity between sequences; Yarza *et al.* 2014) with known taxa. Our results contrast with other culture-independent studies in where a significant percentage of sequences remained unclassified (Vaz-Moreira *et al.* 2011; Bodenhausen, Horton and Bergelson 2013). Our success may be related to the fact that most of the microbiota thriving in the phylloplane is readily cultivable (Jackson *et al.* 2013; Ruppel, Franken and Witzel 2013), but also because of our identification approach relies on phylogenetic inference (by means of the OPU approach; França *et al.* 2014) and not just on sequence identity clustering (classification by means of OTU clustering and blast matches of their representatives) as commonly used (Bodenhausen, Horton and Bergelson 2013; Jackson *et al.* 2013; Emiliani *et al.* 2014).

Proteobacteria, *Firmicutes* and *Actinobacteria* were the most abundant bacterial phyla associated to the plants studied here, which seem to be common in foliar and root areas (Rastogi *et al.* 2012; Bodenhausen, Horton and Bergelson 2013). The principal OPUs in this study (both determined by culture and pyrosequencing) had been previously observed associated with other plants (*e.g. Cupriavidus* genus; da Silva *et al.* 2012), including halophyte species (Ruppel, Franken and Witzel 2013). Some of the genera detected here had been observed as associated with the rhizosphere of halophytes as *Brevibacterium, Chromohalobacter, Halobacillus, Halomonas, Kushneria, Marinococcus, Zungonwangia* (Shiba *et al.* 1991; Sgroy *et al.* 2009; Al-Mailem *et al.* 2010; Gontia *et al.* 2011; Mapelli *et al.* 2013). We just detected one archaeal species, which always occurred as epiphytic and affiliated with *Hcc. hamelinensis*. Similarly, *Halococcus* spp. was reported to thrive in the rhizosphere of *Halonemum strobilaceum* (Al-Mailem *et al.* 2010). On the other hand, species as *A. salilacus* (Jeon *et al.* 2005), *R. celullosilytica* (Weon *et al.* 2009) and *S. dokdonensis* (Bae *et al.* 2010) have not been previously reported in association with plants, but have been isolated from hypersaline environments.

Despite the plants originated in very different geographical locations, we observed a common trend among the same compartments of the different plants as an indication that there might be a host specificity for the moderately halophiles detected here. Contrarily to what has been observed for the phyllosphere of *Tamarix* (Finkel *et al.* 2011), we could find shared species as major microbiota between plants of different origins. However, we never found identical clonal varieties of the same species shared by two different plants. RADP fingerprinting showed a very low degree of clonality, and when clonality was observed (just in 14 OTUs), this occurred always in the same plant. Different clonal varieties may be assumed to be different ecotypes that despite colonize the same environment, may exhibit distinct phenotypes with distinct ecologic advantages (Antón *et al.* 2013). On the other hand, we found clear differences between both compartments in the same plant, which may respond to the different environmental and nutritional conditions between the plant surface and inside the vegetal. While the external part of the plant is subjected to pressures such as pluvial precipitation, solar radiation and wind; internal tissues are relatively stable environments. Therefore, epibionts need strategies to prevail on plant surface, as the formation of mats (Morris and Soule 2005) or the production of biologically active compounds and generation of microcolonies (Rao, Webb and Kjelleberg 2005).

Finally, halotolerant and halophylic microorganisms might be an important resource helping in the crop production system in where soils or irrigation waters contain high salt concentrations [56]. In fact, members of *Chromohalobacter*, *Halomonas*, *Halobacillus* and *Kushneria* species have been reported with plant growth promoting activity under salt stress (until 320 mM NaCl; Mapelli *et al.* 2013; Ramadoss *et al.* 2013; Desale *et al.* 2014), and have been expected to be useful in microbial assisted phytoremediation of saline soils (Newman and Reynolds 2005). Consequently, other microorganisms (i. e. *Salinicola*, *Pseudomonas*, *Roseivivax* and *Marinococcus*) isolated in this study could also have positive effects and their relevance in the development of the plant may be addressed in the future.

Chapter 2. Endophytic microbial diversity of the halophyte *Arthrocnemum macrostachyum* across plant compartments

Abstract

In the present study, the microbial community structures of the endosphere of the halophyte *Arthrocnemum macrostachyum* were evaluated from two locations in Mallorca, Spain, focusing on three plant compartments (roots, green and red stems) compared to the rhizospheric soil where the plants grew. The physicochemical parameters of the rhizospheric soils differed between locations, and the soils were characterized by different microbial community structures. Accordingly, the endophytic community composition, mainly composed of putatively halophilic organisms, was highly influenced by the rhizospheric soil microbiota, as revealed by the co-occurrence of the major endophytic taxa in the endosphere and the rizospheric soils. Moreover, the reduction of diversity from the endorhizosphere towards the red leaves may support the fact that part of colonization of the plant by bacteria could have an origin in the rhizospheric soils through the roots and subsequent migration to the aerial parts of the plant. Finally, there were certain relevant ubiquitous taxa, such as *Chromohalobacter canadensis, Rudaea cellulosilytica* (never reported before as endophytic), *Psychrobacter* sp., *Bradyrhizobium* sp. and *Halomonas* sp., that, due their moderate halophilic nature, seemed to find an optimal environment inside the plants. Some of these relevant endophytes were not always detectable in their respective soils, and were probably part of the soils' rare biosphere, which would gain preponderance in a favorable endophytic environment.

Introduction

The microbial communities associated with the internal tissues of plants (endosphere) are classified depending the kind of interaction established as pathogens or endophytes (Newman and Reynolds 2005a; Mapelli *et al.* 2013). Briefly, endophytes are microorganisms which produce no apparent damage to the plant and their role can be reflected positively as contribution of nutrients, protection against pathogens and plant growth promoting activity (PGPA) (Rosenblueth and Martínez-Romero 2006; Hardoim, van Overbeek and Elsas 2008; Andreote, Azevedo and Araújo 2009; Mercado-Blanco and Lugtenberg 2014). Nevertheless, the internal tissues of the plant can be an hostile environment (Mercado-Blanco and Lugtenberg 2014), and therefore, specific molecular communication is necessary between microorganisms and plants for the colonization process (Schikora, Schenk and Hartmann 2016). There have been some evidence of this colonization processes in different structures of the plant (Lugtenberg, Chin-A-Woeng and Bloemberg 2002; Seghers *et al.* 2004; Rosenblueth and Martínez-Romero 2006; Li *et al.* 2008; Ryan *et al.* 2008), but in general the rhizosphere has attracted most of the research (Hurek *et al.* 2002; Vessey 2003; Lugtenberg and Kamilova 2009; Segura *et al.* 2009; Bringel and Couée 2015). After the colonization processes, the density of the endophytic community is determined by different factors being the most important the availability of nutrients for the microorganisms, the genotype and phase of development of the plant, the environmental conditions (Kuklinsky-Sobral *et al.* 2005; Andreote, Azevedo and Araújo 2009) and, the presence of antibacterial substances such as terpenoids, benzoxazines, flavonoids and isoflavonoids (Hardoim, van Overbeek and Elsas 2008).

As mentioned, most studies of endophytes and their relevance have been focused on rhizobacteria of commercial plants and specifically, their capability to undergo nitrogen fixation (Hurek *et al.* 2002), phosphate solubilization, increment of mineral and nutrient availability, production of indoleacetic acid (IAA) and acetyl-CoA carboxylase (ACC) (Germida *et al.* 1988; Zinniel *et al.* 2002; Seghers *et al.* 2004; Kuklinsky-Sobral *et al.* 2005; Ryan *et al.* 2008; Doty *et al.* 2009; Manter *et al.* 2010; Weber, Videira and Simões de Araujo 2013; Amaresan, Jayakumar and Thajuddin 2014). However, the topic has recently broaden including other plants such as metal-accumulating (Belimov *et al.* 2005) or halophytes (plants which can survive in saline soils) (Sgroy *et al.* 2009; Glick and Glick 2012; Ruppel, Franken and Witzel 2013; Mora-Ruiz *et al.* 2015, 2016). Endophytes of halophytes have been considered an interesting alternative as biofertilizers of plants under stress conditions (salt stress and drought) (Ruppel, Franken and Witzel 2013a; Mercado-Blanco and Lugtenberg 2014). In fact, in front of the current world-wide high demand for food produced principally due to the increment of human population and the constant awareness of environmental damage and protection, new agricultural practices have

been necessary towards a more sustainable and environmentally friendly agriculture (Glick and Glick 2012).

The study of endophytic communities has been principally addressed using molecular techniques with culture-independent approaches, and in some cases including just few isolates (Andreote, Azevedo and Araújo 2009; Manter *et al.* 2010; Weber, Videira and Simões de Araujo 2013). Although NGS (Next Generation Sequencing) techniques allow exploring the richness associated to plants, it also limits the study of the colonizing microbial cells in detail (Zengler *et al.* 2002). Currently, the large scale cultivation combined with mass spectrometry techniques as the Whole-Cell Matrix Assisted Laser Desorption Ionization – Time Of Flight Mass Spectrometry (WC MALDI-TOF MS) has opened the possibility to a better understanding of complex microbial ecosystems (Viver et al., 2015). WC MALDI-TOF MS identification has been recommended for large scale studies because it is efficient, fast and cost-effective in comparison with other identification tools such as 16S rRNA sequencing (Munoz *et al.* 2011; Mora-Ruiz *et al.* 2015).

In our previous studies, we explored the endophytic diversity of *Arthrocnemum macrostachyum* using both 454 amplicon pyrosequencing and culturing of moderately halophilic microorganisms (Mora-Ruiz *et al.* 2015, 2016). The results pointed as the most relevant taxa *Chromohalobacter canadensis, Salinicola halophilus, Kushneria indalinina* and *Rudaea cellulosilytica* (Mora-Ruiz *et al.*, 2015, 2016). In those studies, we recovered by culture an important fraction of the richness observed by massive sequencing (~60%), but other abundant groups (*e.g. Halomonas meridiana, Pseudomonas seleniipraecipitans, Pseudomonas alcaliphila, R. cellulosilytica* and *Cupriavius gilardii*) were not cultured in part because we used hypersaline culture media (from 5 to 30%). Therefore, in order to reveal whether the remaining uncultured ~40% could be also mesophilic, we focused on the recovering of the mesophilic and halotolerant culturable microorganisms inhabiting *A. macrostachyum*. Also, we selected some halophilic and mesophilic isolates to analyze their potential PGPA on *A. thaliana*.

Materials and methods

Plant sample collection and surface sterilization

Six halophytes, identified as A. macrostachyum, were collected from Campos (39º21’03.3’’ N, 3º00’44.3’’ E, Spain) and Albufera d’Alcúdia (39º47’49’’ N, 3º6’24’’ E, Spain) in April 2014. Both locations are in Mallorca and are separated by a distance of approximately 60 km. Three plants were chosen from each location and all plants exhibited green and red (mature) stems (Figure A 2.2). Individual plants were collected whole and stored in zip-lock plastic bags using sterile gloves for immediate processing on arrival at the laboratory. Green and red fractions, and

roots were excised from each halophyte, obtaining three samples in triplicate for each location. Excision areas were first hot cauterized using an incandescent metal loop to avoid the loss of sap.

Approximately 40 g of each section of plant were selected (fragments of 7-10 cm). Once the plant material had been excised and removed, it was sterilized and macerated. Plant material surfaces were washed with the following sequential steps: initial incubation for 5 min in 0.2% Triton X-100 with sterile distilled water, followed by 5 min in sterile distilled water alone, 5 min in 2% bleach (NaClO), and finally 5 min in sterile distilled water. The root samples were rinsed six times with sterile water (5 min each step) in order to completely remove the soil attached to their surfaces. After the last wash, the plant material was dried using sterile paper towels. To disaggregate the plant tissues, 5 to 25 mL of PBS 1X were added to the plant material and the mixture was macerated using a sterile mortar and pestle (Mora-Ruiz et al. 2015).

Separation of the microbial fraction from vegetal debris, and microbial DNA extraction

Fifteen mL of macerated biomass were subjected to differential centrifugation and sucrose density gradient centrifugation, as previously reported (Mora-Ruiz et al. 2015). All layers recovered from the centrifugation gradient were numbered according to their density and stored at -20ºC. The layers with higher density were used for DNA extraction, which was performed as previously reported (Mora-Ruiz et al. 2015).

Soil collection and microbial DNA extraction

For each plant, its respective rhizospheric soil was collected by shaking the roots vigorously in order to separate the loosely adhered soil. The soil tightly adhered to the roots was also collected and the samples were immediately transferred to the laboratory for bacterial DNA extraction following an existing protocol (Nogales et al. 1999).

PCR amplification and 454-pyrosequencing

PCR amplification with the bacterial primers GM3 and S (Table A 2.1) was performed as previously described (Lane et al. 1986). A second PCR was performed using 5 µL of the product as template, in triplicates of 25 µL, using the primers GM3-PS and a variant of 907-PS (from position 8 to 907: V1-V4 regions of 16S rRNA; Table A 2.1) to tag and link the amplicon, with the same annealing temperature. The products were observed in 1% agarose gel electrophoresis run in 1X TAE buffer (at 25 V for 50 min). The band with a size of ~960 bp was excised and eluted using the ZymocleanTM Gel DNA Recovery Kit (Zymo Research,

California, USA) following the manufacturer's instructions. The NanoDropTM Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA) and MassRulerTM Express Forward DNA Ladder Mix (Thermo Scientific, Massachusetts, USA) were used to measure the concentration of the barcoded amplicons. An equimolar mix of the amplicons was sent to Macrogen Inc. (Seoul, Korea) for sequencing using 454 GS-FLX+ Titanium technology. Trimmed sequences have been deposited in the ENA sequence repository under the study accession number SAMEA3928333-SAMEA3928356.

Sequence trimming, OTU grouping, phylogenetic affiliation and OPU design

Sequence data were processed using Mothur (Schloss et al. 2009) software to trim and remove chimeras. Low-quality and short sequences were discarded (<300 pb). Trimming was performed with a window size of 25, and average quality score of 25, and no mismatches or ambiguities between primer pairs and barcodes were allowed. Chimeras were removed using Chimera Uchime (Mothur) and sequences were clustered in OTUs at 99% using the UCLUST tool included in QIIME (Caporaso et al. 2010). The longest read of each OTU was selected as representative and was added to the non-redundant SILVA REF115 database using the ARB software package (Ludwig et al. 2004). Sequences were initially aligned with SINA (Pruesse et al. 2012) and inserted using the parsimony tool (ARB package) in a default tree. Chloroplast and mitochondrial sequences were discarded. The closest relative sequences of adequate quality were selected and merged with the LTPs115 database (Yarza et al. 2010). A phylogenetic reconstruction was performed using the neighbor-joining algorithm and the Jukes-Cantor correction with the selected closest relative sequences and type strain representatives. Finally, the OTU representatives were inserted into the final reconstruction using the parsimony tool, and then clustered in OPUs (França et al. 2014) based on the visual inspection of the final tree. An OPU is the smallest monophyletic group of sequences containing OTU representatives together with the closest reference sequence, including the sequence of a type strain when possible.

Soil sample collection and physicochemical parameters

Six soil samples were collected from locations Campos and Alcúdia where the sampled plants were growing. Physicochemical parameters were analyzed at the Conselleria d'Agricultura, Medi Ambient i Territori (Govern de les Illes Balears, Mallorca, Spain). The analyzed physicochemical parameters were pH, electrical conductivity (at 25ºC), oxidizable organic matter (O.M.), total nitrogen, carbonates (CO_3^{2-}), available phosphorus, and changeable potassium (K^+), sodium (Na^+) and magnesium (Mg^{2+}).

Ecological indices and statistical analysis

Rarefaction curves and equitability-J index were calculated from OPUs using PAST v 3.01 software (Hammer, Harper and Ryan 2001). Jost index (q=1) was computed by a Monte-Carlo re-sampling (1,000 simulations) the OPU matrix to the minimum sequences. Non-parametric Kruskal-Wallis tests were performed in order to compare ecological indices and soil physicochemical parameters in each location and internal fraction, as they did not satisfy assumptions of normality and homogeneity of variances. The multivariate community structure in relation to sampling location, plant compartment and inter-individual variability of OPUs was statistically analyzed using non-metric multidimensional scaling (NMDS; Kruskal and Wish 1978). The goodness of the NMDS was evaluated according to a stress value smaller than 0.263, which is considered acceptable for 18 samples (Sturrock and Rocha 2000). Fitted vectors were represented as arrows pointing in the direction of the most rapid change in the environmental variables (Díaz-Gil et al. 2014; Oksanen et al. 2016). Additionally, a permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) was used to test the statistical significance of the differences between bacterial communities, as well as the environmental parameters, in order to determine their influence on the variability in the microbial communities. Hierarchical cluster analysis was performed using the Sørensen distance (Sørensen 1948) and Ward's linkage (Ward 1963). An indicator species analysis (ISA; De Cáceres and Legendre 2009) was used to identify the indicator taxa responsible for the differences between groups identified with the cluster analyses. The correlation matrixes were obtained by comparing the diversity at the phylum level in the endophytic and rhizospheric community with their respective soil. Analyses were performed with the packages vegan (Oksanen et al. 2016) and indicspecies in R v 3.1.1. (www.r-project.org).

Results

Analysis of 454-pyrosequencing data

This study included plant biomass in triplicate and their respective rhizospheric soil collected in Campos and Alcúdia, both located in Mallorca, Spain. A total of 97,872 sequences were generated that, after trimming and removing short, bad or chimera sequences, resulted in a dataset of 58,517 sequences with a mean of 2,438 ± 1,825 per sample (Table A 2.1). The clustering into OTUs at 99% rendered a total of 5,393 with a mean of 225 ± 296 representatives that after phylogenetic inference were grouped into a total of 657 OPUs (78 ± 70; Table 2.1). From these, 249 OPUs were present in the endosphere of the plants studied, and 408 were exclusive to soils.

Table 2.1 Raw data and sequence distribution in OTUs and OPUs from pyrosequencing. Jost indices in q=0 and q=1 are shown per sample. SD=standard deviation.

	Raw seqs	Removed seqs	Final seqs	OTUs	q=0 (OPUs)	q=1
Endosphere						
ALCÚDIA						
Green	2,656	320	2,336	31	23	14.9
Green	2,304	64	2,240	27	18	10.8
Green	1,704	288	1,416	34	22	8.9
Red	2,120	44	2,076	63	27	50.
Red	1,672	40	1,632	54	29	7.2
Red	1,740	24	1,716	68	27	6.2
Root	2,240	32	2,208	114	56	19.3
Root	1,600	8	1,592	70	39	12.1
Root	1,966	30	1,936	115	57	14.5
CAMPOS						
Green	1,224	15	1,209	31	22	9.4
Green	2,035	34	2,001	269	79	8.1
Green	3,251	2,245	1,006	58	40	14.4
Red	18,679	15,266	3,413	187	72	10.6
Red	14,878	12,330	2,548	175	84	11.0
Red	12,898	5,916	6,982	211	74	4.2
Root	3,027	75	2,952	237	93	16.3
Root	4,993	835	4,158	187	94	19.0
Root	8,659	85	8,574	358	90	10.0
Rizospheric soil						
Alcúdia	1,654	275	1,379	778	218	81.8
Alcúdia	2,506	499	2,007	1,286	300	127.1
Alcúdia	1,358	368	990	683	212	114.1
Campos	2,022	490	1,532	120	82	18.5
Campos	1,429	415	1,014	97	60	34.1
Campos	2,048	448	1,600	140	70	11.7
TOTAL	**98,663**	**40,146**	**58,517**	**5,393**	**657**	
Mean	4,110.96	1,672.75	2,438.21	224.71	78	
SD	4,729.69	3,955.98	1,825.03	295.52	70	

Soil characterization.

Soil physicochemical parameters indicated that the pH was alkaline and nearly the same in both locations (8.28 ± 0.39 and 8.29 ± 0.18; $p>0.05$). In addition, carbonates (%), exchangeable Na^+ and Mg^{2+} (%) displayed similar values ($p>0.05$) in both places. However, the values of salinity (%), exchangeable K^+, oxidizable organic matter (%), total N (%) and available P (%; Figure 2.1 and Figure 2.2) showed differences depending on the location, with salinity and K^+ being higher in C ($p<0.05$), whereas an inverse pattern ($p<0.05$) was observed for the remaining measured parameters (Figure 2.1 and Figure 2.2).

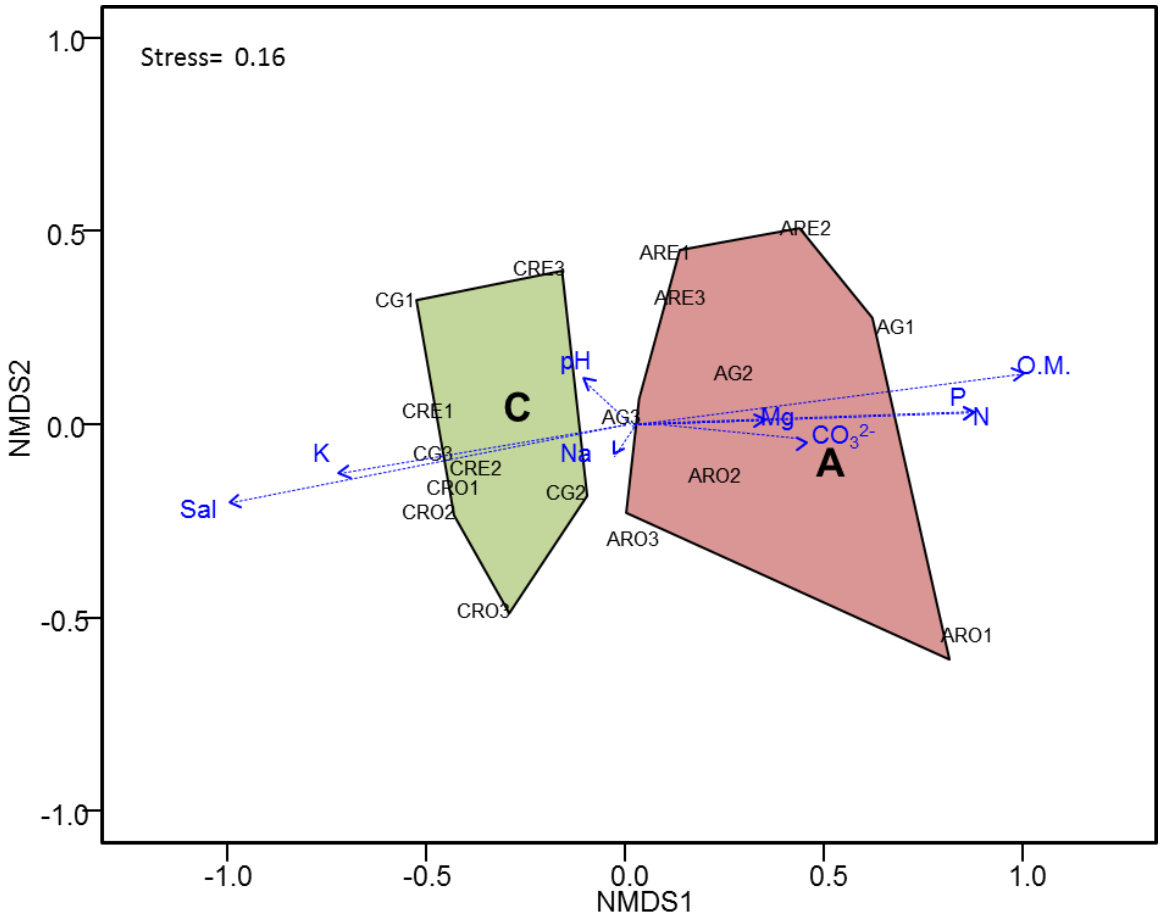

Figure 2.1 Two dimensional Non-metric multidimensional scaling (NMDS) of endophytic bacterial communities associated with *Arthrocnemum macrostachyum*. C= Campos, A= Albufera d'Alcúdia. Dotted lines show the gradient for the soil environmental parameters.

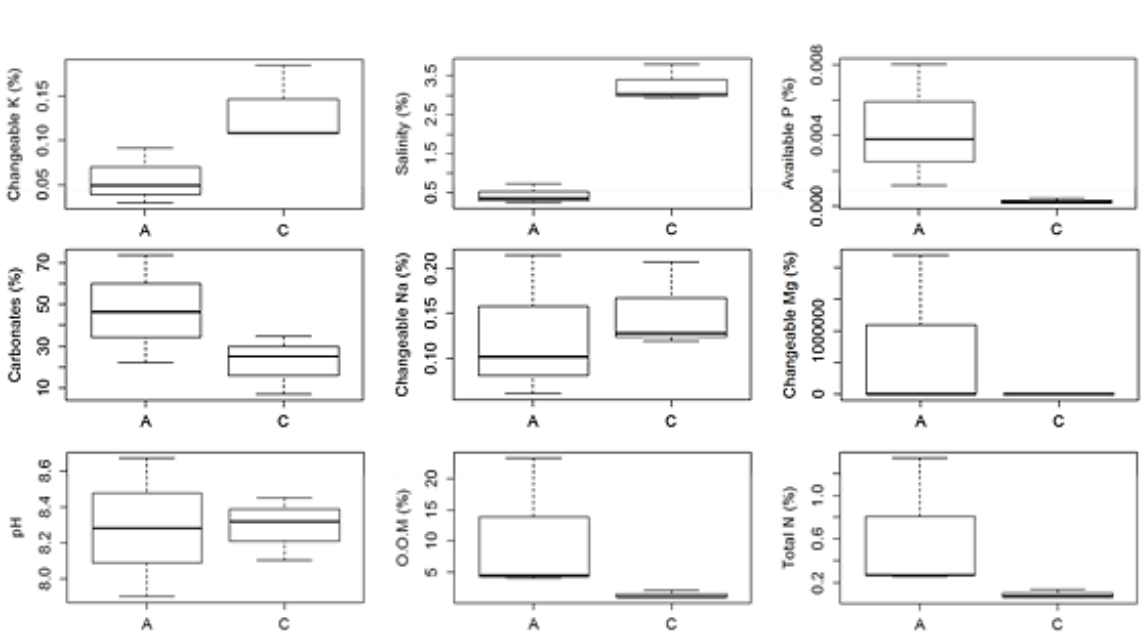

Figure 2.2 Boxplot of analyzed physicochemical parameters in each location. A=Alcúdia and C=Campos.

Analysis of the total bacterial endophytic community structures.

The majority of sequences in the endosphere represented 249 OPUs affiliated with *Proteobacteria* (86.7% of the total sequences integrated in 151 OPUs). The major proteobacterial taxa affiliated with the classes *Alpha-* (48 OPUs = 7.7% sequences) and *Gammaproteobacteria* (74 OPUs = 76.3% sequences). *Firmicutes* (35 OPUs = 8.8% sequences) and *Bacteroidetes* (28 OPUs = 1.8% sequences) followed *Proteobacteria* in abundance. Minor taxa were detected in 11 additional phyla (35 OPUs=2.6% sequences; Table 2.2).

The top most abundant OPUs affiliated with *Proteobacteria*. OPUs 1 (*Rudaea cellulosilytica*), 6 (*Psychrobacter* spp.), 12 (*Chromohalobacter canadensis*), 13 (*Halomonas* spp.) and 109 (*Bradyrhizobium* spp.) were present in all fractions of all plants (Figure 2.3; Table 2.2), and in high sequence numbers. However, a high number of OPUs were exclusively detected in single compartments (green=10, red=26 and root= 79), all of which, except OPU 163 (*Bhargavaea* spp. Figure 2.3; Table 2.2), were detected in very low abundances of less than 0.48%. The results were similar for the beta-diversity analyses where the Campos compartments did not show differences (*F-statistic*=1.034, *p*=0.413) and the Alcúdia compartments showed results very close to the significance level α=0.05 (*F-statistic*=1.78, *p*=0.049; Table A 2.2). In the same way, richness estimations (*p*>0.05; *q=0*) and rarefaction curves (Table 2.1 and Figure

A 2.3) did no exhibited differences by compartment. However, the Jost *q=1* and equitability-J indices were different between compartments, and were always lower in the red fraction (Figure 2.3). On the other hand, when comparing the beta diversity between both locations the differences were significant (*F-statistic*=1.91, $p<0.001$; Table A 2.2) and similar to the richness estimations ($p<0.05$).

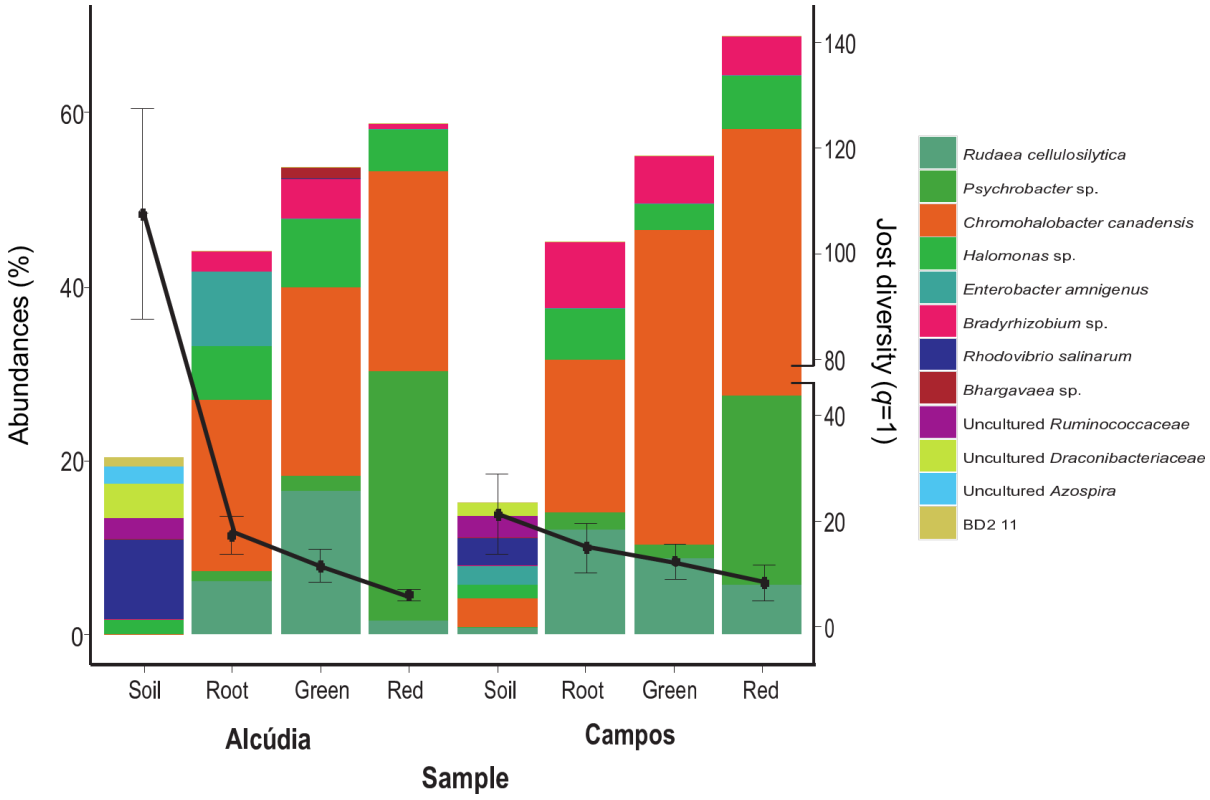

Figure 2.3 Most abundant OPUs in each sample. Rhizospheric soils included (Alcúdia and Campos). Numbers inside the barcharts are the average percentages of sequences detected in each OPU per sample; abundances under 0.5% are not represented. The Y axis and black squares represent the relationship of the values for the Jost diversity index q=1 per group of samples.

Table 2.2 Most relevant OPUs of plants and soil for each sample of the total sequences obtained. First column (from left to right): OPU number; second column: information about the affiliation with the closest relative sequence and the accession number. Other columns contain the percentage of relative abundance of each OPU referenced to the total sequences for each kind of sample.

# OPU	Affiliation/Accession number	CAMPOS								ALCÚDIA							
		GREEN		RED		ROOT		SOIL		GREEN		RED		ROOT		SOIL	
		Mean	Dev.	Mean	Dev.	Mean	Dev.	Mean	Dev.	Mean	Dev.	Mean	Dev.	Mean	Dev.	Mean	Dev.
1	*Xanthomonadaceae, Rudaea cellulosilytica* (EU741687)	8.74	3.94	5.69	2.36	12.14	7.16	0.85	0.74	16.36	1.96	1.54	1.12	6.26	5.37	0.00	0.00
6	*Moraxellaceae, Psychrobacter* sp. (AY513646/ AJ748266)	1.52	1.36	21.73	7.54	1.85	3.10	0.04	0.08	1.79	0.94	28.62	3.81	0.90	0.96	0.00	0.00
12	*Halomonadaceae, Chromohalobacter canadensis* (AJ295143)	36.21	18.20	30.76	17.13	17.55	9.16	3.27	2.05	21.81	16.10	23.11	16.35	19.73	17.74	0.00	0.00
13	*Halomonadaceae, Halomonas* sp. (AM945689/ GQ903435)	3.11	2.69	6.11	3.33	5.93	2.62	1.58	0.86	7.88	6.38	4.84	4.11	6.20	6.46	1.81	0.89
15	*Halomonadaceae, Kushneria indalinina* (JF820663)	0.72	0.65	0.61	0.21	0.20	0.15	0.13	0.15	1.69	2.94	5.12	6.89	0.59	1.02	0.00	0.00
16	*Pseudomonadaceae, Pseudomonas* sp. (AB060135/D84019)	0.51	0.71	0.21	0.03	12.11	8.39	1.39	1.85	1.58	1.10	0.51	0.06	4.80	6.81	0.28	0.26
18	*Alteromonadaceae, Marinobacter persicus* (HQ433441)	0.02	0.03	0.04	0.06	2.06	2.41	0.00	0.00	0.91	1.58	0.08	0.13	0.22	0.21	0.13	0.15
29	*Oceanospirillaceae, Marinomonas* sp. (DQ492749/AJ843079)	0.29	0.34	0.25	0.16	0.22	0.24	0.00	0.00	1.37	2.37	0.00	0.00	0.12	0.21	0.00	0.00
30	*Oceanospirillaceae, Marinomonas* sp.	0.00	0.00	0.00	0.00	3.66	6.32	0.35	0.60	0.00	0.00	0.00	0.00	1.75	3.03	0.00	0.00
31	*Pseudoalteromonadaceae, Pseudoalteromonas* sp. (FJ200652/AY682201)	0.00	0.00	0.36	0.63	0.85	1.44	0.00	0.00	0.00	0.00	3.18	2.76	0.00	0.00	0.00	0.00
32	*Shewanellaceae, Shewanella* sp. (AF500078/FM887037)	0.00	0.00	0.00	0.00	0.18	0.31	0.61	1.05	0.00	0.00	0.00	0.00	1.84	3.10	0.00	0.00
38	*Vibrionaceae, Vibrio* sp. (AB562592/AY332401)	0.46	0.75	0.03	0.01	0.13	0.19	1.35	1.90	1.37	2.37	0.00	0.00	0.23	0.25	0.00	0.00
40	*Vibrionaceae, Vibrio fortis* (AB257333)	0.00	0.00	2.85	4.77	0.01	0.01	0.00	0.00	9.27	8.93	19.30	29.10	0.06	0.10	0.00	0.00
42	*Vibrionaceae, Vibrio rumoiensis* (AB013297)	0.00	0.00	0.01	0.02	10.99	19.00	0.22	0.38	0.00	0.00	0.00	0.00	0.96	1.67	0.00	0.00
45	*Enterobacteriaceae, Erwinia* sp. (HM008943/FJ611860)	0.00	0.00	0.00	0.00	0.01	0.01	0.61	1.05	0.00	0.00	0.00	0.00	1.93	3.34	0.00	0.00
50	*Enterobacteriaceae, Enterobacter hormaechei* (JN645954)	1.76	3.06	0.08	0.14	0.70	0.56	0.00	0.00	0.75	1.30	0.30	0.36	1.32	1.36	0.00	0.00
57	*Enterobacteriaceae, Enterobacter amnigenus* (AM062693)	0.00	0.00	0.00	0.00	0.00	0.00	2.29	2.16	0.00	0.00	0.00	0.00	8.67	7.51	0.00	0.00
65	*Enterobacteriaceae, Serratia marcescens* (FM213391)	0.02	0.03	0.00	0.00	0.00	0.00	2.09	3.61	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
66	*Enterobacteriaceae, Serratia* sp. (HQ326819/JF431270)	0.02	0.03	0.00	0.00	0.00	0.00	1.05	1.82	0.00	0.00	0.00	0.00	12.38	10.95	0.00	0.00
68	*Enterobacteriaceae, Rahnella aquatilis* (JX867757)	0.00	0.00	0.00	0.00	0.00	0.00	0.74	0.72	0.00	0.00	0.00	0.00	0.06	0.10	2.40	1.95
76	*Burkholderiaceae, Burkholderia* sp. (EU219865/HQ698908)	2.21	3.27	0.42	0.47	0.35	0.25	0.21	0.36	0.48	0.82	0.48	0.48	0.62	1.08	0.00	0.00
109	*Bradyrhizobiaceae, Bradyrhizobium* sp. (FJ025111/AY039016)	5.44	1.67	4.50	3.49	7.68	6.05	0.00	0.00	4.56	6.05	0.60	0.37	2.34	2.33	0.00	0.00
128	*Sphingomonadaceae, Sphingomonas rhizogenes* (AY962684)	5.29	6.28	2.06	2.58	2.20	0.81	0.00	0.00	0.00	0.00	0.06	0.11	0.03	0.06	0.00	0.00
139	*Rhodobacteraceae, Falsirhodobacter halotolerans* (HE662814)	0.00	0.00	0.00	0.01	0.07	0.06	0.00	0.00	2.74	4.75	0.00	0.00	0.00	0.00	0.00	0.00
145	*Rhodospirillaceae, Rhodovibrio salinarum* (FM177506)	0.22	0.38	0.16	0.20	0.08	0.14	3.29	3.35	0.00	0.00	0.00	0.00	0.00	0.00	9.06	7.87
156	*Bacillaceae, Alkalibacillus salilacus* (EU377478)	3.26	2.86	1.81	1.12	1.75	1.43	0.24	0.31	3.28	4.55	1.65	0.61	3.25	3.07	0.00	0.00
157	*Bacillaceae, Marinococcus halotolerans/M. tarijensis* (AY817493/JQ413413)	0.82	0.17	1.80	2.25	1.62	1.11	0.47	0.42	1.87	0.82	0.33	0.56	3.78	3.78	0.00	0.00
165	*Planococcaceae, Sporosarcina* sp. (AB243859/JX840395)	0.25	0.43	0.01	0.02	0.01	0.01	0.35	0.38	4.96	2.48	3.79	1.53	1.80	1.27	0.00	0.00

171	*Carnobacteriaceae, Marinilactibacillus psychrotolerans* (AB083413)	3.31	5.73	6.88	11.55	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.26	0.00	0.00	0.00	0.00
176	*Streptococcaceae, Streptococcus* sp. (HG315101/JX861483)	8.38	14.52	0.45	0.45	0.23	0.14	0.84	0.63	0.00	0.00	0.16	0.28	0.00	0.00	0.00	0.00
190	*Ruminococcaceae*, uncultured *Ruminococcaceae* (HQ716315)	0.00	0.00	0.03	0.05	0.01	0.01	2.40	2.73	0.00	0.00	0.00	0.00	0.00	0.00	2.60	1.20
204	*Flavobacteriaceae*, uncultured *Flavobacteriaceae* (U87104)	0.15	0.26	0.00	0.00	0.00	0.00	0.07	0.11	1.41	1.43	0.71	0.67	1.44	1.30	0.03	0.06
219	*Chitinophagaceae, Gracilimonas* sp. (JN038257)	0.00	0.00	0.00	0.00	0.00	0.00	2.02	1.50	0.00	0.00	0.08	0.13	0.00	0.00	1.43	0.53
246	*Deinococcaceae, Deinococcus* sp. (Y11331/CP002536)	0.12	0.20	0.00	0.00	0.00	0.00	0.00	0.00	3.67	5.17	1.27	1.46	0.60	0.45	0.03	0.06
251	*Acidobacteria*, ABS-19 (JQ801025)	0.00	0.00	0.00	0.00	0.00	0.00	5.11	6.24	0.00	0.00	0.00	0.00	0.00	0.00	1.50	1.02
278	OM1 clade (KF964596/GQ263220)	0.00	0.00	0.00	0.00	0.00	0.00	32.96	19.32	0.00	0.00	0.00	0.00	0.00	0.00	0.79	0.59
297	*Hyphomicrobiaceae*, uncultured *Cucumibacter* (JQ800771)	0.00	0.00	0.00	0.00	0.00	0.00	1.76	2.40	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.09
408	Uncultured *Draconibacteriaceae* (KJ817690/DQ899885)	0.00	0.00	0.00	0.00	0.00	0.00	1.35	1.90	0.00	0.00	0.00	0.00	0.00	0.00	3.85	0.26
432	*Rhodocyclaceae*, uncultured *Azospira* (DQ337003)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.96	3.31
536	*Staphylococcaceae, Salinicoccus carnicancri* (ANAM01000001)	0.00	0.00	0.00	0.00	0.00	0.00	2.03	2.85	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
559	*Ectothiorhodospiraceae*, uncultured *Thioalkalispira* (FR828704)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.48	2.57
638	uncultured *Saccharibacteria* (AB015558)	0.00	0.00	0.00	0.00	0.00	0.00	2.21	2.16	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.10

Regarding the community structures in relation to the soil physicochemical properties, salinity and O.M. were the parameters that better correlated with the endophytic differences between locations (Figure 2.1). However, only O.M. showed a significant value ($p<0.05$) for the effect of discriminating the communities by location. Additionally, it was notable that the correlation analyses (data not shown) indicated that none of the soil chemical parameters measured were good predictors of the bacterial diversity indices.

Description of the bacterial communities by plant compartment and location.

The most abundant OPUs 1 (*R. cellulosilytica*), 6 (*Psychrobacter* sp.), 12 (*C. canadensis*), 13 (*Halomonas* spp.) and 109 (*Bradyrhizobium* sp.) were present in all fractions (Table 2.2). *Psychrobacter* sp. (OPU 6; up to 37.5%) was especially abundant in red and *R. cellulosilytica* (OPU 1; up to 12.1%) was remarkably abundant in green and root (Figure 2.3). In addition, there were compartment-exclusive OPUs, especially noticeable in the root fraction dominated principally by *Enterobacter* species, and uncultured *Sodalis* sp. in red. The 10 OPUs exclusive to green were homogeneously distributed between *Bacteroidetes* (*Hymenobacter* sp.) and *Actinobacteria* (*Corynebacterium* sp.; Table 2.2).

The differences between locations were marked by 45 exclusive OPUs in Alcúdia and 120 in Campos. The latter showed the highest richness values, which were principally observed in the root compartments (Table 2.1). In general, the abundances of the exclusive OPUs were low and ranged between 0.01-1.70%, with the exception of *Enterobacter amnigenus* (OPU 57) that accounted for 8.67% of the total sequences in Alcúdia roots (Figure 2.3). In the Campos location, the most common families were *Moraxellaceae*, *Bradyrhizobiaceae*, *Sphingomonadaceae* and *Flavobacteriaceae*, whereas in A they were *Enterobacteriaceae* and *Planococcaceae*. Among these, according to the ISA, there were 27 indicator species for Campos and 3 for Alcúdia ($p<0.05$; Table A 2.3). Additionally, all the representatives of *Epsilonproteobacteria* (OPUs 101 and 102), *Tenericutes* (OPUs 180 and 181) and *Gemmatimonadetes* (OPU 229) were only detected in Alcúdia, with all *Planctomycetes* (OPUs 224 and 225), *Armatimonadetes* (OPU 244) and *Chloroflexi* (OPU 249) only in Campos.

Rhizospheric soil microbial community structures

Alcúdia samples exhibited higher richness than those in Campos, contrary to that observed for the endophytic community (Table 2.1), and both locations showed different community structures (Figure A 2.4 and Table A 2.2). Rhizospheric soils from Alcúdia and Campos shared 17 (4.8 ± 2.5% of the total sequences) and 39 (20.2 ± 3.3% of the total sequences) OPUs with the respective endospheres. On the other hand, these shared OPUs represented approximately

8.9 ± 5.0% and 53.5 ± 17.3% in Campos of the sequences belonging to the endophytic communities (Figure 2.4). No special abundance of the shared OPUs was detected in any specific fraction of the plant. *Halomonas* sp. and *Pseudomonas* sp. (OPUs 13 and 16), were the most important OPUs shared between rhizospheric soil and endosphere in both locations.

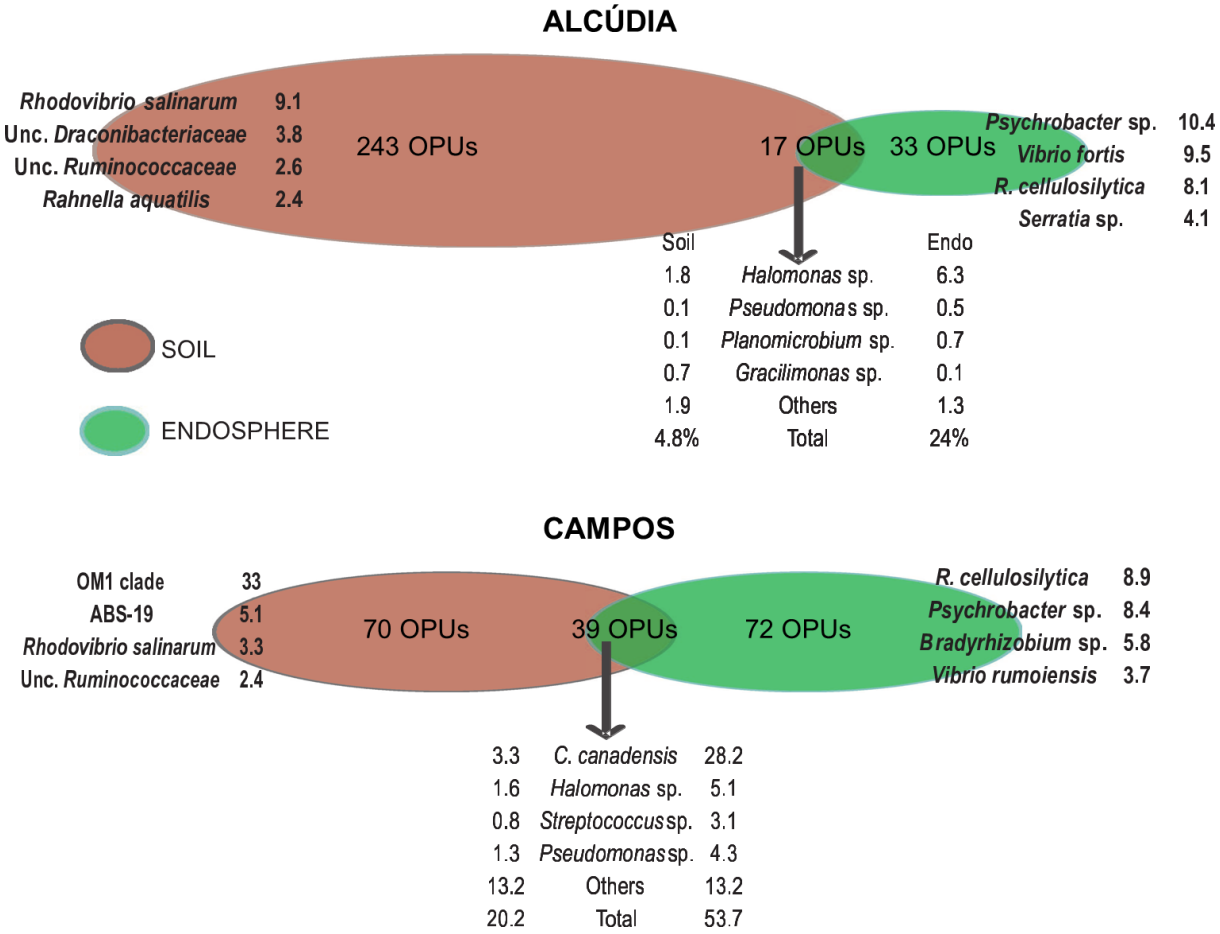

Figure 2.4 Diagram showing the comparisons between the bacterial communities of the rizhospheric soils and the endospheres of the halophytes in Alcúdia and Campos. The size of the ellipses represents the total number of OPUs. The percentages of abundances of the most relevant OPUs in each group of samples (rhizospheric soil and endosphere) and shared OPUs are given below.

Despite the fact that the rhizospheric communities were distinct in both locations, they were both principally represented by *Proteobacteria* (52.7% of the total sequences for Alcúdia, and 33.9% for Campos), with *Gammaproteobacteria* being the most abundant class. Another 21 phyla were additionally detected but, of these, only *Acidobacteria*, *Actinobacteria*,

Bacteroidetes, *Gemmatimonadetes*, *Firmicutes*, *Planctomycetes* and *Saccharibacteria* exhibited abundances higher than 2% in Alcúdia or Campos (Table 2.2).

The most abundant OPUs in Alcúdia soil were *Rhodovibrio salinarum* (OPU 145; representing up to 3-18% of the sequences), *Halomonas* sp. (OPU 13), uncultured *Ruminococcaceae* sp. (OPU 190), *Acidobacteria* Subgroup 6 sp. (OPU 254), *Draconibacteriaceae* sp. (OPU 408), and uncultured *Azospira* sp. (OPU 432; these latter five clades together represented between 10-16% of the total sequences; Table 2.2). Only the first OPUs co-occurred in their respective endophytic fraction, and *Halomonas* sp. (OPU 13) occurred in all fractions.

On the other hand, Campos soils were characterized by high abundances of OM1 clade sp. (OPU 278; between 11 to 48% of the total sequences), *C. canadensis* (OPU 12), *E. amnigenus* (OPU 57), *R. salinarum* (OPU 145; uncultured *Ruminococcaceae* sp. (OPU 190), and ABS-19 (OPU 251; these latter five clades together represented between 10-19% of the total sequences). From these, only *C. canadensis* and *R. salinarum* co-ocurred in some of their respective endophytic fractions (Table 2.2; Figure 2.3). In addition, *R. cellulosilytica* also co-occurred but with abundances lower than 1.4%, whereas *C. canadensis* occurred in all fractions from plants and soil, which was similar to *Halomonas* sp. for the Alcúdia location.

Discussion

To our knowledge, this is the first report where compartmentalization of endophytic bacterial communities from the phyllosphere and rhizosphere of halophytes has been analyzed at a micro- and mesoscale. OPUs could be identified as taxa at the species and genus levels because the sequences were of higher quality (lengths of over 800 bp) compared to other similar studies (Gottel *et al.* 2011; Bodenhausen, Horton and Bergelson 2013), and also because the OPU approach by means of phylogenetic inferences produced more accurate identifications (92.8% of the OPUs could be affiliated at the genus level; Vidal *et al*. 2015)

We are aware that the sample number may be low, but our results appear consistent with our previous studies (Mora-Ruiz *et al.* 2015), where differences in endosphere composition were dependent on the location, but the major key players were common in all plants. Most of the principal elements in the endosphere were moderate halophiles. The presence of *C. canadensis* was previously reported in *A. macrostachyum* and the other halophyte *Allenrolfea vaginata* (Mora-Ruiz *et al.* 2015).

The differences detected between the locations may respond to the combination of the soil physicochemical properties and microbial composition, and the host genotype (Bulgarelli *et al.* 2012). However, relevant host genotype differences were discarded as the plants were only 60 km apart. In this regard, the soils studied here exhibited considerable physicochemical

differences that could condition their bacterial diversity (Fierer and Jackson 2006), despite the fact that pH was alkaline and similar to other reported values (Pereira, Vicentini and Ottoboni 2014; Gao *et al.* 2015). However, the soil in Campos exhibited 10-fold higher salinity (considered to be a strongly saline soil ≥ 2%; Richards 1954) and differences in other parameters depending on the location were observed. These physicochemical parameters might directly influence the microbial composition of soils (Gao *et al*. 2015), and the rhizospheric soil of Alcúdia (more mesophilic environment and organic rich) showed nearly 3-fold more OPUs than Campos. In addition, a direct correlation was detected between CO_3^{2-} and salinity concentrations with the presence of *Bacteroidetes*, *Deltaproteobacteria* and *Chlorobi* (higher in Alcúdia), and K^+ concentrations with *Actinobacteria* (higher in Campos). K^+ has been considered as one of the principal chemical parameters influencing distinct abundances of phyla in soil bacterial communities (Pereira *et al*. 2014). However, there may be other environmental traits (*e.g.* aeration, pore size, temperature and water) not registered in the current study that could control microbial diversity patterns (Nemergut *et al.* 2011; Bokulich *et al.* 2014).

Accordingly, the soils from the two locations were different in their taxa compositions. For both locations, only *R. salinarum* and uncultured *Ruminococcaceae* sp. were part of the most abundant OPUs in Campos and Alcúdia. The former OPU, already reported in hypersaline sediments (Mack *et al.* 1993), was shared by both soils in similar quantities. However, it was remarkable that *C. canadensis* remained undetectable in the Alcúdia soils, this OPU being one of the most abundant in endosphere. In the same way, *Bradyrhizobium* sp. was abundant in the endosphere from the two locations and not detected in their corresponding soils, contrary to previously reported in *Populus deltoides*, where it was principally detected in rhizosphere (Gottel *et al.*, 2011).

The soil salinity also influences the halophytes colonizing them. As other halophytes, *A. macrotachyum* accumulates distinct ion concentrations in its vacuoles in amounts directly correlated with the increase of external (soil) salt concentrations due to increased uptake by osmoregulation (Redondo-Gómez *et al*. 2010). As Campos soil was more saline than Alcúdia soil, the plants may have had different saline contents in their endospheres, which would influence the composition of each endosphere. There were some major ubiquitous organisms in the endosphere, and some were also detected in the soils of both locations, such as *Halomonas* sp., or only in the Campos soils. Their presence in the endosphere could originate after root colonization due to chemotaxis (Bulgarelli *et al*. 2013) from the soil communities (Wulff, van Vuurde and Hockenhull 2003; Chi *et al.* 2005), followed by migration to the aerial parts. However, some relevant taxa were undetectable in Alcúdia soils, although they probably formed part of the seed-bank or rare biosphere of these soils (Pedro-Aliós 2006), and would gain preponderance in a favorable endophytic environment. These were not isolated cases, as other organisms (not observed as ubiquitous), such as *Marinomonas* sp. (also endophytes of marine

plants; Espinosa *et al*. 2010) or *Sphingomonas* sp., were not detected in the soils. An alternative origin could also be related to aerosol transmission through the aerial parts (Fahlgren *et al.* 2010), or by vertical transmission (bacteria already occurring in the seeds; Truyens *et al.* 2015). However, the fact that between 9% (for Alcúdia) and 53% (for Campos) of the total endospheric taxonomic composition was shared with their corresponding rhizospheric soil communities, led us to believe that root colonization may be the major contributor to the endosphere composition (Bulgarelli *et al*. 2013). The lower salinity of the Alcúdia soils may explain the low abundances of the ubiquitous and relevant endophytic, halophilic bacteria, such as *C. canadensis* that would gain preponderance once colonizing the plant with higher saline levels in its endosphere.

A clear diversity gradient was observed between the three compartments in each plant, being higher in the rhizosphere (as already reported for *A. thaliana*; Bodenhausen *et al*. 2013) and decreasing towards the more mature aerial parts, with the latter having less equitability. In general, the diversity values detected were very similar to previous studies of leaves (Emiliani *et al.* 2014; Jin *et al.* 2014). No clear trend could be detected in the distinct occurrence of specific OPUs in the different compartments, some of which (generally a minority) seemed to be specifically associated with one fraction of the plant. Others, like most of the representatives of the *Enterobacteriaceae* (especially *E. amnigenus*), were notably abundant in the root's endosphere, some of which have been reported as having plantgrowth-promoting effects principally associated with roots (Whipps 2001). Additionally, two of the most important OPUs, *Psychrobacter* sp. and *R. cellulosilytica,* had notable abundance variations in the red fractions, being higher and lower (respectively) in these more mature parts of the plant in comparison with other areas of the endosphere. The reduction of diversity from the roots towards the more mature parts of the plant, apart from reinforcing a rhizospheric origin of the endogenous microbiota, may respond to factors such as passive mobility following water fluxes (Taghavi *et al.* 2010), specific selection due to plant-microbe interaction, such as chemotaxis (Bulgarelli *et al*. 2013) or plant defensive systems (Jones and Dangl 2006), and specialization in niche colonization (Bulgarelli *et al*. 2013).

In summary, the results presented here indicated that the endosphere of the halophyte *A. macrostachyum* may be strongly influenced by the microbial composition of the soils where this plant grows, and the soils themselves by their environmental physicochemical parameters. In this regard, the higher saline conditions of the Campos soil led to reduced taxa richness in accordance with what occurs in extreme saline environments (Hollister *et al.* 2010). The influence of the soil on plant microbial colonization was supported by its sharing of taxa with the plant compartments, and with reduction of diversity from the roots towards the mature aerial parts. On the other hand, there were several ubiquitous major microbial components, moderately halophilic in their metabolism, which seemed to be plant-related and not always detectable in the rhizospheric soils. Their presence may indicate a probable relevant role in the plant-microbe

interaction, and the absence of detection in some soil samples could point to other alternative origins (*e.g.* presence in plant seeds prior to germination). Finally, the endophytic lifestyle of some taxa is reported here for the first time, such as the known cellulose-degrading microorganism *R. cellulosilytica*, which although present in some soils has not yet been reported as part of the endomicrobiota of plants (Weon *et al*. 2009). The study provides an insight into understanding the possible mechanisms of dispersion and colonization in different plant lifecycle stages (seeds, seedlings and adults) and the endosphere microbial community dynamics associated with halophytes.

Chapter 3. Non-halophilic endophytes associated to the euhalophyte *Arthrocnemum macrostachyum* and their potential plant growth promoting activity

Abstract

Numerous microbial taxa establish natural relations with plants, and specifically endophytes can be relevant in the development of the host. In this work we explore the diversity of non-halophilic microorganisms inhabiting the endosphere of the halophyte *Arthrocnemum macrosthachyum* to complement our previous studies on halotolerant and extreme halophilic endophytes. A total of 1,045 isolates were recovered and clustered into 22 Operational Phylogenetic Units (OPUs) including 7 putative new species and 13 OPUs not previously detected as endophytes. The most abundant isolates corresponded to close relatives of *Kushneria indalinina/K. marisflavi*, *Providencia rettgeri*, *Pseudomonas zhaodongensis* and *Bacillus safensis*, which made up to ~ 62% of the total isolates. We also isolated OPUs not detected previously by the culture-dependent approach reinforcing the need of cultures to reveal the microbial diversity associated to plants. Additionally, the plant growth promoting activity was evaluated in representative strains of the most abundant OPUs (total= 94 strains) including also some previously isolated halophiles from the same plants. Under both saline and non-saline conditions, some strains principally those affiliated to *Paenibacillus borealis*, *Staphylococcus equorum*, *Salinicola halophilus* and *Marinococcus tarijensis*, exhibited growth promoting activity in *Arabidopsis thaliana* expressed as an increment of the weight and root length.

Keywords: *Arthrocnemum macrostachyum*, Operational Phylogenetic Unit, plant growth promoting activity

Introduction

The microbial communities associated with the internal tissues of plants (endosphere) are classified, depending on the kind of interaction established, either as pathogens or endophytes (Newman and Reynolds 2005a; Mapelli *et al.* 2013). Briefly, endophytes are microorganisms that produce no apparent damage to the plant and their role can be reflected positively as contribution of nutrients, protection against pathogens and plant growth promoting activity (PGPA; Rosenblueth and Martínez-Romero 2006; Hardoim, van Overbeek and Elsas 2008; Andreote, Azevedo and Araújo 2009; Mercado-Blanco and Lugtenberg 2014). However, the internal tissues of the plant can be a hostile environment (Mercado-Blanco and Lugtenberg 2014), and therefore, specific molecular communication might be necessary between microorganisms and plants for the success of the colonization processes (Schikora, Schenk and Hartmann 2016). There are some evidence of colonization processes in different structures of the plant (Lugtenberg, Chin-A-Woeng and Bloemberg 2002; Seghers *et al.* 2004; Rosenblueth and Martínez-Romero 2006; Li *et al.* 2008; Ryan *et al.* 2008), but in general the rhizosphere has attracted most of the attention (Hurek *et al.* 2002; Vessey 2003; Lugtenberg and Kamilova 2009; Segura *et al.* 2009; Bringel and Couée 2015). After the colonization processes, the abundances of the endophytes are influenced by different factors being the most important the availability of nutrients for the microorganisms, the genotype and phase of development of the plant, the environmental conditions (Kuklinsky-Sobral *et al.* 2005; Andreote, Azevedo and Araújo 2009) and the presence of antibacterial substances such as terpenoids, benzoxazines, flavonoids and isoflavonoids (Hardoim, van Overbeek and Elsas 2008).

As mentioned, most studies of endophytes and their relevance have been focused on rhizobacteria of commercial plants and specifically, their capability to undergo nitrogen fixation (Hurek *et al.* 2002), phosphate solubilization, increment of mineral and nutrient availabilities, production of indoleacetic acid (IAA) and acetyl-CoA carboxylase (ACC; Germida *et al.* 1988; Zinniel *et al.* 2002; Kuklinsky-Sobral *et al.* 2004; Seghers *et al.* 2004; Ryan *et al.* 2008; Doty *et al.* 2009; Manter *et al.* 2010; Weber, Videira and Simões de Araujo 2013; Amaresan, Jayakumar and Thajuddin 2014). However, the topic has been recently broadened including other plants such as metal-accumulating species (Belimov *et al.* 2005) or halophytes (plants which can survive in saline soils; Sgroy *et al.* 2009a; Glick and Glick 2012; Ruppel, Franken and Witzel 2013; Mora-Ruiz *et al.* 2015, 2016). Endophytes of halophytes have been considered an interesting alternative as biofertilizers for plants under stress conditions (salt stress and drought) (Ruppel, Franken and Witzel 2013a; Mercado-Blanco and Lugtenberg 2014). In fact, in front of the current world-wide high demand for food derived principally from the increment of human population and the constant awareness of environmental damage and protection, new

agricultural practices have been necessary towards a more sustainable and environmentally friendly agriculture (Glick and Glick 2012).

The study of endophytic communities has been principally addressed by culture-independent approaches, and in some cases including just few isolates (Andreote, Azevedo and Araújo 2009; Manter *et al.* 2010; Weber, Videira and Simões de Araujo 2013). Although next generation sequencing (NGS) techniques allow exploring the richness associated to plants, they also limit the study of the colonizing microbial cells in detail (Zengler *et al.* 2002). Currently, the large scale cultivation combined with mass spectrometry techniques as the Whole-Cell Matrix Assisted Laser Desorption Ionization–Time Of Flight Mass Spectrometry (WC MALDI-TOF MS) has opened the possibility to a better understanding of complex microbial ecosystems (Viver *et al.* 2015). WC MALDI-TOF MS identification has been recommended for large scale studies because it is efficient, fast and cost-effective in comparison with other identification tools such as 16S rRNA sequencing (Munoz *et al.* 2011; Mora-Ruiz *et al.* 2015).

In our previous studies, we explored the endophytic diversity of *Arthrocnemum macrostachyum* using both 454 amplicon pyrosequencing and culturing of moderately halophilic microorganisms (Mora-Ruiz *et al.* 2015, 2016). The results pointed as the most relevant taxa *Chromohalobacter canadensis, Salinicola halophilus, Kushneria indalinina* and *Rudaea cellulosilytica* (Mora-Ruiz *et al.* 2015, 2016). In those studies, we recovered by culture an important fraction of the richness observed by massive sequencing (~60%), but other abundant groups (e.g. *Halomonas meridiana, Pseudomonas seleniipraecipitans, Pseudomonas alcaliphila, R. cellulosilytica* and *Cupriavius gilardii*) were not cultured in part because we used culture media for halophiles (from 5 to 30%). Therefore, in order to reveal whether the remaining uncultured ~40% could be also non-saline or halotelerant organisms we focused on the recovering of the non-halophilic and halotolerant culturable microorganisms inhabiting *A. macrostachyum*. Finally, we selected some halophilic strains from our previous studies (Mora-Ruiz et al., 2015), and non-halophilic strains isolated here to analyze their potential PGPA on the model plant *Arabidopsis thaliana*, a salt sensitive plants species.

Material and methods

Collection of samples and plant material treatment

Three plants identified as *A. macrostachyum* were collected in Salinas de Levante S.A. (39º21’03.3’’N, 3º00’44.3’’E) in Mallorca (Spain) in January 2015. The samples were collected with sterile gloves, stored in individual sterile zip-lock bags and brought to the laboratory to be immediately processed. Forty grams of green stems from each plant were excised, their surface sterilized and the plant tissues disaggregated following the protocol previously described in

Mora-Ruiz *et al.* (2015). The obtained plant extracts were used as the endophyte inoculum (EI) for the culture-dependent approach using different media (see next section). Fragments of each treated plant were also used to prove the efficacy of the plant surface sterilization process. The sterile plant material was submerged in R2A media and gently shaken for three minutes. This suspension was used as the sterile inoculum (SI) and consequently inoculated in the different culture media by triplicate.

Isolation of mesophilic and halotolerant heterotrophic bacteria

To achieve a high number of isolated microorganisms, six bacteriological culture media with different composition were selected: Reasoner's 2A (R2A) medium for oligotrophs (Reasoner, Blannon and Geldreich 1979); Bacteria Screening Media 523 (M523) for organisms present in plants (Kado and Heskett 1970); a poor nitrogen medium (NP; Cavalcante and Dobereiner 1988); a thioglycolate containing medium to detect the presence of microaerophilic bacteria (TH; Leijh *et al.* 1984); and the modified Burk's (BM) (Atlas 2010) and Rennie (RMR; Rennie 1981) media to detect nitrogen-fixing microbes (Sturz and Kimpinski 2004). Also, a medium prepared with sterile plant extract (EX) was used trying to resemble the plant conditions and nutrients. For the EX medium a total of 398 g of sterilized and macerated plant tissue were mixed with 380 ml of sterile phosphate buffered saline (PBS) PBS 4x. The mix was separated in 50 mL Falcon tubes and disaggregated using ultrasound (Omni-Ruptor 400 Ultrasonic Homogenizer, Omni International Inc, Georgia, U.S.A.; 3 cycles of 60% and potency 10, 2 min) to lyse the vegetable cells. Then, the samples were centrifuged (3,300 x*g*, 5 min, room temperature-RT). The supernatant was collected in 50 mL Falcon tubes and the pellet was discarded. Centrifugation was repeated twice and the supernatant filtered through 0.5 μm membranes (Sterivex Millipore, Darmstadt, Germany). A new and last centrifugation was performed (51,275 x*g*, 15 min, RT) and the supernatant was finally sterilized through filtration using 0.22 μm membranes (Sterivex Millipore, Darmstadt, Germany). The final volume of 160 mL of sterile plant extract was used to prepare the medium. To control the sterile conditions of the filtered plant extract, three of these plates were kept non-inoculated to verify absence of growth.

From each of the three plants, the EI and the SI were inoculated in all media by triplicate using dilutions from 10^{-1} to 10^{-4}. With the aim of recovering the maximum richness we used two different methods of selection. The first consisted on a visual selection of colonies according to their morphology; at least four colonies per morphotype were further analyzed. In all cases, a minimum of 30 colonies were isolated by plant and medium. Alternatively, we performed a random selection of colonies dividing the plates in quarters and isolating all colonies from one single quarter. All colonies were reinoculated in the same original medium

but complemented with 100 µL of Itraconazol (2 mg/mL; Bexal Farmacéutica, Madrid, Spain) to minimize fungal contamination (Mora-Ruiz *et al.* 2015).

WC MALDI-TOF MS screening, 16S rRNA gene sequencing and phylogenetic reconstruction.

The identification of the isolates was performed using the tandem approach WC MALDI-TOF MS with 16S rRNA gene sequencing and phylogenetic inference (Munoz *et al.* 2011; Viver *et al.* 2015). All MALDI-TOF MS spectra were manually supervised with the Maldi Biotyper v 3.2 (Bruker Daltonics, Germany) and compared with the 3,995 profiles of its database for their identification, obtaining scores from 1.703 to 2.379. The minimum sampling size was calculated for one plant by the sequential addition of MS in the dendrogram until the Operational Taxonomic Unit (OTU) number tended to the asymptotes. Each WC MALDI-TOF MS cluster was considered as a single OTU, and for those that could not be identified already with the MS database between 1 and 4 isolates were selected for 16S rRNA gene sequencing. The DNA extraction was performed by osmotic shock using MilliQ water followed by a thermal shock consisting in three cycles of 100ºC to -20ºC during 5 min. The extract was used to amplify the 16S rRNA gene following the 5PRIME Mastermix protocol (2.5X master Mix, 10 mM of each primer, DNA≤250 ng and distilled water), in a final volume of 50 µL. The amplification was performed with the universal primers for the *Bacteria* domain: GM3 (5′-AGAGTTTGATCATGGCTCAG-3′) and S (5′-GGTTACCTTGTTACGACTT-3′) following previously reported conditions (Mora-Ruiz *et al.* 2015). The amplicons were visualized in 1% agarose gels and stained with ethidium bromide. PCR products were purified with MSB® Spin PCRapace (Invitek GmbH, Berlin) and they were sent for sequencing to Secugen S.L. (Madrid, Spain).

Sequences were reviewed, corrected and assembled using Sequencher v 4.9 software (Genes Corporation, Michigan), and aligned using the SINA software (SILVA; Quast *et al.* 2013) implemented in the ARB v 5.5 program package (Ludwig *et al.* 2004). Alignments were finally manually improved. The phylogenetic reconstruction of the almost complete sequences was performed using the RAxML algorithm also implemented in the ARB software package, while partial sequences were later added to the reconstructed tree using the parsimony tool in ARB. The conservational filter for the bacterial domain implemented in the database was used in all cases. The manual inspection of the tree topology allowed grouping the sequences in Operational Phylogenetic Units (OPUs) with identity scores ranging from 88.6% to 100% with the closest relative type strains from the LTPs123 database (Yarza *et al.* 2010). For OPUs with no close relative type strain, the closest relative sequence was selected from the SilvaRef_NR99 (Quast *et al.* 2013a).

DNA extraction and Random Amplification of Polymorphic DNA (RAPD) fingerprints

Five to six organisms within each OTU were randomly selected and their DNA extracted as mentioned above. The RAPD fingerprints were generated using MasterMix 5PRIME (2.5X MasterMix, 10 mM of primer, DNA≤250 ng and distilled water), in a final volume of 25 µL. The primer used was RAPD1 in the same conditions as previously reported (Peña *et al.* 2005). The amplicons were visualized in 1.5% agarose gels stained with ethidium bromide.

Plant Growth Promoting Activity (PGPA)

Each strain was homogeneously inoculated at $1x10^4$ colony forming units (CFU) in 0.8% agar, $MS^1/_2$ plates (Murashige and Skoog 1962) and seeds of *Arabidopsis thaliana* Col-0 ecotype plants were sown in the agar surface. The plant growth in vertically arranged plates was monitored during 21 days in thermoregulated growth chambers at 22ºC with a 16h/8h light/dark photoperiod. *Paraburkholderia phytofirmans* strain PsJN was used as positive PGPA control since it has been proved to act as a PGPA-microbe in different plants including *A. thaliana* (Poupin *et al.* 2013), maize (Naveed *et al.* 2014) and grape (Trdá *et al.* 2014). For those bacterial isolates that had a positive effect on the plant in the first assay, a second assay was carried out measuring the production of the auxin indoleacetic acid (IAA), that promotes cellular division and radicular ramification of the plant (Naveed *et al.* 2014), and the production of the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), that promotes growth in plants influenced by environmental stress, decreasing ethylene concentrations and increasing those of ammonia in the rhizosphere, thus delaying the senescence of the plant (Glick 2014).

Statistical analysis and ecological indices

The total abundances between plants and culture media were compared using a permutation test (PERMANOVA; Anderson 2001) followed by a post-hoc pairwise permutational group comparison because the data complied with the precept of homoscedasticity, but not for normality even after transformation. Additionally, the ecological indices of "real diversity" of Jost (Jost 2006) were calculated in order q=1 and q=0 to describe the communities in each culture media. With the aim to compare the bacterial communities from each culture media, a Morisita index was calculated (Chao *et al.* 2008) and a multivariate cluster analysis (dendrogram) with Euclidian distance was also carried out. All the statistical analysis and ecological indices were obtained with the software R v 3.3 using the packages vegan (Oksanen 2010), vegetarian (Charney and Maintainer 2015) and spaa (Zhang 2013). For plant growth promotion assays, statistically significant differences among bacteria-inoculated and non-

inoculated control plants for each measured parameter, were calculated using one way ANOVA Tukey's HSD tests, set with a fixed $p < 0.05$ threshold value. These were estimated using the MINITAB Software, release 13.31 (Minitab Inc., State College, Pennsylvania). One way ANOVA was performed to evaluate the PGPA of the strains. Statistically significant differences with non-inoculated control plants for each measured parameter (One way ANOVA Tukey's HSD tests; $p < 0.05$).

Results

Viable cell counts of endophytic aerobic bacteriaof *Arthrocnemum macrostachyum*

Sterile tests performed on the vegetal fragments did not show growth in any culture media used during one month of incubation. Therefore, the viable cell counts reported here were considered true endophytes. Colonies were already observed after 72 h, being R2A, TH and M523 the media in where the growth was faster. Conversely, RMR showed the most delayed growth (> 10 days later). Endophytic abundances ranged between 10^3 to 10^6 colony forming units (CFU) g^{-1} (Figure 3.1), and the differences depended on the culture media ($p<0.05$; Table A 3.1). Regarding CFU g^{-1} yields, three groups were determined (Figure 3.1). group A formed by the media M523, R2A, TH and NP showed the highest yields; group B formed by the media BM and EX showed intermediate yields; whereas group C showest the lowest values when using RMR culture media.

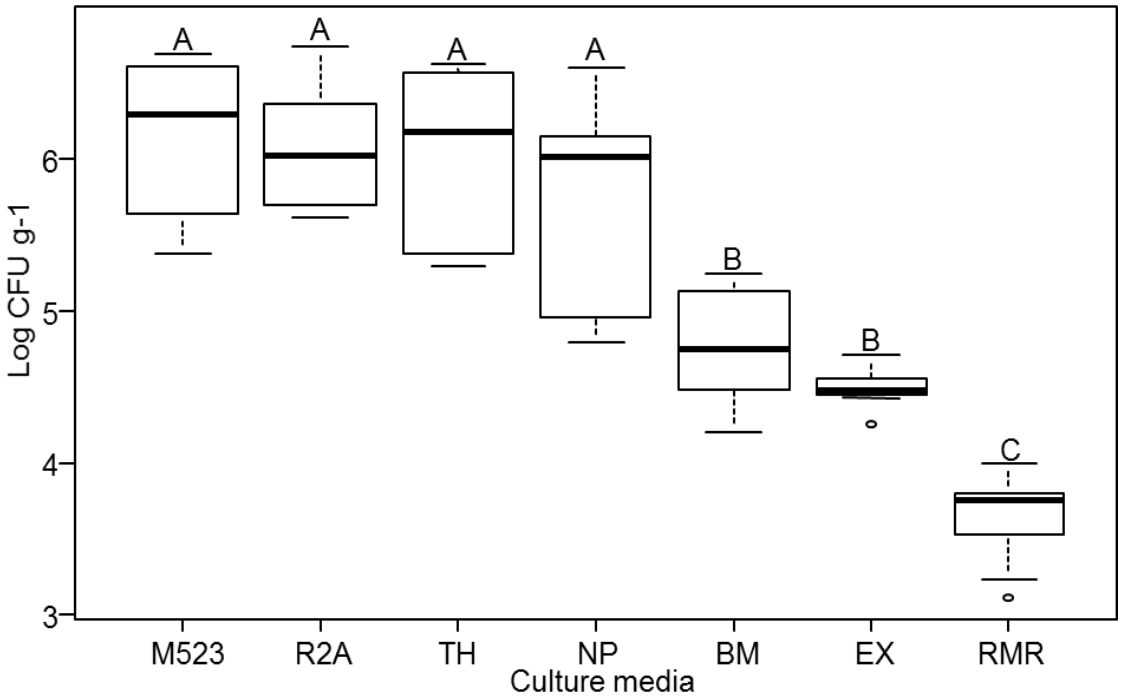

Figure 3.1 Boxplot of total abundances of cultivable microorganisms in the different culture media. Three groups can be distinguished (A to C). Letters on each plot are groups formed based on the permutation analysis. Error bars indicate standard deviation.

Identification of the isolates from the endosphere of *Arthrocnemum macrostachyum* by a tandem MALDI-TOF MS and 16S rRNA gene sequence inference

A total of 1,096 strains were isolated and analyzed using WC MALDI-TOF MS (Table A 3.2). The manual inspection of the spectra rendered a total of 1,045 good quality spectra, which were subsequently used. The minimum number of strains isolated by plant/culture media ranged from 23 in the EX medium to 35 in R2A medium (Table A 3.3). The spectra obtained were clustered in a dendrogram generating 36 OTUs at 700 distance (Figure 3.2). A random selection of representative organisms from each OTU was used for sequencing and identification.

Table 3.1 Endophytic OPUs detected in *Arthrocnemum macrostachyum* including the type species closest. * OPUs identified by WC MALDI-TOF MS and their respectively values with the Bruker Daltonics database. The additional OPUs correspond to 16S rRNA and the similarity value with the closest strain and the accession number. + OPUs identified to genus level and potential new species.

OPU	Type species	Identity	Accesionnumber	Number of isolates
OPU1	*Proteobacteria. Gammaproteobacteria. Halomonadaceae. Kushneria indalinina/K. marisflavi*	98.9-99.7	AM941745/KF359966	235
OPU2	*Proteobacteria. Gammaproteobacteria. Enterobacteriaceae. Providencia rettgeri*	99.6-99.9	KC456547	144
OPU3	*Proteobacteria. Gammaproteobacteria. Enterobacteriaceae. Pantoe aeucrina*	99,6-100	EU216736	74
OPU4	*Proteobacteria. Gammaproteobacteria. Pseudomonadaceae.*	99.7-99.8	AJ575816	7

	Pseudomonas psychrotolerans			
OPU5	*Proteobacteria. Gammaproteobacteria. Pseudomonadaceae. Pseudomonas zhaodongensis*	91.4-99.0	JQ762275	126
OPU6	*Proteobacteria. Gammaproteobacteria. Pseudomonadaceae. Pseudomonas graminis*	98.0-99.9	Y11150	75
+OPU7	*Proteobacteria. Gammaproteobacteria. Pseudomonadaceae. Pseudomonas cichorii*	97.6	Z76658	56
*OPU8	*Proteobacteria. Gammaproteobacteria. Moraxellaceae. Acinetobacter johnsonii*	1.9		1
*OPU9	*Proteobacteria. Alphaproteobacteriaia. Sphingomonadaceae. Sphingomonas desicabilis*	1.7		2
OPU10	*Proteobacteria. Alphaproteobacteriaia. Rhodobacteraceae. Paracoccus chinensis*	99.5	EU660389	1
+OPU11	*Actinobacteria. Actinobacteria. Sanguibacteraceae. Sanguibacter keddieii*	88.6	X79450	8
+OPU12	*Actinobacteria. Actinobacteria. Intrasporangiaceae. Janibacter sanguinis*	91.5-96.4	XC019204	1
+OPU13	*Actinobacteria. Actinobacteria. Microbacteriaceae. Curtobacterium flaccumfaciens pv. flaccumfaciens*	95.4	AJ312209	6
OPU14	*Actinobacteria. Actinobacteria. Micrococcaceae. Nesterenkonia halotolerans*	99.9	AY226508	1
OPU15	*Firmicutes. Bacilli. Bacillaceae. Bacillus safensis*	99.6-99.8	AF234854	143
+OPU16	*Firmicutes. Bacilli. Bacillaceae. Bacillus pumilus*	96.3	KF532968	1
OPU17	*Firmicutes. Bacilli. Staphylococcaeae. Staphylococcus saprophyticcus subs. saprophyticcus*	99.9	AP008934	56
OPU18	*Firmicutes. Bacilli. Staphylococcaeae. Staphylococcus equorum*	98.9	AB009939	19
OPU19	*Firmicutes. Bacilli. Carnobacteriaceae. Marinilactibacillus piezotolerans*	98.9-99.3	AY485792	29
+OPU20	*Firmicutes. Bacilli. Paenibacillaceae. Paenibacillus borealis*	97.3-98.6	AJ011321	34
+OPU21	*Firmicutes. Bacilli. Paenibacillaceae. Paenibacillus tundrae*	98.2	EU558284	7
OPU22	*Firmicutes. Bacilli. Paenibacillaceae. Paenibacillus taichungensis*	99.3	EU179327	3

The phylogenetic reconstruction based on the representative sequences of the 36 OTUs generated a total of 22 OPUs (Table 3.1). Only OPU8 was not recovered from the random sampling strategy, whereas four (OPU10, OPU14, OPU16 and OPU22) were not recovered from the morphology selection strategy (Table A 3.2). From the 22 OPUs, ten (representing 70% of the total abundance of strains) affiliated with members of *Proteobacteria*, four (16% of abundance) with *Actinobacteria* and eight with *Firmicutes* (28% of abundance). When using the 98.7% identity as the lowest and conservative cutoff to discriminate species (Stackebrandt and Ebers, 2006), 13 OPUs affiliated with known species (Table 3.1 and Figure 3.3) and seven with known genera but representing new species (OPU7, OPU11, OPU12, OPU13, OPU15, OPU20 and OPU 21). The most abundant OPUs in the collection were OPU1 (*Kushneria indalinina/K. marisflav*i; 232 isolates), OPU2 (*Providencia rettgeri*; with 144 isolates), OPU5 (*Pseudomonas zhaodongensis*; with 126 isolates) and OPU15 (*Bacillus safensis*; with 142 isolates). Additionally, OPU1 and OPU15 were the unique OPUs isolated from all culture media (Table A 3.1).

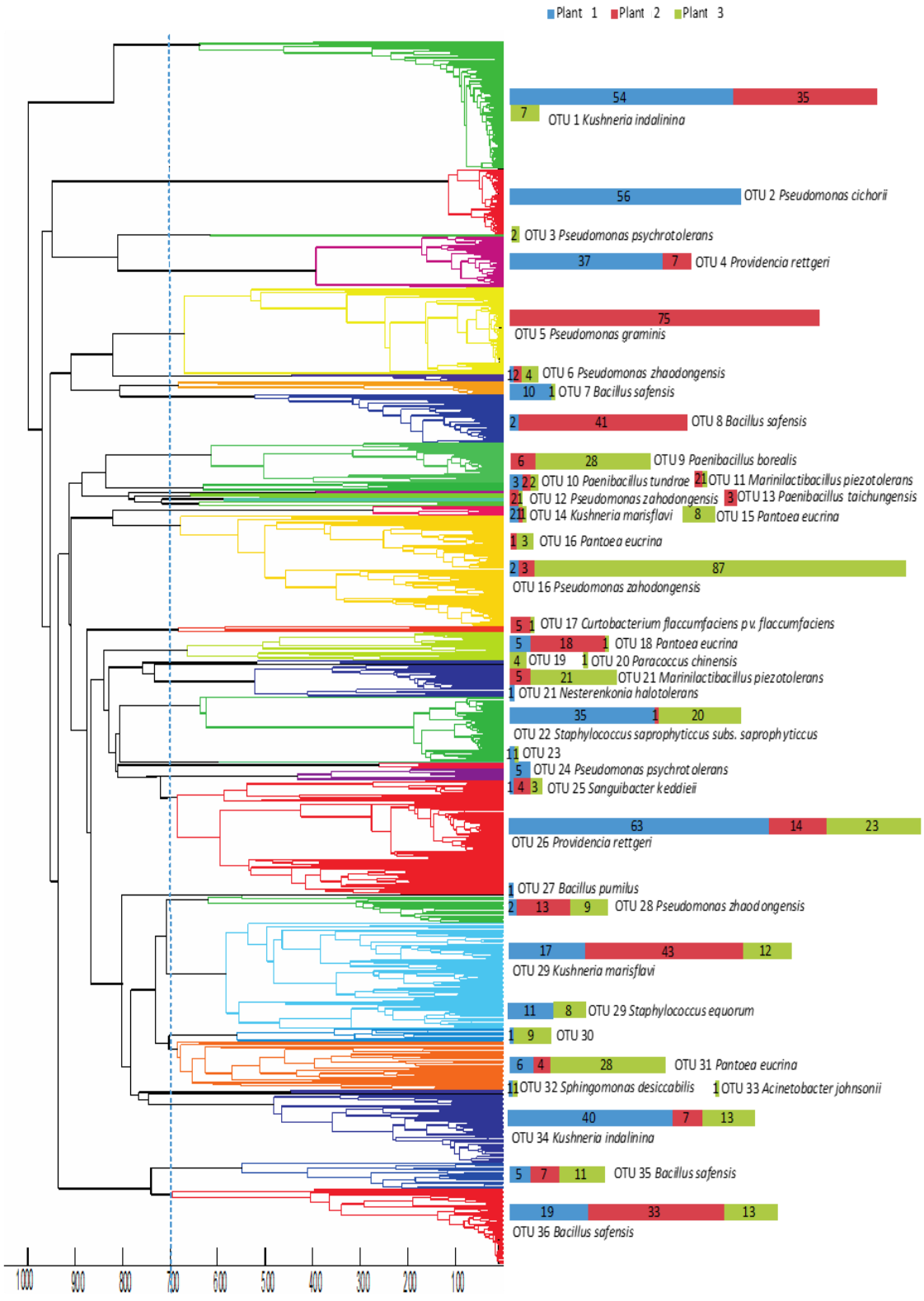

Figure 3.2 Dendrogram of 1,045 Main Spectra (MSP) showing the clustered OTUs with a cutoff of 700. Colors of the barcharts correspond to the plant sample number and the value inside indicates the number of isolates of each OTU, in each plant.

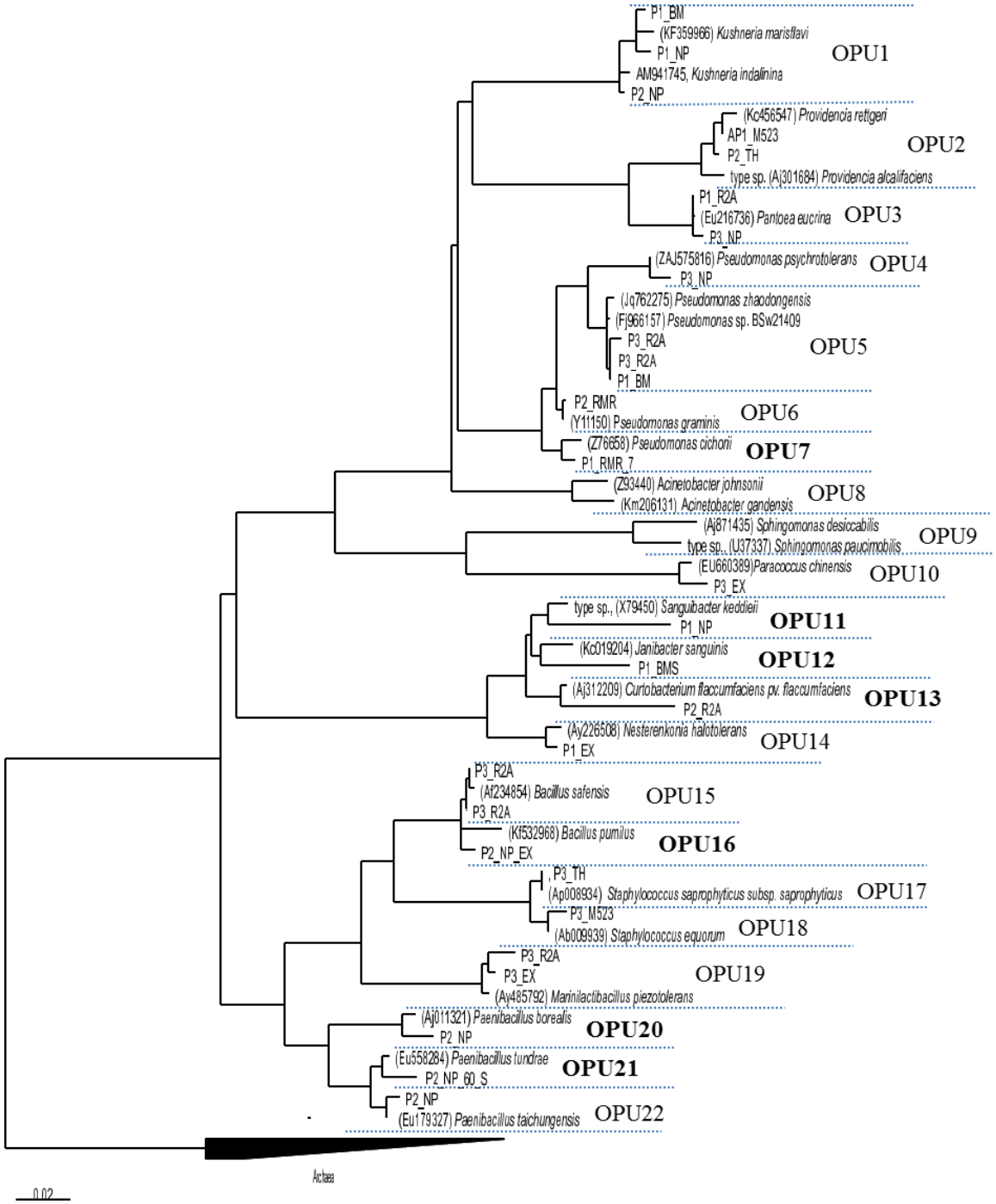

Figure 3.3 16S rRNA phylogenetic reconstruction of representative isolates, and their close relative type strains and additional reference sequences. Bold letters in OPUs include the potential new species.

The analysis of the yields by culture media comparing Morisita overlap indices showed a higher similarity (94.5%) between NP and M523. Contrarily, RMR was markedly dissimilar from the rest (ranging between 2.0 to 13.2%; Table A 3.4). These results were corroborated by a multivariate clustering where RMR was relatively more similar to BM and EX, but distinct from the other samples (Figure A 3.1). The dissimilarity of RMR medium was mainly due to a

notable dominance of *Pseudomonas* spp.: OPU6 (*P. graminis*) and OPU7 (*P. cichorii*). In addition, the Jost index revealed R2A and NP as the culture media with higher diversity, and RMR with lower diversity (Table A 3.5).

PGPA of isolates on *Arabidopsis thaliana*

The isolates for the PGPA analysis were selected avoiding clones using RAPD fingerprints generated for at least two organisms of each OTU. A total of 98 isolates were analyzed and we selected at one representative from each banding pattern. The profiles showed between 2 and 9 bands, with product lengths ranging from 250 to 4000 bp (Figure A 3.2). A total of 72 strains of 12 OPUs (1 to 6 per group; Supplementary Table S6) with different patterns were finally selected for the PGPA test. In addition, 22 strains from the five most representative moderate halophilic OPUs isolated in a previous study (Mora-Ruiz *et al.* 2015) were also incorporated to the PGPA tests (Table A 3.6).

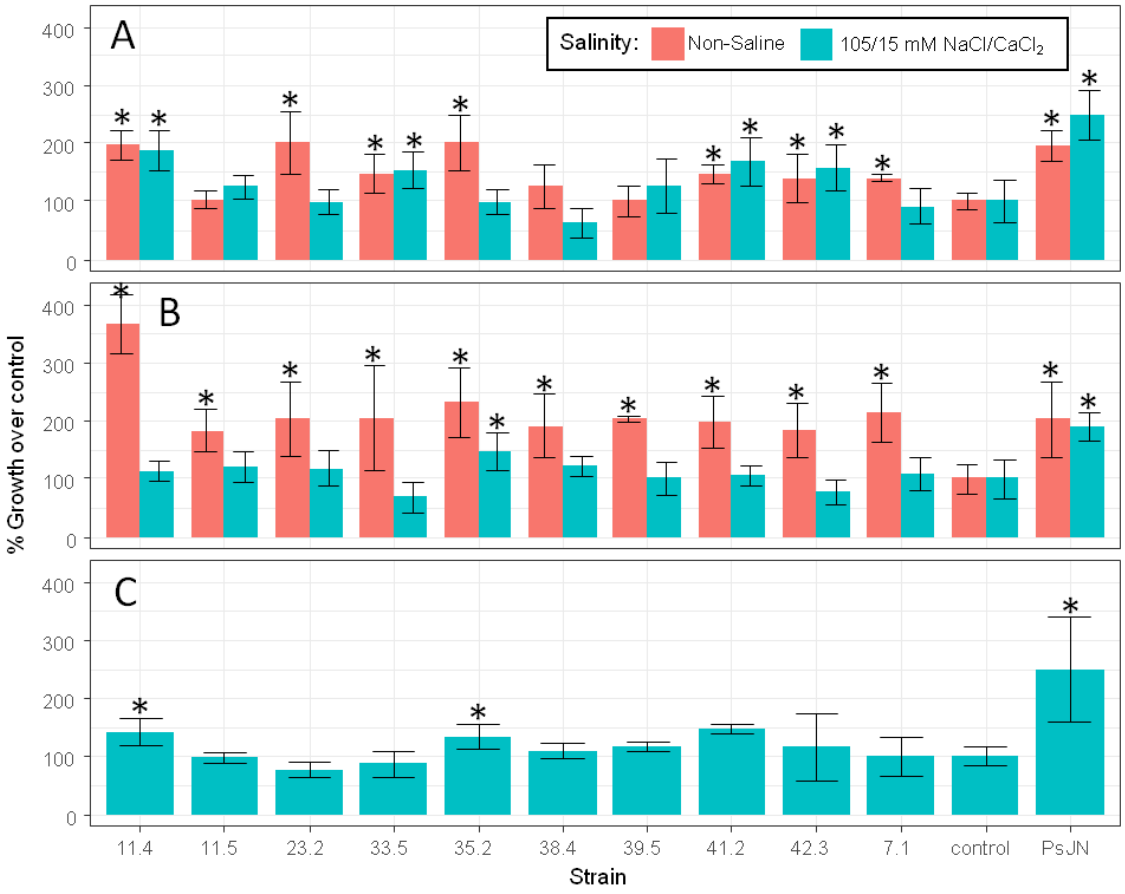

Figure 3.4 (A) Root length, (B) fresh weight and (C) Dry weight of *Arabidopsis thaliana* plants inoculated with growth promoting isolates under salt stress. *indicates significant differences with the control. *P. graminis* (strain 7.1), *P. borealis* (strain 11.4), *P. borealis* (strain 11.5), *S. equorum* (strain 23.2), *S. equorum* (strain 33.5), *K. indalinina* (strain 35.2), *Pseudomonas zhaodongensis* (strain 38.4), *B. safensis* (strain 39.5), *S. halophilus* (strain 41.2), *M. tarijensis* (strain 42.3) and *P. phytofirmans* (strain PsJN). Data are normalized relative to control (non-inoculated) plants.

The PGPA test on *A. thaliana* showed only 10 strains with higher PGPA than the control (200%), which were identified as members of the eight OPUs. From these strains, seven exhibited a significant increment of the root length in comparison with the non-inoculated control ($p<0.05$; Figure 3.4 A). The strains showing a higher PGPA affiliated with *P. borealis* (strain 11.4); *Staphylococcus equorum* (strain 33.5), *S. halophilus* (strain 41.2) and *Marinococcus tarijensis* (strain 42.3). All four consistently increased root length of *A. thaliana* both in non-saline and saline conditions. However, their performances under salt stress were all lower than the positive control *Paraburkholderia phytofirmans* PsJN (Figure 3.4, A). All the ten strains analyzed increased fresh weight under non-saline conditions (Figure 3.4, B), having *P. borealis* strain 11.4 a remarkable effect (350%). Again, their effects under salinity were much lower than those of *P. phytofirmans* PsJN, being non-significantly different from the control, except for *K. indalinina* strain 35.2 (Figure 3.4, B). Dry weight measured for the inoculated plants showed a significant increase when inoculated with *P. borealis* strain 11.4, *K. indalinina* strain 35.2 and *S. halophilus* strain 41.2 (Figure 3.4, C). No putative growth promotion activity functions, such as IAA production and the ability to use ACC as a nitrogen source (ACC deamination) were detected in any of the growth promoting isolates (Figure A 3.3).

Discussion

The endosphere of halophyte has been recently described as a hypersaline environment being the habitat of several halophilic microorganisms (Sgroy *et al.* 2009a; Mora-Ruiz *et al.* 2015). However, here we explore the possibility of finding non-halophilic in the endosphere of the halophyte *A. macrostachyum* by culture-dependent analysis as well as the PGPA of the strains isolated. Although other studies have attempted to culture the endophytic microbiota using different culture media, the analyses and identification of the isolates is usually reduced to < 200 strains (Li *et al.* 2012; Jackson *et al.* 2013; Jin *et al.* 2014). We evidenced that the use of different culture media results in a better recovery of the cultivable fraction of the plant associated endophytic microbial communities. To our knowledge this is the first study where the non-halophilic bacterial community structure from halophytes is analyzed with a large-scale culturing approach including six different kind of culture media and more than 1,000 isolates. We could recover up to 22 OPUs that can be equalized to species (Mora-Ruiz *et al.* 2016), including the isolation of taxa yet unreported as associated with plants such as *Paracoccus chinensis* (OPU10) and *Paenibacillus taichungensis* (OPU22) as well as some strains with PGPA. Moreover, we have isolated representatives of seven putative new species, demonstrating that the large-scale cultivation is a valuable tool for the retrieval of novel members of the endophytic microbiota.

From the 22 OPUs detected in this study, only eight (OPU1: *K. indalinina* / *K. marisflavi*; OPU2: *P. rettgeri*; OPU18: *S. equorum*; OPU4, OPU5 and OPU6: affiliating with

the *Pseudomonas* genus; and OPU15 and OPU16: affiliating with the *Bacillus* genus) belonged to genera that have been previously reported as endophytes in halophytes, as well as other coastal plants (Sgroy *et al.* 2009a; Amaresan, Jayakumar and Thajuddin 2014; Mora-Ruiz *et al.* 2015, 2016). The OPU1 (*K. indalinina/K. marisflavi*) was here confirmed as one of the more common taxa present in the endosphere of *A. macrostachyum* (Mora-Ruiz *et al.* 2015, 2016). In previous studies, some species of *Pseudomonas* such as *P. seleniipraecipitans, P. alcaliphila, P. pseudoalcaligenes* and *P. geniculata* were isolated from the endosphere of plants (Mora-Ruiz *et al.* 2015). However, in this work we obtained a broader species collection (i.e. *P. psychrotolerans* OPU4, *P. zhandongensis* OPU5, *P. graminis* OPU6 and *P. cichorii* OPU7). In both cases, *Pseudomonas* members presented important abundances (~22% and 25% of the total in the previous and this study, respectively) evidencing the relevance of these members in the endospheric halophyte environment.

Although our study was not successful in recovering the most abundant groups detected by culture-independent approaches (e.g. *Rudaea cellulosilytica and Cupriavius gilardii, P. seleniiparaecipitans*; Mora-Ruiz et al. 2015, 2016), we cultured nearly 5.5% of the total abundance observed by culture-independent sequencing, that with the already cultured 60% using media for moderate halophiles (Mora-Ruiz et al. 2015) represents up to 66% culturing success. In addition, we recovered groups yet unreported as endophytes such as OPU12 (*Janibacter sanguinis*), OPU14 (*Nesterenkonia halotolerans*), OPU19 (*Marinilactibacillus piezotolerans*) and OPU22 (*P. taichungensis*). All such OPUs have been previously found in sediments and soils (Newman and Reynolds 2005a; Toffin *et al.* 2005; Ryan *et al.* 2008; Li *et al.* 2009; Singh, Singh and Dubey 2013; Arora *et al.* 2014; Khessairi *et al.* 2014; Mercado-Blanco and Lugtenberg 2014; Bringel and Couée 2015; Sharma, Kaul and Dhar 2015) and they could have entered through the roots to colonize the endosphere of the plant as hypothesized for other bacteria (Chi *et al.* 2005; Rosenblueth and Martínez-Romero 2006; Liu *et al.* 2014a; Mora-Ruiz *et al.* 2016).

Here, we recovered up to 10^6 CFU g^{-1}, which are lower yields than when using seawater culture media (SW) for moderate halophiles tested in previous studies, that reached values up to 10^7 CFU g^{-1} in SW5% and SW15% (Mora-Ruiz *et al.* 2015). This one order of magnitude difference suggests that the cultivable endospheric environment might be dominated by moderately halophilic bacteria as previously hypothesised (Mora-Ruiz *et al.* 2016). In this context, the oligotrophic medium R2A produced the highest richness and diversity, and it contains 3 to 15 times lower concentrations of carbon sources (i.e. glucose, starch and malic acid) and proteins (*i.e.* peptone and casein) than the other media we used here. This fact is in accordance with nutrient-poor media being a better source for cultivable diversity (Aagot *et al.* 2001), as dominant strains with slower growth rate are not outcompeted by those fast-growing (Connon and Giovannoni 2002).The plant extract (EX) yielded three additional exclusively

OPUs; i.e. *P. chinensis* (OPU10), *N. halotolerans* (OPU14) and *B. pumilus* (OPU16). Similar studies using plant extracts despite observing an increment of abundances, did not report an increment in bacterial richness (Eevers *et al.* 2015).

Nearly 62% of the total isolates were included in four OPUs identified as *K. indalinina/K. marisflavi* (OPU1), *P. rettgeri* (OPU2), *P. zhaodongensis* (OPU5) and *B. safensis* (OPU15). Members of these species had been reported with potential nitrogen fixation capability (Mapelli *et al.* 2013), similarly to some species of the *Pseudomonas* genus (Doty *et al.* 2009). The beneficial effects of these endophytic bacteria on their host plant can occur through similar mechanisms described for plant growth-promoting rhizobacteria as previously suggested by Kloepper, Ryu and Zhang (2004) and Mercado-Blanco and Lugtenberg (2014). In accordance, our results of PGPA activity revealed a positive effect of *K. indalinina/K. marisflavi* isolates on *A. thaliana* despite no significant ACC deamination and IAA production was found. This is not necessarily intriguing because, although these functions are usually active and effective in plant growth promotion *Proteobacteria*, such as *P. phytofirmans* (Ledger *et al.* 2016), the bacterium used as positive control in this work, ACC deamination and IAA production are not necessarily responsible for the observed plant growth effects. Other functions can be linked to the PGPA of these strains such as the solubilization of phosphates (Glick and Glick 2012; Mapelli *et al.* 2013), production of volatile compounds and some enzymes as pectinase (Ledger *et al.* 2016). The latter highlights the need of using plant-bacteria assays to assess growth promotion potential, rather than screening for ACC deamination and IAA production as a first (sometimes unique) choice (Mayak, Tirosh and Glick 2004; Glick 2014).
Other strains principally isolated from a previous work (Mora-Ruiz *et al.* 2015), such as; *S. equorum* strain 33.5, *S. halophilus* strain 41.2 and *M. tarijensis* strain 42.3, also exhibited important increments of the root lengths on *A. thaliana*, with similar yields as the PGPA positive *P. phytofirmans* strain PsJN (Sessitsch *et al.* 2005). Although these strains, together with others isolated in this work showed to be efficient under non-saline conditions, their effects decreased as increase of salinity. This was not unexpected, as bacterial growth promotion of a particular growth parameter under non-stressed conditions does not necessarily imply a positive effect under saline stress. As we used the model plant organism *A. thaliana* and the strains were isolated from a halophyte, the results of lower activity in saline conditions do not imply a lack of effect on the plant host (*A. macrostachyum*). However, the election of performing the PGP analysis in the premiere model for plant biology (*A. thaliana*) is principally explained by the important features of this plant including a short generation time, small size that limited the requirement for growth facilities and prolific seed production through self-pollination. Additionally, the high amount of research publications related with this plant allows the comparison of our results with other studies (Koornneef and Meinke 2010).

Chapter 4. Halophilic endophytic *Archaea* in the halophyte *Arthrocnemum macrostachyum*.

Abstract

Endophytic microorganisms play an important role in the association with their host plants providing some metabolic products such as nutrients for the hosts and even acting as growth promoters, which can have a positive impact on their health, growth and fitness. This may be the case of halophytes, plants that can grow in hypersaline soils, partially due to their physiological adaptations to control the high osmotic pressure, but possibly also due to their endophytic microorganisms. Moreover, the endosphere of halophilic plants has recently been described as a hypersaline environment in where members of the bacterial domain (such as *Chromohalobacter canadensis, Rudaea cellulosilytica* and *Salinicola halophilus*) have been identified. However, archaeal members have not been identified properly as inhabitants of the endosphere of many plants, probably due to their low abundances and the problems associated with cultivation. In this study, we find *Archaea* associated with the endosphere of *Arthrocnemum macrostachyum*, our model halophyte. We studied plants collected in two different locations of Mallorca Island (Western Mediterranean Sea), one of them located in a solar saltern and the other in a wetland. Regarding the richness detected in our samples by culture-independent analysis, most of them affiliated with the genera *Halococcus*, *Halorubrum*, *Haloquadratum* and *Halonotius* and members of the *Nanohaloarchaeaota* phylum. The microorganisms studied in this work can be relevant in the lifestyle of the *A. macrostachyum* and we consider that the exploration of endophytic *Archaea* is necessary to unravel their role in the endosphere of plants and the effects produced in this interaction.

Keywords: *Archaea*, halophyte, *Arthrocnemum macrostachyum*

Introduction

The study of endophytic microorganisms has increased along the last decades, principally helped by culture-independent and microscopy approaches (Kloepper and Ryu 2006; Natalia V. Malfanova 2013; Weber, Videira and Simões de Araujo 2013; Bogas *et al.* 2015). The interactions between plants and their endophytic communities have been described as mutualistic, in where both organisms obtain benefits from such relation (Andreote, Azevedo and Araújo 2009). The plant would act as a source of nutrients, substrates and protection for the endophytes, whereas these endophytes might have an essential role in the development of the plant introducing nutrients, protecting against pathogens and promoting growth (Hardoim, van Overbeek and Elsas 2008; Mercado-Blanco and Lugtenberg 2014). In fact, some previous studies pointed out the difficulty to culture transplants of different plant species in the absence of bacteria (Hardoim, van Overbeek and Elsas 2008).

Although the exploration of the endosphere has been principally focused on prokaryotes, other groups like fungi or protists have been recently investigated. However, members of the archaeal domain have not received much attention despite it has been demonstrated that *Archaea* establish symbiotic relations with other organisms such as other prokaryotes, protists, arthropods, marine mollusks, marine sponges and primates (including humans) as reviewed by Wrede *et al.* (2012). The exploration of endophytic *Archaea* associated with plants has been only recently reported in a few plants such as maize (*Zea mays*) (Chelius and Triplett 2001), rice (*Oryza sativa*) (Sun *et al.* 2008) and *Phragmites australis* (Ma *et al.* 2013). All these studies were conducted by culture-independent approaches, while Reinhold-Hurek and Hurek, (2011) remarked the need to gain microscopic evidence of those microorganisms in the internal part of the plant to confirm their presence.

While *Archaea* have been found in non-extreme systems, such as seawater, terrestrial soils, lakes, marine and freshwater sediments (DeLong 1998), during the last century they were commonly related to extreme environments considering them as acidophilic, thermophilic, halophilic, etc. (Baker-Austin and Dopson 2007; Yihwa Yang, Daniel T. Levick 2007; Andrei, Banciu and Oren 2012). Among the habitats in where *Archaea* have been detected, hypersaline environments are one of the most studied. Several taxa have been detected for the first time in hypersaline environments, most of them included as members of the *Halobacteriaceae* family (Benlloch *et al.* 2002; Podell *et al.* 2014; Oren *et al.* 2016). In addition, haloarchaea had been reported as resident microbiota of some pelagic birds as the shearwater (Brito-Echeverría *et al.* 2009).

Here, we revealed and explored the presence and diversity of endophytic *Archaea* in the endosphere of plants growing in hypersaline soils, known as halophytes. We selected the halophyte *Arthrocnemum macrostachyum* because this plant can tolerate hypersaline conditions

with a mechanism based on the ion accumulation to control the osmotic pressure (Song and Wang 2014), being an excellent model to study endophytic halophilic *Archaea*. Previous studies have described the presence of halophilic bacteria within the internal tissues of *A. macrostachyum* but to our knowledge, this is the first study where endophytic (halo)archaea have been detected as inhabitants of a plant's endosphere by a combination of culture-dependent and -independent approaches. In this work, we reveal and explore the presence and diversity of *Archaea* inhabiting the endosphere of the halophyte *A. macrostachyum* by amplicon sequencing analysis, microscopy evidences and cultures.

Material and methods

Halophytes collection

Eight specimens of the halophilic plant *A. macrostachyum* were collected from Salines d'Es Trenc, in Campos (39º21'03.3'' N, 3º00'44.3'' E, Mallorca, Spain), and Alcúdia (39º50'30.11'' N, 3º7'0.09'' E, Mallorca, Spain) during January and April 2014 being the samples previously used to study the bacterial composition (Mora-Ruiz *et al.* 2016). All selected plants exhibited mature (red; RE) and green (G) shoots and were entirely collected with their respective roots (RO). Whole individual specimens were introduced in zip-lock plastic bags using sterile gloves for further processing in the laboratory.

Surface sterilization, macerated and separation of microbial fraction for DNA extraction

Three different fractions (RE, G and RO) were separated from each halophyte and between 40-90 g of each fraction were collected for further analyses. A sterilization and maceration protocol was applied to the individual parts (Mora-Ruiz *et al.* 2016). After the maceration, 15 mL were subjected to consecutive differential centrifugation and a subsequent sucrose density gradient centrifugation was performed, as previously described in Mora-Ruiz *et al.* (2015), to isolate the microbial fraction from the vegetal fraction.

PCR amplifications and 454 pyrosequencing of archaeal DNA

The first PCR amplification was performed with archaeal primers Arch21F and 1492R as previously described (Lane *et al.* 1986) and then a second PCR of five cycles with 5 μL of the initial product as a template in a final volume of 25 μL in triplicate to incorporate tags and linker into the amplicons, using the primers Arch21F-PS as forward and a variant of 907-PS as reverse primers. The resulting products were observed in 1% agarose gel electrophoresis run in 1X TAE buffer (at 50V during 45 min). All the samples showed specific bands belonging to *Archaea* (Figure 4.1) but the yields of some of these samples were insufficient for

pyrosequencing and were discarded at this point. The band of approximately 960 pb was excised and purified using the Zymoclean™ Gel DNA recovery Kit (Zymo Research, California, USA) following the manufacturer's instructions. The concentration of these barcoded amplicons was measured both with NanoDrop™ and MassRuler™ Express Forward DNA Ladder Mix (Thermo Fisher Scientific Inc., Massachusetts, USA). An equimolar mix of samples was sent to Macrogen Inc., Seoul, Korea, for sequencing using 454 GS-FLX+ Titanium technology.

Sequence trimming, OTU clustering, OPU grouping and taxonomic affiliation

Sequences were trimmed using the Mothur (Schloss et al. 2009) software under the same specifications of Mora-Ruiz et al., 2016, reducing the minimum length of quality sequences at 200 bp. Chimeras were removed with Chimera Uchime implemented in Mothur and then the sequences were grouped at 99% of identity using the UCLUST tool included in Qiime (Caporaso et al. 2010). Trimmed samples with less than 200 sequences were also discarded because subsequent analysis could not be comparable; finally ten samples were used for the analysis (G1, G3, G4, G5, RE2, RE3, RO2 and RO4 of Campos and ARE and ARO of Alcúdia). The longest read of each OTU was selected as a representative, aligned with SINA (Pruesse et al. 2012), and introduced in SILVA REF115 database in a default tree with the parsimony tool implemented in the ARB software package (Ludwig et al. 2004). The closest relative sequences were merged with the LTP115 database (Yarza et al. 2010) and then a phylogenetic reconstruction performed following Mora-Ruiz et al. 2016. Finally, OTU representatives were inserted at the final tree, in which these were clustered in OPUsby visual inspection (França et al. 2014; Mora-Ruiz et al., 2016).

Visualization of endophytic archaea using CARD-FISH

The CARD-FISH approach was performed in red and green shoots of *A. macrostachyum.* The samples were fixed in two different ways, both yielding positive results. First, complete shoots of the plant were submerged in formaldehyde (final concentration, 4% [vol/vol]) for 24 hours at 4ºC. These shoots were macerated to obtain the plant extracts. The second method consisted of fixing 0.5 ml of plant extract in formaldehyde (final concentration, 4% [vol/vol]) for 24 hours at 4ºC. After incubation, samples were centrifuged (10 min, 15,700 *xg*, 4ºC). The supernatant was discarded and the pellet resuspended in 1 mL of 4X PBS. The process was repeated and 500 µL of 100% ethanol added. After 30 min incubation at –20ºC, ethanol was discarded by centrifugation (10 min, 15,700 *xg*, 4ºC). To minimize the interferences due to the presence of residues of chlorophylls, 5 µl of plant extract were mixed in 10 ml of 1x saline phosphate buffer

(PBS) and filtered through 0.2-µm-pore-size isopore polycarbonate membrane filter (diameter 47 mm; GTTP Millipore, USA). CARD-FISH was performed using a slightly modified standard protocol (Pernthaler et al., 2002), and the commonly used probes for *Bacteria* (EUB338-I (Amann et al., 1990); EUB338-II and –III; Daims et al., 1999), *Archaea* (ARCH915; Raskin et al., 1994) and negative control (NON338; Wallner et al., 1993) were used using the recommended stringency conditions.

Isolation of endophytic archaea

The halophyte's macerated biomass was also used for the isolation of endophytes. Fifty mL of plant extract was mixed with 20 mL of 25% Sea Water (SW) culture media (Rodríguez-Valera et al. 1985). To remove vegetal fragments, this mixture was filtered through 40 µm Cell Strainer (Biologix Research Company, USA) and centrifuged (15,700 xg, 5 min, RT) to concentrate the cells. The supernatant was discarded and 2 mL of SW 25% were added to the precipitated, which was used as inoculum. One hundred µL of each sample were inoculated in 25% SW and 30% SW (Rodriguez-Valera *et al.* 1985a) culture media supplemented with 0.05% yeast extract. Serial dilutions were performed until 10^{-2} and plates were incubated at room temperature during two months. Additionally, 200 µL were inoculated in 25% SW and 30% SW liquid media for an enrichment of fast growing organisms. All liquid cultures were incubated at 30ºC with shaking at 125 rpm with a Kuhner Shaker X (ISF1-X, Climo Shaker). Sterilized water was used as a negative control both for solid and liquid cultures. All samples were inoculated by triplicate.

Statistical analysis and ecological indices

The diversity was measured using Jost index (Jost 2006) order q=0 (richness) and q=0 (diversity). With the aim to compare the archaeal communities a Principal Coordinates Analysis (PCOA) was performed using all the OPUs matrix and calculating Euclidean distance. PCO was obtained with PAST *v* 3.01 software (Hammer, Harper and Ryan 2001).

Results

Sequences analysis and Operational Taxonomic Units (OPU) approach

Our study generated a total of 38,893 sequences, which were initially clustered into 981 OTUs and finally grouped in 56 OPUs, making a range per sample from 7 to 34 OPUs (Table 4.1, Table 4.2, and Table A 4.1). Except for two OPUs (OPUs 33 and 39), all sequences affiliated to known genera and a 73.0 ± 16.1% of them to known species. With the exception of OPU56, classified into the *Nanohaloarcheaota* phylum, all OPUs were affiliated with the *Euryarchaeota* phylum. The distribution at the genus level showed a dominance of eight genera (Figure 4.1), being *Halococcus, Halorubrum, Haloquadratumm* and *Halonotius* the most abundant. At the

species level, the most abundant OPUs were OPU26 (*Haloquadratum walsby*), OPU41 (*Halococcus qingdaonensis*), OPU40 (*Hcc. dombrowskii*), OPU11 (*Halorubrum californiense*) and OPU7 (*Hrr. orientale*). Although these were the most abundant OPUs, none of them was detected in all samples; in fact in any case one OPU was detected as ubiquitous. However, for the samples taken in Campos we found five OPUs ubiquitous in all plants: OPU1 (uncultured *Halonotius*), OPU6 (uncultured *Halorubrum*), OPU9 (uncultured *Haloquadratum*), OPU11 (*Hrr. californiense*) and OPU26 (*Hqr. walsby*).

Regarding the diversity indices, diversity was higher in the green shoots (max. value of 10.2), while red and root fractions showed a lower diversity (max. value of 7.4). On the other hand, the ordination analysis showed that Campos samples were distributed closer than Alcúdia samples (Figure 4.2). Samples from Alcúdia were apparently more different, especially ARO, which seemed to be the most different sample. Alcúdia samples presented a dominance of OPU40 (*Hcc. dombrowskii*). Additionally, ARE also had elevated abundances of OPU26 (*Hqr. walsby*) as well as Campos samples. Regarding the Campos samples, we observed a higher similarly among green shoots and with RE2, all of them dominated by OPU6 (uncultured *Halorubrum*) and OPU26 (*Hqr. walsby*). The additional samples from Campos presented an elevated abundance of OPU41 (*Hcc. qingdaonensis*).

Table 4.1 Number of OTUs and Jost diversity indices and number of sequences per sample.

Sample	# Sequences	OTUs	Jost=0	Jost=1
G1	1100	76	34	9.37
G3	1344	273	27	8.5
G4	16607	101	26	8.7
G5	3618	163	31	10.22
RE2	1062	78	24	6.99
RE3	1680	75	22	2.42
RO2	6457	156	28	4.37
RO4	1435	23	16	2.03
ARE	260	22	14	7.4
ARO	240	17	7	4.4

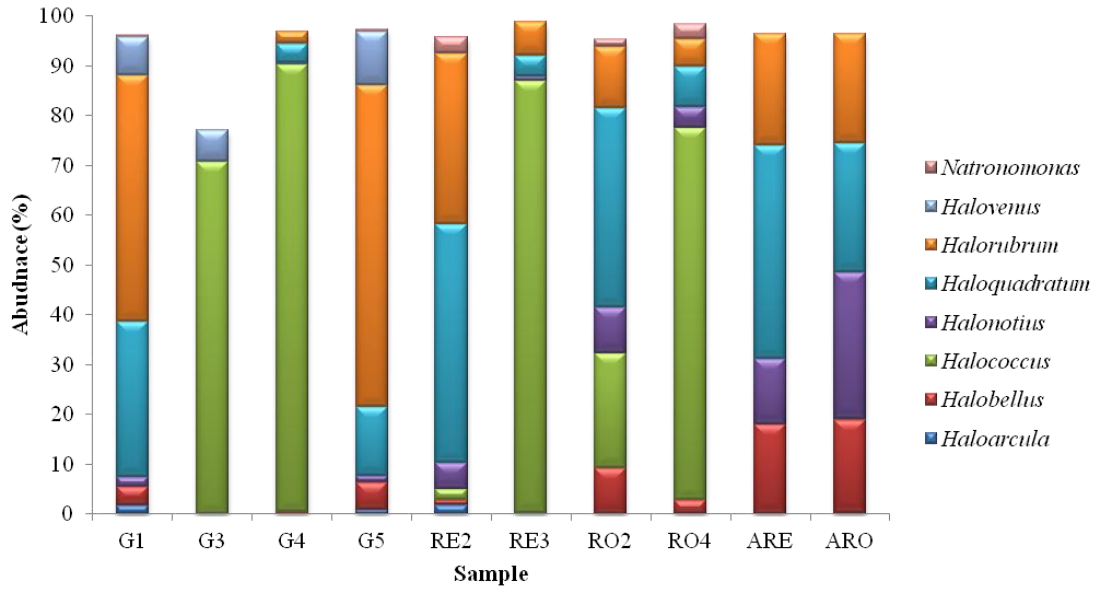

Figure 4.1 Distribution of the most abundant genera of the endophytic *Archaea* in *Arthrocenmum macrostachyum*.

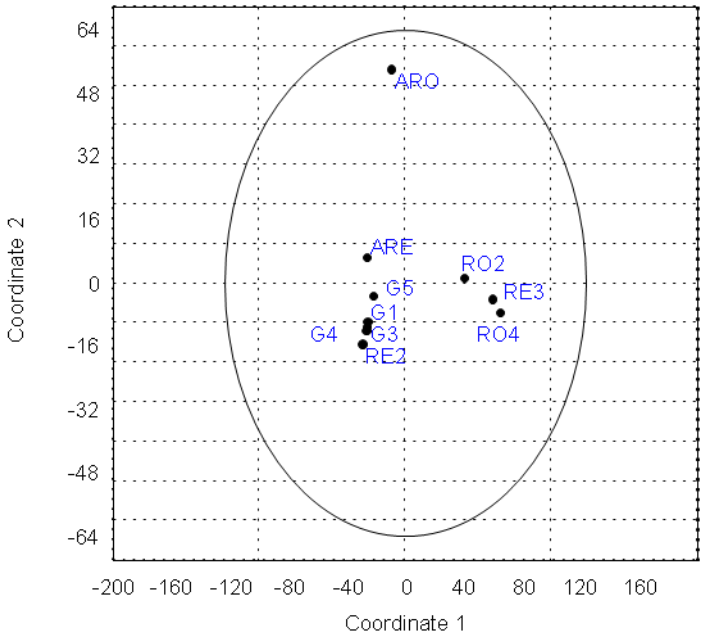

Figure 4.2 *Principal* Coordinates Analysis (PCO) of relative abundances of endophytic *Archaea* in *Arthrocnemum macrostachyum*. Values of coordinates: 1: 66.3%; 2: 17.8%

Table 4.2 Most relevant OPUs with total abundances (above 90% of the total pyroquencing data). From left to right: the number of OPU (in bold) and the affiliation of the sequences with the closest relative sequence; accession number; identity value; and samples with their relative abundances regarding to the total sequences for each sample.

			Samples									
OPU and affiliation	**accession number**	**% identity**	**G1**	**G3**	**G4**	**G5**	**RE2**	**RE3**	**RO2**	**RO4**	**ARE**	**ARO**
1 Uncultured *Halonotius*	FN391233/FN669147	>89% <99.9%	1.5	5.9	5.9	1.0	2.9	0.3	0.3	0.4	1.5	0.0
2 *Halonotius pteroides*	AY498641	>99.4% <99.9%	0.2	0.8	14.0	0.0	0.0	0.1	1.7	0.0	4.6	0.0
3 Uncultured *Halonotius*	CU467165/AM947475	>93.4% <99.7%	0.0	3.0	4.5	0.0	0.0	0.0	0.9	0.0	3.1	0.0
4 Uncultured *Halorubrum*	AM947495/AYLI01000322	>90% <98.7%	7.8	0.1	0.1	6.0	0.1	1.0	0.0	0.0	0.0	0.0
5 Uncultured *Halorubrum*	KF234325/GQ375011	>98%	0.2	0.0	0.0	0.3	7.2	0.0	0.0	0.0	0.0	0.0
6 Uncultured *Halorubrum*	KJ546109	>86.5%	10.4	19.1	19.2	7.6	22.3	4.5	4.7	0.4	6.2	0.0
7 *Halorubrum orientale*	AM235786	>94.2% <99.4%	0.0	0.0	0.0	0.2	3.6	0.4	0.0	0.4	0.0	0.0
9 Uncultured *Haloquadratum*	FN669145/AY987833	>92% <99.7%	0.9	2.5	0.4	0.5	4.4	0.8	0.3	0.4	0.0	0.0
11 *Halorubrum californiense*	LN649801	>87.3% <98.6%	0.6	0.1	0.1	32.9	0.1	0.4	0.1	0.4	0.0	0.0
13 Uncultured *Halorubrum*	AM947498/CU467133	>98.2% <99.5%	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	6.2	0.0
14 *Halorubrum* sp. MG525	GU361142	>99% <99.9%	0.2	2.5	1.7	6.4	0.0	0.0	0.0	0.0	0.0	0.0
16 *Halorubrum tebenquichense*	FR870448	>98.4% <99.6%	0.2	0.0	0.0	5.7	0.0	0.0	0.0	0.0	0.0	0.0
17 *Halorubrum* sp. halo-10	KJ573440	>92.7% <99.1%	26.0	0.0	0.0	0.9	0.0	0.0	0.7	1.1	0.0	0.0
19 *Halorubrum* sp. ST_22S88	LN649893	>99.1%	2.7	0.0	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0
23 Uncultured *Natronomonas*	HQ425159	>95% <99.3%	0.4	0.0	0.0	0.5	2.1	0.0	0.0	0.0	1.5	0.0
26 *Haloquadratum walsbyi*	AM180088	>96% <99.9%	28.0	26.8	23.8	13.1	42.8	2.7	7.4	3.5	38.5	0.0
27 Uncultured *Haloquadratum*	GQ375018/AM947441	>93.4% <99.2%	1.6	11.8	0.2	0.3	0.3	0.4	0.1	0.0	0.0	0.0
29 Uncultured *Halonotius*	AM947444/CU467178	>92.8% <99.7%	0.0	3.5	4.8	0.0	2.4	0.6	1.3	0.0	0.0	0.0
30 *Halobellus salinus*	HQ451075	>88.3% <99.9%	3.1	16.0	16.1	5.2	0.0	0.1	0.1	0.0	4.6	0.0
31 Uncultured *Halobellus*	DQ103672/FN391240	>95.5% <99.5%	0.7	2.0	2.6	0.2	0.9	0.0	2.6	0.4	4.6	0.0
40 *Halococcus* sp. NCIMB 734 / *H. dombrowskii*	AB074302/AJ420376	>87.4% <99.7%	0.0	0.1	0.1	0.0	1.3	5.9	11.1	1.4	21.5	54.2
41 *Halococcus qingdaonensis*	AY243109	>91.7% <99.6%	0.0	0.0	0.0	0.0	0.9	80.5	62.0	86.1	1.5	6.3
42 *Halococcus hamelinensis*	DQ017835	>93% <97.6%	0.0	0.0	0.1	0.0	0.0	0.1	0.0	1.1	0.0	0.0
43 *Halococcus agarilyticus*	AB748562	>88.5% <99.5%	0.0	0.0	0.0	0.0	0.0	0.4	1.8	1.4	0.0	10.4
44 Uncultured *Halomarina*	HQ400562	>96.7% <98.5%	0.0	0.0	0.0	0.0	0.0	0.1	0.0	2.4	0.0	6.3
54 Uncultured *Halovenus*	AM947493	>94.7% <99.8%	2.2	0.0	0.0	4.3	0.0	0.0	0.0	0.0	0.0	0.0
55 *Halovenus aranensis*	HQ197980	>95.9% <99.8%	5.6	0.0	0.0	6.6	0.0	0.0	0.0	0.0	0.0	6.3
56 Uncultured *Nanohaloarchaeota* / Candidatus *Haloredivivus*	GQ374934	>99.1%	0.9	0.0	0.0	0.0	0.4	0.0	0.2	0.0	0.0	12.5
		TOTAL	**91.5**	**92**	**90.8**	**94.8**	**90.3**	**98.2**	**92.4**	**98.6**	**93.9**	**95.8**

Microscopic evidence of endophytic archaea

For the observation of endophytic *Archaea* we used CARD-FISH with the Alexa 633 dye to minimize the chlorophyll interference. Very low occurrence of *Archaea* was observed in different tissues, being principally observed in green shoots (Figure A 4.2). The microbial morphologies observed were mainly pleomorphic and squared. Additionally, we also present micrographs of cells, which hybridized with the bacterial probe (Figure A 4.3).

Isolation of endophytic archaea

No growth was observed in any of the negative controls. Growth was observed after 40 days in 25% SW culture at 10^{-1} dilution in both locations. Regarding the liquid media, they exhibited light yellowish color firstly in 25% SW after one month. Colonies principally presented a circular or irregular form, raised elevation, entire and undulate margin with pink, red or white colors. For microscopic observation, some colonies of the plates cultures from Campos and Alcúdia were selected. A total of eight isolates were visually inspected using bright field microscopy. The results exhibited two principal morphologies: bacilli shape (white color) and coccids (red color). The identification process of the isolates by Sanger Sequencing of the 16S rRNA is currently in process.

Discussion

The present work is focused on the exploration of possibility to detect archaeal endophytes within the tissues of *A. macrostachyum* because this plant is able to tolerate hypersaline conditions growing in substrates with salinities up to 1,030 mM NaCl (Redondo-Gómez *et al.* 2010). *A. macrostachyum* can tolerate repeated exposure to seawater (Breckle, 2002; Flowers and Colmer, 2008) by a mechanism based on the compartmentalized ion accumulation to control the osmotic pressure (Song and Wang, 2015). Furthermore, some studies showed that the associations formed between the host plant and the endophytic microbial communities, allow them to deal with the environmental stress, such as saline stress (Ruppel, Franken and Witzel 2013; Gupta, B. & Huang 2015). Thus, *A. macrostachyum* is a proxy for the study of halophilic microorganisms as previously reported by Mora-Ruiz *et al.* 2015, 2016.

The presence of endophytic *Archaea* had been already suggested or even detected by culture-independent approaches (Ma *et al.* 2013; Müller *et al.* 2015). However, no microscopy evidence had been reported yet. Our results using FISH indicated the presence of pleomorfic and squared *Archaea* (by CARD-FISH), However, we could retrieve cultures of extreme halophiles from our samples. Unfortunately, the identification by 16S rRNA gene sequencing is still in process, which will define the identity of the isolates. Here we rely on the affiliation process performed by the OPU approach based on the NGS results.

Regarding to the total distribution of our samples, those from Alcúdia, and specially ARO, seem to be different in community structure to Campos samples. This is unexpected since geographic distance has been reported to be a crucial factor affecting the structure of endophytic microbial communities (e.g. short distance (<100 km) in Mora-Ruiz *et al.* 2016). Additionally, the endophytic archaeal communities (as in bacteria) have been suggested to be principally influenced by the diversity hosted in the soil, through colonization processs (Reinhold-Hurek and Hurek 2011), and therefore, the differences in the microbial composition of the soil may be directly reflected in the endophytic community. Although diversity in the soils from Alcúdia was higher than in Campos (as seen in Chapter 3), endophytic richness of both *Bacteria* (Chapter 3) and *Archaea* (reported here, Table 4.1) were higher in Campos plants. This suggests that the endosphere colonization is a selective process only achieved by several specific taxa: for example the study of Lemanceau *et al.* (1995) where flax (*Linum usitatissinum)* and tomato (*Lycoperiscon esculentum*) attracted specific strains of *Pseudomonas*; or the study of Kloepper et al., (2004) where *Arabidopsis thaliana* recruited selectively *Bacillus* spp. to enhance immunity and prevent pathogenic attack. Moreover, Müller *et al.* (2015) suggested the presences of *Thaumarchaeota* (Soil Crenarchaeota Group) in the endosphere of olives due to the colonization of those *Archaea* from the roots to the endophyllosphere. Although no *Thaumarchaeota* OPUs were found in our study, we detected taxa that have been commonly described inhabiting hypersaline sediments and brines (López-López *et al.* 2010; Mora-Ruiz,

submitted). Although the origin of the endophytic *Archaea* may be principally from the soil, we cannot exclude the hypothesis that some taxa may have an origin related with the sea spray (in Alcúdia) or crystallizer brines (in Campos), which may be constantly influencing the exophyllosphere of the plants living in the studied regions such as *A. macrostachyum*.

An interesting example was the presence of *Halococcus* spp., one of the most abundant genera detected in this study. Although there are some problems related with the DNA extraction of *Halococcus* spp. (Kandler and König 1998; Fendrihan *et al.* 2009), in our case an important proportion of reads affiliated with this genus that principally accumulated in red fractions. Although *Halococcus* has been principally detected in salterns, members of this taxa have also found in other environments such as the nostrils of the seabird *Calonectris diomedea*. Brito-Echeverría *et al.* (2009). There the major members affiliated the two species *Hcc. morrhuae* and *Hcc. dombrowskii*. Other environments with presence of *Halococcus* include stromatolites in Shark Bay, Australia (Goh et al. 2006), Permian alpine salt deposits (Legat et al. 2002), Thailand fish sauce (Namwong et al. 2007), salted-ripened anchovy (Felix et al. 2016) and salted curate fish (Pasad y Seenayya, 2000). In this sense, the presence of this genus is another evidence of their versatility, being capable to colonize a wide range of environments (Brito-Echeverría et al., 2009).

On the other hand, the results of the diversity indices exhibited a higher diversity in green shoots while previous reports in *Bacteria* found the opposite behavior, with higher diversity in roots and a consecutively decrement to the red shoot (Mora-Ruiz *et al.* 2016). Finally, although the number of sequences reported for Alcúdia were low. However, those sequences are evidence that halophilic *Archaea* can be found in the endosphere of such plants not directly located close to salterns. Therefore, the endosphere of these plants seem to be a suitable habitat for the halophilic microorganisms.

Microbial diversity in salterns

No había más que la inmovilidad y el silencio en las tinieblas, en la noche. Estaba también solo El Creador, El Formador, El Domador, El Serpiente cubierta de Plumas. Los que engendran, los que dan la vida, están sobre el agua como una luz creciente.

Popol Vuh

Chapter 5. Biogeographical patterns of bacterial and archaeal communities of distant hypersaline environments

Abstract

Microorganisms are globally distributed, however new evidences show that the microbial structure of the communities can vary due to their geographical location and environmental parameters. We analyzed 50 samples including brines and sediments from Europe, Africa and South America applying the Operational Phylogenetic Unit (OPU) approach in order to understand whether microbial community structures in hypersaline environments exhibit biogeographical patterns. The fine-tuned identification of about 1000 OPUs (almost equivalent to "species") using multivariant analysis revealed regionally distinct taxa compositions. This segregation was more diffuse at genus level, pointing to a phylogenetic and metabolic redundancy at higher taxa level, where their different species acquired distinct advantages related to the regional physicochemical idiosyncrasies. We also evidenced the presence of groups not previously described in such environments as Parcubacteria, or the presence of members of *Nanohaloarchaeota* in anaerobic hypersaline sediments. Finally, we observed an important OPU overlap between anoxic sediments and their overlaying brines, pointing to a versatile metabolism of the pelagic organisms.

Keywords: *Archaea*, *Bacteria*, brines, hypersaline sediments, Operational Phylogenetic Units, salterns.

Introduction

In the last decades, the paradigm of the global distribution of microorganisms ("everything is everywhere") has been constantly questioned (de Wit and Bouvier 2006; O'Malley 2008). Traditional explanations for cosmopolitan distributions are large microbial population sizes, high probability of dispersion and low probability of extinction (Fenchel 2003). The actual conception of microbial biogeography and the possibility of non-random distribution of microorganisms are currently a hot topic (Fierer and Jackson 2006; Jeffries *et al.* 2015; Roeselers *et al.* 2015). The analysis of microbial communities sampled at different distant locations can facilitate the understanding of the underlying drivers causing the differentiation of population/communities (Habel *et al.* 2014). There are a few studies performed on the global distribution of microorganisms, mostly focused on their possible pathogenic effects (Duron, Cremaschi and McCoy 2016; Limmathurotsakul *et al.* 2016). Nonetheless, the attention has drifted out from clinical microbiology towards global environmental studies including oceanic sediments (Kallmeyer *et al.* 2012), ocean waters (Cabello *et al.* 2016) and hypersaline environments such as salterns (Benlloch *et al.* 2002; Whitaker, Grogan and Taylor 2003; Gomariz *et al.* 2014).

Extreme environments, due to their isolated nature, often scattered in different geographical points without direct connexions are excellent systems to evaluate biogeographical patterns and allopatric speciation (Whitaker 2003; Rosselló-Mora *et al.* 2008b). Hypersaline environments are globally distributed in different climatic regions including some of the most extreme environments such as the Atacama desert, or the Arctic and Antarctic regions (Niederberger *et al.* 2010; Farías *et al.* 2014; Williams *et al.* 2014; Fernández *et al.* 2016). Therefore, they offer an excellent opportunity to compare complex halophilic communities in very distant yet similar hypersaline environments around the world. Depending on the origin of the ionic composition of their brines, salterns can be divided in thalassohaline and athalassohaline. Briefly, thalassohaline salterns present a similar ionic composition to seawater and ultimately occur upon evaporation of seawater or dissolution of evaporite rocks. On the other hand, athalassohaline hypersaline environments show multiple different ionic compositions, distinct from seawater, which depends directly on the composition of the surrounding substrate (Rodríguez-Valera, Acinas and Antón 1998).

Generally, diversity in hypersaline environments is dominated by halophillic microorganisms that belong to the bacterial and the archaeal taxa such as *Bacillus* (Kim *et al.* 2012) and *Salinibacter* (Antón *et al.* 2002), and *Haloquadratum, Halorubrum* (Dillon *et al.* 2013) and the candidate division *Nanohaloarchaeota* (Andrei *et al.* 2015) respectively. Despite the local descriptions of hypersaline environments, a global vision of these environments is still necessary to understand microbial adaptation to different environmental conditions and its

functioning (Lozupone and Knight 2007). Also, the analysis of physico-chemical characteristics is necessary for achieving a better understanding of the habitats, as well as the response of the microbial communities to environmental variations (López-López *et al.* 2010; Podell *et al.* 2014; Patadia 2015).

Recently, Filker *et al.* (2017) using some of the samples of the present study found a high degree of novel genetic diversity and also a strong effect of the geographical distance in the protistan communities. We consider salterns as suitable systems to test the hypotheses of biogeographic heterogeneity for prokaryotic communities and the possible effect of environmental parameters on them. To complement the biogeographical findings on the protist global patterns we characterized the structure of the bacterial and archaeal communities in a larger set of hypersaline sediments and brines in geographically distant salterns from Europe, Africa and South America. We also compared coastal seawater-fed (thalassohaline) and inland endorheic (athalasohaline) systems.

Materials and methods

Sampling sites and sample collection

Between January and March of 2011, a total of 17 brines and 33 sediment samples were collected from 27 sites in ten locations from Spain, Argentina and Chile (Table 5.1; Figure 5.1).). From the samples collected, six were located in the Mediterranean region: four from insular coastal salterns (Balearic Islands Mallorca (SP-IB1to SP-IB5), Ibiza (SP-IB8), and Formentera (SP-IB6 and SP-IB7); and two from the peninsular coastal salterns (Santa Pola (SP-VC1 and SP-VC2) and La Trinidad (SP-AR1and SP-AR2). One sampling site was located in the inland saltpan Peñahueca (SP-CM1 to SP-CM7) situated in the centre of the Spanish Peninsula, at about 500 meters above sea level (masl). In the North Atlantic region two insular salterns were sampled in the islands of Fuerteventura (SP-CN1 and SP-CN2) and Lanzarote (SP-CN3 and SP-CN4) (Canary Islands, Spain), also at the sea level and adjacent to the coast. Nine sampling sites were located in the Argentinean Altiplano, all situated between 3000 and 5000 masl, considered athalassohalines and located in the regions of Salta and Catamarca (ARG1 to ARG23). Finally, one sampling site in the Chilean coast of the Pacific Ocean was located in the Boyeruca salterns (CHL1 to CHL4), at the sea level and adjacent to the coastline. In all cases, sediments were extracted using methacrylate cores and brines filling sterilized bottles. All samples were stored at 4°C until processing.

Table 5.1 Location and parameters analyzed for samples from Spain (SP), Argentina (ARG) and Chile (CHL). Coun.=Country.

ID	Location	Coun.	Region	Type	Altitude (masl)	Sample source	Salinity [%]	Coordinates Lat	Coordinates Lon	Cl^-	SO_4^{2-}	Br^-	NO_3^-	NO_2^-	F^-	PO_4^{3-}	Na^+	Mg^{2+}	Ca^{2+}	K^+	NH_4^+	Li+
SP-IB1	S´Avall 1	Spain	Mediterranean	Coastal	0	S	27	39.32	2.99	175698.8	26248.8	633.8	202.5	0	31.3	0	95903.8	14640	3732.5	4667.5	0	0.000
SP-IB2	S´Avall 2				0	S	28	39.32	2.99	184931.3	23160	775	112.5	0	27.5	0	97333.8	17770	1257.5	5816.3	0	0.000
SP-IB3	Campos 1				0	S	28	39.35	3.01	34421.3	34947.5	152.5	136.3	0	32.5	0	18387.5	3033.8	15038.8	1046.3	0	0.000
SP-IB4	Campos 2				0	S	27	39.35	3.01	27323.8	21148.8	146.3	150	0	35	176.3	13776.3	3376.3	8931.3	1112.5	0	0.000
SP-IB5	Campos 2				0	B	31	39.35	3.01	195503.8	26541.3	1096.3	137.5	0	21.3	22.5	95475	20203.8	1476.3	6706.3	0	0.000
SP-IB6	Formentera 1				0	S	24	38.73	1.42	164018.8	74026.3	1112.5	96.3	0	30	0	86150	29996.3	66.3	7856.3	0	0.000
SP-IB7	Formentera 2				0	S	25	38.73	1.42	170176.3	18033.8	517.5	131.3	0	33.8	0	94937.5	12626.3	1357.5	3653.8	0	0.000
SP-IB8	Ibiza				0	S	27	38.85	1.40	185046.3	30596.3	1206.3	171.3	0	26.3	0	90132.5	22675	101.3	9086.3	0	0.000
SP-VC1	Santa Pola 1				0	S	31	38.19	-0.59	166725	40533.8	1013.8	168.8	0	25	0	71036.3	30328.8	121.3	8456.3	0	0.000
SP-VC2	Santa Pola 2				0	S	32	38.19	-0.59	191635	60063.8	1340	137.5	0	30	0	76691.3	39893.8	197.5	14483.8	0	0.000
SP-AR1	Salines Trinidad 1				0	S	29	40.53	0.69	179445	17003.8	571.3	135	0	30	0	96600	15395	265	3958.8	0	0.000
SP-AR2	Salines Trinidad 2				0	S	29	40.58	0.69	179287.5	21631.3	687.5	112.5	0	28.8	0	91645	19183.8	192.5	5177.5	0	0.000
SP-CM1	Peña Hueca 1		Central Spain	Inland	529	S	35	39.52	-3.34	42980	18980	53.8	167.5	10	32.5	6211.3	25080	5580	5843.8	1705	108.8	0.000
SP-CM2	Peña Hueca 2				529	S	36	39.52	-3.34	40030	15981.3	57.5	170	0	47.5	6535	23130	5303.8	5448.8	1838.8	115	0.000
SP-CM3	Peña Hueca 3				529	S	37	39.52	-3.34	35595	15416.3	55	170	23.8	43.8	4052.5	20856.3	5355	4727.5	1467.5	83.8	0.000
SP-CM4	Laguna Azul				529	S	39	39.51	-3.34	448855	85268.8	305	123.8	13.8	33.8	0	13146.3	165922.5	1205	10285	47.5	0.004
SP-CM5	Laguna Azul				529	B	29	39.51	-3.34	64495	17506.3	91.3	233.8	12.5	25	4632.5	34803.8	6796.3	6940	1438.8	0	0.000
SP-CM6	Laguna Amarilla				529	S	30	39.50	-3.35	89196.3	43541.3	16.3	236.3	11.3	25	91.3	48657.5	12171.3	9246.3	572.5	0	0.004
SP-CM7	Laguna Amarilla				529	B	43	39.50	-3.35	244046.3	69350	100	318.8	11.3	21.3	72.5	23258.8	87410	556.3	3830	0	0.005
SP-CN1	Fuerteventura 1		Atlantic	Coastal	0	S	35	28.36	-13.86	164988.8	102095	911.3	132.5	0	30	0	72770	37341.3	66.3	32205	0	0.000
SP-CN2	Fuerteventura 2				0	S	36	28.36	-13.86	183323.8	24813.8	665	153.8	0	27.5	0	94801.3	19527.5	57.5	5483.8	0	0.000
SP-CN3	Lanzarote 1				0	S	32	28.94	-13.82	183455	41887.5	1318.8	116.3	0	32.5	0	76897.5	33230	192.5	10022.5	0	0.000
SP-CN4	Lanzarote 2				0	S	31	28.94	-13.82	194198.8	59712.5	1787.5	115	0	31.3	0	68753.8	45125	75	15000	0	0.000
ARG1	Salar de COIPA	Argentina	Altiplano	Inland	3650	S	39	-24.52	-68.21	82696.3	69511.3	108.8	192.5	11.3	75	57.5	73306.3	6151.3	11795	9511.3	201.3	0.062
ARG2	Salar de COIPA				3650	B	31	-24.52	-68.21	136435	461.3	1622.5	151.3	0	46.3	97.5	86382.5	826.3	1671.3	2311.3	0	0.007
ARG3	Laguna Santa María				3508	S	45	-24.09	-67.36	116960	11570	225	143.8	0	212.5	2490	86503.8	156.3	278.8	32800	38.8	0.055
ARG4	Laguna Santa María				3508	B	43	-24.09	-67.36	24782.5	3673.8	153.8	252.5	10	75	95	18198.8	586.3	4208.8	3060	0	0.071
ARG5	Ojo Naranja Antofalla				3338	S	29	-25.57	-67.60	177070	18762.5	2710	271.3	0	103.8	172.5	120201.3	210	196.3	12162.5	0	0.082
ARG6	Ojo Naranja Antofalla				3338	B	28	-25.57	-67.60	815193.8	22668.8	622.5	118.8	0	31.3	43.8	541450	262.5	3440	5395	0	0.132
ARG7	Ojo Naranja Antofalla				3338	B	29	-25.58	-67.59	195152.5	21312.5	107.5	232.5	0	18.8	96.3	132423.8	825	197.5	5555	0	0.062
ARG8	Ojo Blanco de				3338	S	34	-25.56	-67.59	177121.3	13616.3	0	113.8	0	12.5	0	115555	597.5	1766.3	2592.5	0	0.079
ARG9	Ojo Blanco de				3338	B	35	-25.56	-67.59	915851.3	63235	0	196.3	0	33.8	141.3	600277.5	1406.3	26301.3	1927.5	0	0.007
ARG10	Ojo Seco de Antofalla				3338	S	34	-25.55	-67.57	49442.5	114915	0	140	0	40	0	29250	486.3	48786.3	317.5	0	0.009
ARG11	Ojo Seco de Antofalla				3338	B	32	-25.55	-67.57	30570	4838.8	12.5	190	10	17.5	23.8	16497.5	1553.8	1985	1220	50	0.087
ARG12	Laguna Diamante				4560	S	33	-26.03	-67.04	121300	88563.8	202.5	136.3	0	12.5	23.8	95535	8875	801.3	11323.8	38.8	0.044
ARG13	Laguna Diamante				4560	B	32	-26.03	-67.04	170348.8	25778.8	102.5	212.5	11.3	25	36.3	102081.3	3630	7451.3	10178.8	281.3	0.082
ARG14	Laguna Cabe				4255	S	35	-26.25	-67.06	193632.5	23362.5	36.3	606.3	0	13.8	0	111347.5	6913.8	1230	14267.5	0	0.082
ARG15	Lagua Cabe				4255	B	31	-26.25	-67.06	160251.3	20597.5	51.3	186.3	12.5	30	0	81285	3337.5	26446.3	2517.5	36.3	0.116
ARG16	Salar de Pocitos				3673	S	31	-24.37	-66.98	383667.5	3498.8	173.8	595	0	125	0	195950	11973.8	21776.3	7606.3	0	0.027
ARG17	Salar de Pocitos				3673	B	43	-24.37	-66.98	146721.3	18447.5	226.3	218.8	0	263.8	3345	136601.3	293.8	845	36996.3	0	0.008
ARG18	Tolar Grande				3508	S	40	-24.55	-67.49	142837.5	14677.5	276.3	390	12.5	328.8	3583.8	103825	418.8	116.3	41258.8	43.8	0.859
ARG19	Tolar Grande				3508	B	37	-24.55	-67.49	166895	1386.3	2033.8	165	0	67.5	103.8	101936.3	1836.3	3795	8047.5	356.3	0.644
ARG20	Salar de Llullialliaco				3677	S	36	-24.8	-68.29	189111.3	1962.5	1387.5	190	0	16.3	0	113386.3	1375	915	7513.8	0	0.016
ARG21	Salar de Llullialiaco				3677	B	36	-24.8	-68.29	190227.5	1855	13.8	187.5	0	15	198.8	113901.3	1250	1213.8	7721.3	0	0.062
ARG22	Laguna Negra,					S	35	-27.63	-68.55	106406.3	1193.8	42.5	118.8	0	48.8	43.8	62748.8	1266.3	2236.3	6536.3	0	0.021
ARG23	Laguna Negra,					B	43	-27.63	-68.55	913750	62500	171.3	195	0	35	137.5	600000	1405	26250	1925	0	0.003
CHL1	Boyeruca 1	Chile	Pacific coast	Coastal	0	S	44	-34.70	-72.01	138202.5	24825	868.8	111.3	0	26.3	0	64036.3	20525	480	6643.8	0	0.000
CHL3	Boyeruca 1				0	B	44	-34.70	-72.01	188280	59313.8	1603.8	130	0	35	0	62703.8	47750	125	8897.5	0	0.000
CHL2	Boyeruca 2				0	S	44	-34.69	-72.00	171256.3	30156.3	956.3	106.3	0	28.8	0	78196.3	25953.8	120	4966.3	0	0.000
CHL4	Boyeruca 2				0	B	44	-34.69	-72.00	193606.3	67442.5	1977.5	155	0	32.5	0	57317.5	54163.8	67.5	10718.8	0	0.000

S: Sediment; B: Brine. masl: above medium sea level (expressed in meters). The coordinates are given using the decimal degrees system. Ion concentration values are shown in ppm.

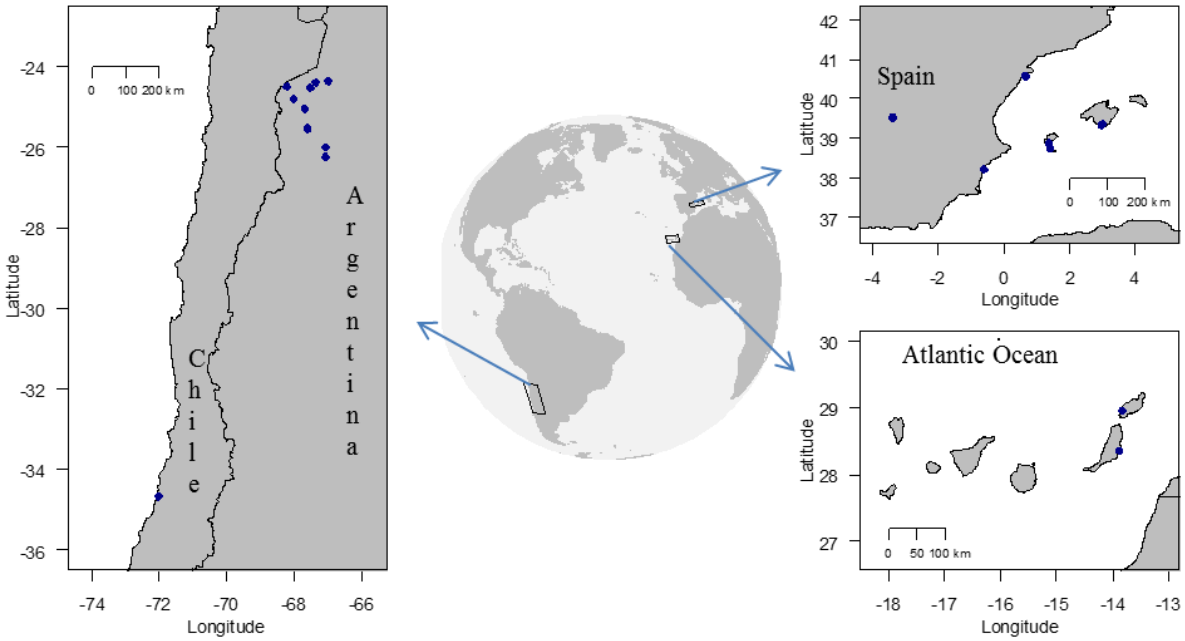

Figure 5.1 Sampling locations in Chile, Argentina and Spain.

Measurement of the ionic composition

Ionic concentration quantifications were done at the Research Technical Services of the University of Alicante by ionic chromatography with a Metrohm, 850 ProfIC AnCat- MCS equipment. Sodium carbonate (3.6 mM) was used as eluent for anion detection, with a flow of 0.8 mL/min, with a Metrosep A SUPP 7-250 (Metrohm) column plus a Metrosep ASSUP 4/6 as a pre-column, whereas nitric acid (3.5 mM) was the eluent for cation detection (flow of 1.9 mL/min), employing a Metrosep C3-250 (Metrohm) column plus a Metrosep C3 pre-column. Column temperature for both anion and cation determinations was 40ºC. Total carbon and carbonates were measured using the Bernard's calcimeter method (Lamas *et al.* 2005).

Retrieval of microbial biomass and DNA extraction

Sediment samples were processed as described by López-López et al. (2010) with the difference of retrieving microbial biomass from 120 g of a homogenized sample (six subsamples, each one of 20 g of sediment of different horizons and cores up to 20 cm below sea floor -bsf). Pellets were stored at -20ºC for DNA extraction. In the case of brines, 250 mL were filtered throughout 0.22 µm pore size membrane filters (Durapore, Millipore), which were stored at -80ºC until DNA extraction.

Environmental DNA was extracted from sediment pellets and thawed cut filters from brine samples. Pellets and cut filter pieces were separately vortexed in 2 mL of extraction buffer (100 mM Tris-HCl, 100 mM EDTA) in 50 mL polypropylene centrifuge tubes. Then, the

supernatant was transferred to a new tube and 20 µl 10 mg/mL of proteinase K (Roche), 24 µl 300 mg/mL of lysozyme (Roche) and 20 µl 1000 u/mL of mutanolysin (Roche) were added, and the tubes incubated for 1 h in an orbital shaker (Thermo Electron Corp.) at 15,700 xg and 37ºC. After the incubation period, 10% sodium-dodecyl-sulphate (Panreac) was added to a final concentration of 1% and incubated at 55ºC for 30 min. Lysates were extracted with phenol-chloroform-isoamyl alcohol as previously described (López-López et al. 2010). Next, the DNA was precipitated overnight with 0.7 (v/v) isopropanol, centrifuged 30 min at 15,700 xg, at 4ºC, rinsed with 70% ethanol (v/v) and centrifuged again for 15 min. After air-drying nucleic acids were resuspended in 50 µl of sterile nuclease-free water (Sigma), and stored at -20ºC.

PCR amplification and pyrosequencing of 16S rRNA

16S rRNA gene sequences of environmental samples were amplified using the primer pairs GM3 and S for *Bacteria* and 21F and 1492R for *Archaea* (Weisburg *et al.* 1991; DeLong 1992; Muyzer *et al.* 1995). The PCR reactions were performed as previously described by Mora-Ruiz *et al.* (2015). A secondary PCR reaction was performed to incorporate barcodes and linkers into the previously obtained amplicons. This was done using 1:10 µL of the original PCR product as a template, using the same PCR conditions but only 5 cycles. Primers GM3-PS and 907-PS were used for *Bacteria* (Mora-Ruiz *et al.* 2015). For *Archaea*, primer 21F-PS was used instead of GM3-PS (Mora-Ruiz *et al.* 2016). This PCR was done by triplicate for each sample, and the final products were mixed and purified using MSB® Spin PCRapace (INVITEK), following the manufacturer's instructions. The samples were sequenced using the 454 GS-FLX+ Titanium technology.

Processing of pyrosequencing data

Data were processed following the Mothur pipeline (Schloss *et al.* 2009). Briefly, low-quality sequences were removed (sequences <500 bp and quality score <25; no ambiguities were allowed and no mismatches in reads with primers and barcodes). The 10-bp barcodes were examined for the assignation of sequences to the samples. Chimeras were detected and removed with the application UCHIME implemented in Mothur (Edgar *et al.* 2011). Sequences were clustered into Operational Taxonomic Units (OTUs) at 98.7% level using UCLUST (Edgar *et al.* 2011) included in QIIME v. 1.9.0 (Caporaso *et al.* 2010). All sequences were submitted to public repositories under the accession number ERR2003672-ERR2003764.

Phylogenetic affiliation

Representative sequences of the bacterial and archaeal OTUs were incorporated separately to the non-redundant SILVA REF 111 database (Quast *et al.* 2013b) using the ARB package

(Ludwig *et al.* 2004). Alignment was performed with the SINA tool (SILVA Incremental Aligner) (Pruesse, Peplies and Glöckner 2012b) using the LTP 111 database as template and manually improved following the reference alignment in ARB-editor (Yarza *et al.* 2010). The closest relative non-type strain SILVA REF 111 sequences of an acceptable quality affiliating with the OTU representatives were selected and merged with the LTP 111 type-strain sequence database. For the final tree reconstruction, the selected representative sequences with an additional set of about 750 supporting sequences (highest quality in the LTP and covering a balanced representation of all major phyla of both *Bacteria* and *Archaea* domains were used for a neighbour joining reconstruction (Munoz, Rosselló-Móra and Amann 2016). To this final topology, all OTU representatives were inserted using the parsimony tool, and clustered in OPUs (Operational Phylogenetic Units; (França *et al.* 2014; Mora-Ruiz *et al.* 2015) based on the visual inspection of the final tree. The thresholds used for taxonomic levels were those suggested previously (Stackebrandt E 2006; Yarza *et al.* 2014).

Diversity and statistical analysis

The ionic composition of the samples was evaluated using a hierarchical cluster analysis using the Bray-Curtis distance and Ward's linkage. Rarefaction analyses for the two domains were conducted using PAST *v* 3.01 software (Hammer, Harper and Ryan 2001). For Jost q=0 (richness) and q=1 (diversity) indices calculation, a re-sampling was conducted by Monte-Carlo method with 1,000 simulations (Mora-Ruiz *et al.* 2016). Pearson correlations were obtained with R Commander (Fox 2005). Beta diversity was calculated using the Whitakker index and ordination analysis such as Non-metric Multidimensional Scaling (NMDS) performed by domain with previous normalization. The goodness of the NMDS was evaluated according to a stress value smaller than 0.366, which is considered acceptable for 50 samples, 0.337 for 33 samples and 0.228 for 17 samples (Sturrochk, K. Rocha 2000). Fitted vectors (environmental variables) were represented as arrows that point in the direction of the most rapid change and the length of the arrow was proportional to the r^2 obtained (Díaz-Gil *et al.* 2014; Oksanen *et al.* 2016). Additionally, a Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson 2001) was used to test the statistical significance of geographic location in the microbial communities. Simple and partial Mantel tests (Mantel 1967) running 5000 randomizations were used to evaluate the significance and correlation coefficients between genetic, spatial and environmental distance matrices. Bray-Curtis distances for paired locations were used to calculate the genetic distances and Euclidean distances were used for geodesic distances based on longitude/latitude. Analyses were performed using the packages *geosphere* (Hijmans 2016) and *vegan* (Oksanen 2011) in R *v* 3.1.1. Finally, a pooled SIMPER analysis by Bray Curtis dissimilarity was conducted to detect the OPUs, which generate the biogeographic

patterns. SIMPER was performed with PAST *v* 3.01 software (Hammer, Harper and Ryan 2001).

Results

Description and ionic characterization of samples

Our study included a total of 50 samples (Table 5.1), 27 from South America and 23 from Spain, among them 33 sediments and 17 brines from ten different locations. Our sites represented coastal seawater-fed (20 samples) and inland endorheic (30 samples) systems. In the same way, high altitude (23 samples between 3500 to 4560 masl) and sea-level sites (27 samples). The ionic composition, location and origin of the different samples are summarized in Table 5.1 (and Table A 5.1). Cl- was always the dominant anion, and Na+ was in most of the cases the dominant cation followed by Mg2+, with the exception of both Chilean brines, Peñahueca brine CM7, and Peñahueca sediment CM4 where the dominance of both cations was inverted (Table A 5.1). Moreover, all Argentinean samples as well as some from Peñahueca contained Li^+, which was not detected in any of the costal samples, and NO_2^-, NO_3^-, PO_4^{3-} and NH_4^+ were higher in inland samples. The sediments IB3 and IB4 (from Campos) exhibited lower values of Br^-, Cl^-, K^+, Mg^{2+} and Na^+ and higher values for Ca^{2+}, than other similar coastal samples, and were more similar to inland samples measured here, especially from Peñahueca (Table A 5.1 In addition, brines from Peñahueca exhibited higher values of F^- and Li^+ more similar to the Argentinean samples, information corroborated by the clustering analysis (Figure A 5.1).). The range of salinities found for the samples was 25%-43% with South American samples showing, in general, the highest salinities (Table 5.1).

Sequencing and OPU design

After trimming, chimera check and removal of low quality sequences, the approach used recovered 462,931 sequences for *Bacteria* and 692,411 for *Archaea* with a mean of 9,259 (±4,664) and 13,848 (±9,766) sequences per sample, respectively, and with a length mean of 644 bp (300 - 898 bp range). The sequences were clustered in OTUs at 98.7% identity rendering a total of 103,616 OTUs for *Bacteria* and 77,839 for *Archaea*. The representative OTU sequences affiliated with a total of 844 OPUs (mean 138 ± 68 per sample) for *Bacteria* and 362 for *Archaea* (mean 84 ± 49 per sample; Table A 5.2). In both cases, and for each individual sample, the diversity of OPUs reached saturation or was close to saturation based on the shape of the rarefaction curves (Figure A 5.2).

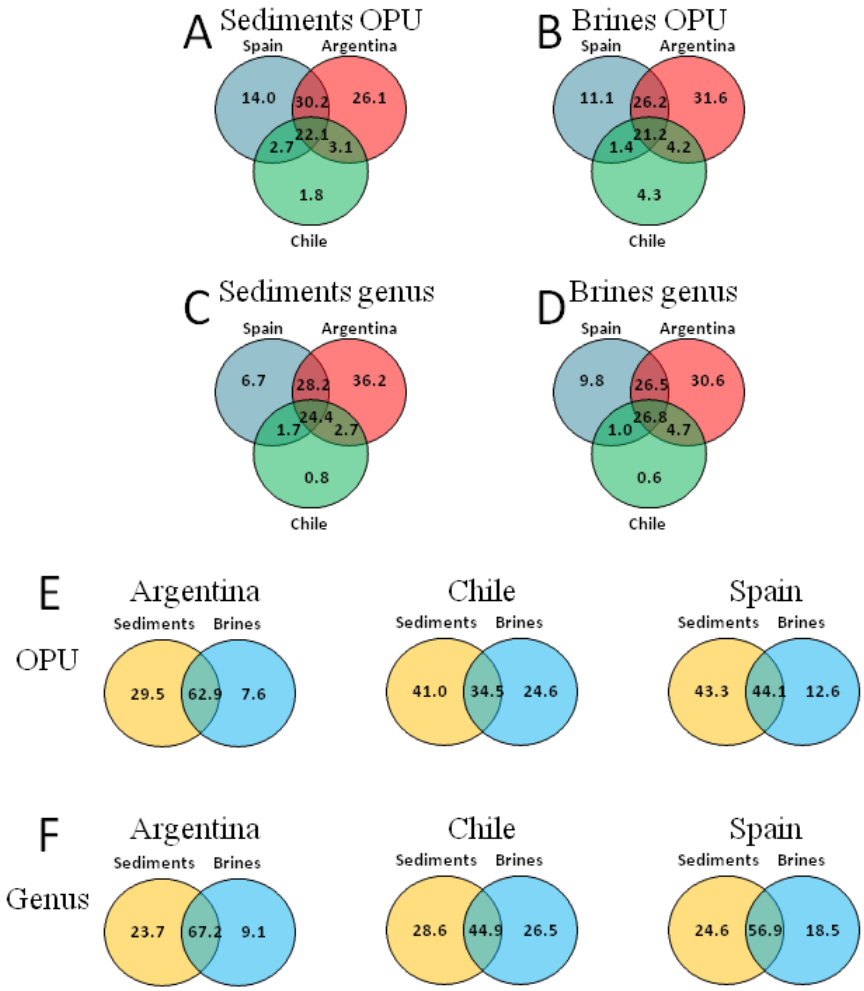

Figure 5.2 Venn diagram with the percentage of taxa detected in sediments (A, C, G, I) and brines (B, D, H, J) samples, at OPU (A, B, G, H) and genus (C, D, I, J) level. Distribution of OPUs (E, K) and genera (F, L) in each country by type of sample (sediments and brines), for *Bacteria* and *Archaea* domains.

Bacterial diversities

The bacterial richness for sediments was composed by a total of 788 OPUs. From them, 471 (60%) affiliated with 326 known genera and 256 with known species (Figure 5.2; Figure A 5.3; Table A 5.3 and Supplementary Spreadsheet S1). Also, these OPUs affiliated with 44 bacterial phyla, including some Candidate Division such as OP11 and TM6 (Table A 5.4 and Supplementary Spreadsheet S2). *Proteobacteria* was the most represented phylum, followed by *Firmicutes*, *Bacteroidetes* and *Cyanobacteria.* However, *Firmicutes* was the most abundant in the inland samples with the exception of SP-IB2 (Figure 5.3). Our determined bacterial OPUs were included in 159 known families and 326 known genera being *Halanaerobiaceae* (phylum *Firmicutes*), *Moraxellaceae*, *Desulfohalobiaceae* and *Commamonadaceae* (the three within *Proteobacteria*) the most important families (Supplementary Spreadsheets S3 and S4). In addition, the most representative genera were *Halanaerobium*, *Acinetobacter*,

Desulfovermiculus and *Halanaerobacter* encompassing 42.1% of the total sequences distributed in 35 OPUs. The genus *Halanaerobium* was also detected as the unique ubiquitous genus in all sediment samples.

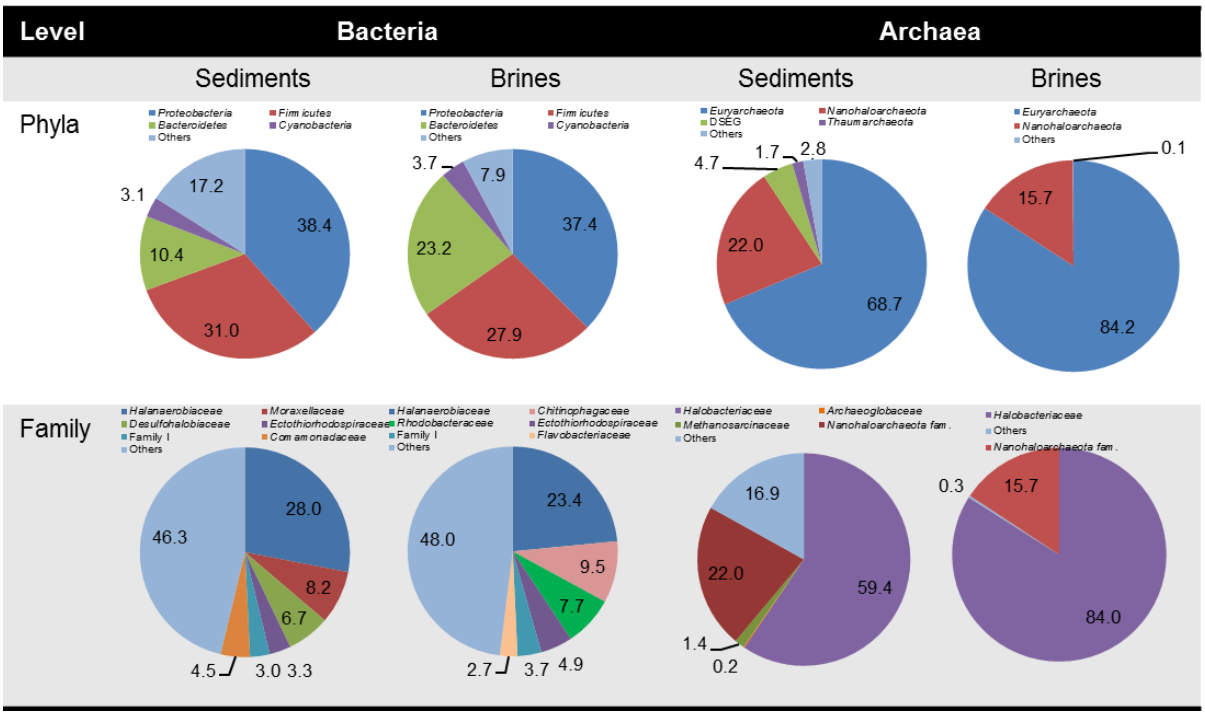

Figure 5.3 Taxonomic distribution of sediments and brines at Phyla and Family level for *Bacteria* and *Archaea* domains. DSEG=Deep Sea Eukaryotic Group), Family I= Family of *Cyanobacteria*).

Despite some genera were ubiquitous (*Halanaerobium, Rhodovibrio, Bacillus and Legionella*) none OPU representing putative single species was detected as ubiquitous in all sediment samples for *Bacteria.* However, we could observe some regionalisms. We detected 41 OPUs exclusive from the Spanish sediments (affiliating with *Moraxellaceae*, dominated by the genus *Acinetobater*), *Rhodobacteraceae* and *Clostridiaceae* families (Figure A 5.4; Supplementary Spreadsheet S5), 83 in Argentinian sediments (within the *Cytophagaceae*, *Hypomicrobiaceae*, *Rhodobacteraceae* and *Rhodothermaceae* families, Figure A 5.4 and Supplementary Spreadsheet S6) and 15 OPUs from Chilean sediments (affiliated with the *Ectothiorhodospiraceae* and *Desulfobacteraceae* families, Figure A 5.4 and Supplementary Spreadsheet S7).

In the case of brines, these were represented by 662 bacterial OPUs where 415 (63%) affiliated with 297 known genera and 218 known species (Figure 5.2; Figure A 5.3; Table A 5.3 and Supplementary Spreadsheet S1). With exception of *Bacteroidetes*, that was more abundant in brines than in sediments, the abundance of the other phyla was similar to that observed in sediments (Figure 5.3). Our analyses detected 146 families and 297 genera. The most important

families were *Halanaerobiaceae (Firmicutes), Chitinophagaceae* and *Flavobacteraceae (Bacteroidetes), Rhodobacteraceae and Ectothirhodospiraceae (Proteobacteria*), and the Family I of *Cyanobacteri*a (Supplementary Spreadsheet S3).

A high number of genera detected in brines were putative anaerobes (81 genera, e.g. *Halanaerobium*) or facultative anaerobic bacteria (94 genera, e.g. *Gracilimonas*) including those that showed the highest number of sequences (*Halanaerobium* and *Halanaerobacter*). In contrast, putative aerobic genera (e.g. *Psychroflexus*) were detected (Supplementary Spreadsheet S4)) in lower abundances (mean of % sequences per sample 15.11% ± 14.4%). *Halanaerobium* was the unique genus detected in all brine samples, but within this genus we detected three putative species distributed in different countries (OPUs 425, 553 and 726). Additionally, we observed a parallel increment of sequence amplicons of the genera *Psychroflexus* with *Roseovarius* (r^2=0.89) as well as *Rhodovibrio* and *Hahellaceae* (r^2=0.88).

Eleven OPUs were exclusively detected in Spanish brines (including two OPUs in *Bacillaceae*), and 42 OPUs (*Gallionellaceae, Cystobacteraceae,* and *Enterobacteriaceae*) were detected exclusively in Argentinean brines and five OPUs in Chile (including unclassified members of *Clostridiales, Lactobacillales* and *Sphingobacteriales*; Figure A 5.4). More detailed information about the OPUs detected in Spain, Argentina, and Chile is given in the Supplementary Spreadsheets S5, S6, and S7, respectively. Moreover, 211 OPUs were detected in a unique location (Salar de Pocitos; ARG17), being the single location without site-specific OPUs. Additionally, 22 OPUs were found in all coastal brines (among the most relevant were the OPU313, uncultured *Sphingobacteriales*; OPU127, uncultured *Rhodobacteraceae*; and OPU040, uncultured *Pseudomonas*), while no OPUs were detected as ubiquitous in the inland brines. Since both inland and coastal sample types were represented in Spanish locations, we analysed their shared groups. Four and seven OPUs were detected in all Spanish coastal and inland sites, respectively; in both cases affiliated principally to the genus *Halanaerobium* (three and four OPUs, respectively).

The members of *Salinibacter* genus, identified as OPU318, OPU319, OPU573 and OPU747, were mainly observed in brines and poorly detected in sediments, with a maximum of 0.2% in abundance for ARG20. *Salinibacter* was present in brines of the three countries, but mainly (up to 17.3%) in the Spanish samples, in contrast with the other Argentinian (up to 0.2%) and Chilean (up to 0.02%) samples. From these four OPUs, OPU318 (uncultured *Salinibacter*) was the most abundant (up to 16.4% in SP-CN4), with OPU319 (*S. ruber*), OPU573 (*S. iranicus/S. luteus*) and OPU747 (uncultured *Salinibacter*) found in low abundances (< 0.23%) in all countries with exception of the Pacific coast (0.85%). No sequences affiliated with the branch related with the putative new species *S. altiplanensis*, despite members of this new species were isolated from ARG6 and ARG21 (Viver et al., unpublished data).

A total of 192 OPUs were exclusive of sediments, principally affiliating with *Rhodobacteraceae* (11 OPUs), *Caulobacteraceae* (five OPUs) and *Thermotogaceae* (four OPUs) (Supplementary Spreadsheet S8. In contrast, 66 OPUs were present exclusively in brines. Five OPUs affiliated with *Bacillaceae*, and three with *Enterobacteraceae* and Family I (of *Cyanobacteria*) each one (Figure A 5.4 and Supplementary Spreadsheet S9).

On the other hand, we detected 596 common OPUs between sediments and brines, for example, OPUs 194, 195 and 196 (affiliated with the Candidatus "Parcubacteria"). However, the OPU188 (uncultured *Rickettsiales*) was the unique present in all sediments and brines of Argentina. Eleven OPUs were common in brines and sediments of Chile (dominated by *Halanaerobiaceae*; two OPUs), and ten OPUs were present in all sediments and brines in Spain (principally *Halanaerobiaceae;* two OPUs and *Comamonadaceae*; one OPU). Additionally, 164 OPUs were site-specific (Figure 5.2; Figure A 5.3; Figure A 5.4; Supplementary Spreadsheet S10).

The highest richness value was for SP-CM5 (312 OPUs) and the lowest for SP-IB4 (30 OPUs). In general, the richness in the South American samples was higher for sediments than their respective brines. However, this pattern was not clearly observed in the Spanish samples. Additionally, none of the Jost´s indices presented a pattern for *Bacteria* by sample kind nor country or inland/coastal.

Archaeal diversities

The total archaeal diversity in sediments was represented by 360 OPUs (Table A 5.5) from 13 different phyla (Figure 5.2; Figure A 5.5; Table A 5.6; For additional information about *Archaea*l OPUs see Supplementary spreadsheets S11 and S12). From the 360 OPUs, 31 (7.5%) were assigned as uncultured or candidate archaeal lineages such as Deep Sea Euryarchaeotal Group (DSEG; seven OPUs), miscellaneous Crenarchaeotic Group (MCG, ten OPUs) and MSBL1 (two OPUs). All of them generally showed low abundances (<11%), with exception of DSEG, The highest dominances (Figure 5.3) were for *Euryarchaeota* and *Nanohaloarchaeota*, observing a negative correlation between their abundances (r^2=-0.72). *Thaumarchaeota* and DSEG were also abundant in Chilean sediments and in two Balearic sediments (IB4 and IB6). Additionally, Mediterranean Sea Brine Lakes 1 (MSBL1), Deep Sea Hydrothermal Vent Group 6 (DHVEG 6) and MCG were principally distributed in Spanish locations. It was remarkable that some populations were restricted to some locations, such as Miscellaneous Euryarchaeotic Group (MEG) and KTK 28A only present in Spanish samples (Table A 5.6).

We identified 16 archaeal families (Fig. 3), with a marked dominance of *Halobacteriaceae* (25 OPUs) and four families of *Nanohaloarchaeota* (113 OPUs; based on the thresholds suggested by Yarza et al., 2014 (Yarza *et al.* 2014) in where the minimal identity

threshold for family was 86.65%), with a notable dominance of *Nanohaloarchaoeta*-1 (12.9% of the sequences and 89 OPUs) in sediments and brines. An exceptional case was ARG18 where *Methanosarcinaceae* was the most representative taxon with 47% of the sequences (Supplementary Spreadsheet S13). From the 105 OPUs (29%) affiliating with known genera (94 OPUs only within the family *Halobacteriaceae*), only 26 OPUs were identified as known species. The most relevant genera were *Halorubrum* (six OPUs), *Halobacterium* (nine OPUs), *Halorhabdus* (20 OPUs), and *Natronomonas* (three OPUs), but none of these, or other genera, were ubiquitous in all sediments. Regionally, *Halomicroarcula* (four OPUs) was higher in Spanish samples (up to 22.4% of the sequences in SP-CM4), while *Haloferax* (one OPUs) was detected principally in Argentinean sediments, with low proportions in some samples from Spain (Supplementary Spreadsheet S14). Besides, an increment of abundances correlated (co-occurrence) between some genera such as *Halobonum* ~ *Halosimplex* (r^2=0.82) and *Halorubellus* ~ *Halomicroarcula* (r^2=0.84). Other correlations were also observed in the abundances for specific genera and ions, such as F^- ~ *Natronomonas*, PO_4^{3-} ~ *Halobonum*, Mg^{2+} ~ *Halorubellus* (r^2=0.89) and Ca^{2+} ~ *Halomicrobium* (r^2=0.88).

The archaeal sediment richness showed OPU106 (uncultured *Natronomonas*), OPU053, and OPU138 (uncultured *Halorubrum*) as the most abundant, but none of them were ubiquitous. Seventy OPUs were exclusively found in Spanish sediments (*Nanohaloarchaeota* and *Methanomicrobia* Group C; Supplementary Spreadsheet S15); 20 OPUs, mainly putative methanogens (*Methanobacteriaceae* and *Methanothermaceae*) were detected exclusively in Argentina (Supplementary Spreadsheet S16), a and three from Chile (mainly included in Candidates TMG III and MBGB; Supplementary Spreadsheet S17).

Richness was lower in brines than in sediments, with 179 OPUs included in seven phyla, also with an important dominance of *Euryarchaeota* (114 OPUs), followed by *Nanohaloarchaeota* (59 OPUs; Figure 5.3) and, as in sediments, their abundances were highly correlated (r^2=-0.99). and, as in sediments, their abundances were highly correlated (r^2=-0.99). The remaining phyla (six OPUs) contributed to the *Halobacteriaceae* (108 OPUs) family was notably more abundant followed by *Nanohaloarchaeota* 1 (51 OPUs) (Figure 5.3 and Supplementary Spreadsheet S13). The most important genera were *Halorubrum* (five OPUs), *Natronomonas* (3 OPUs), and *Halonotius* (four OPUs). It is important to note that *Halorubrum*, *Halorhabdus*, *Halobacterium*, *Halovenus*, and *Natronomonas* were present in all samples (Supplementary Spreadsheet S14), but similarly to the sediments, their respective species were distinctly occurring in the different regions. In relation with the environmental variables, our results also showed an increment of abundances of *Halonotius* when Mg^{2+} increased (r^2=0.86). As in sediments, the most abundant OPUs were OPU053 (uncultured *Halorubrum*) and OPU106 *Natronomonas*).

Although most of the genera were detected in both brines and sediments, at the OPUs level were not coincident (Figure 5.2; Figure A 5.5);, *e.g.* in the case of the most abundant genus *Halorubrum*, four of the six OPUs were exclusively detected in sediments. In the same way, uncultured *Halobacterium* OPU004 was only found in sediments, and *Nanohaloarcheota* OPU399 was only found in brines. However, we observed a large part of archaeal OPUs present in both habitats (178 OPUs). A total of 183 OPUs were found exclusively in sediments, principally affiliated to *Nanohaloarchaeota* (55 OPUs), and Methanomicrobia group C (17 OPUs) and Marine Benthic Group D and DHVEG-1 (seven OPUs; Supplementary Spreadsheet S11).

No ubiquitous OPUs was detected as specific for coastal or inland habitats. On the other hand, Santa María exhibited the lowest richness and diversity value. In general, Spanish samples showed higher diversity, being Lanzarote (SP-CN4) the one with the highest diversity (q1=56 Table A 5.1). Finally, interesting that *Haloquadratum* (two OPUs) in brines were relatively low abundant (0.39% of the reads) in comparison with other genus such as *Halorubrum* and *Halonotius* (with 34.4% and 7.1% of the reads, respectively).

Beta diversity analyses and environmental influences

As it was stated before, we detected some location exclusive OPUs, and based on multivariate analysis, the samples showed segregation by country (Spain, Argentina and Chile Figure 5.4) when OPUs were taken into account. On the other hand, these biogeographic patterns based on genera (Figure 5.5) presented lower resolution as some ubiquitous genera were regionally represented by distinct species. For example, *Halanaerobium* showed distinct occurrence of their species as e.g. OPU413 (uncultured *Halanaerobium*) was more abundant in Spain (both sediments and brines), OPU416 (uncultured *Halanaerobium*) in the Argentinean sediments, and OPU432 (*Halanaerobium lacunarum/H. salinarus*) in the Chilean brines (Figure 5.6, Table A 5.7) The main bacterial genera explaining the distribution by country were *Halanaerobium, Acinetobacter*, and *Desulfovermiculus*, more abundant in Spain; and for brines, *Halanaerobacter* and *Idiomarina* were notably more important in Chile (Figure A 5.4 and Table A 5.8). In contrast, for *Archaea*, *Halorubrum* was the most important genus explaining the dissimilarity by country, being more abundant both in sediments and brines in Argentina followed by *Natronomonas* (Figure A 5.6 and Table A 5.10Table A 5.9).

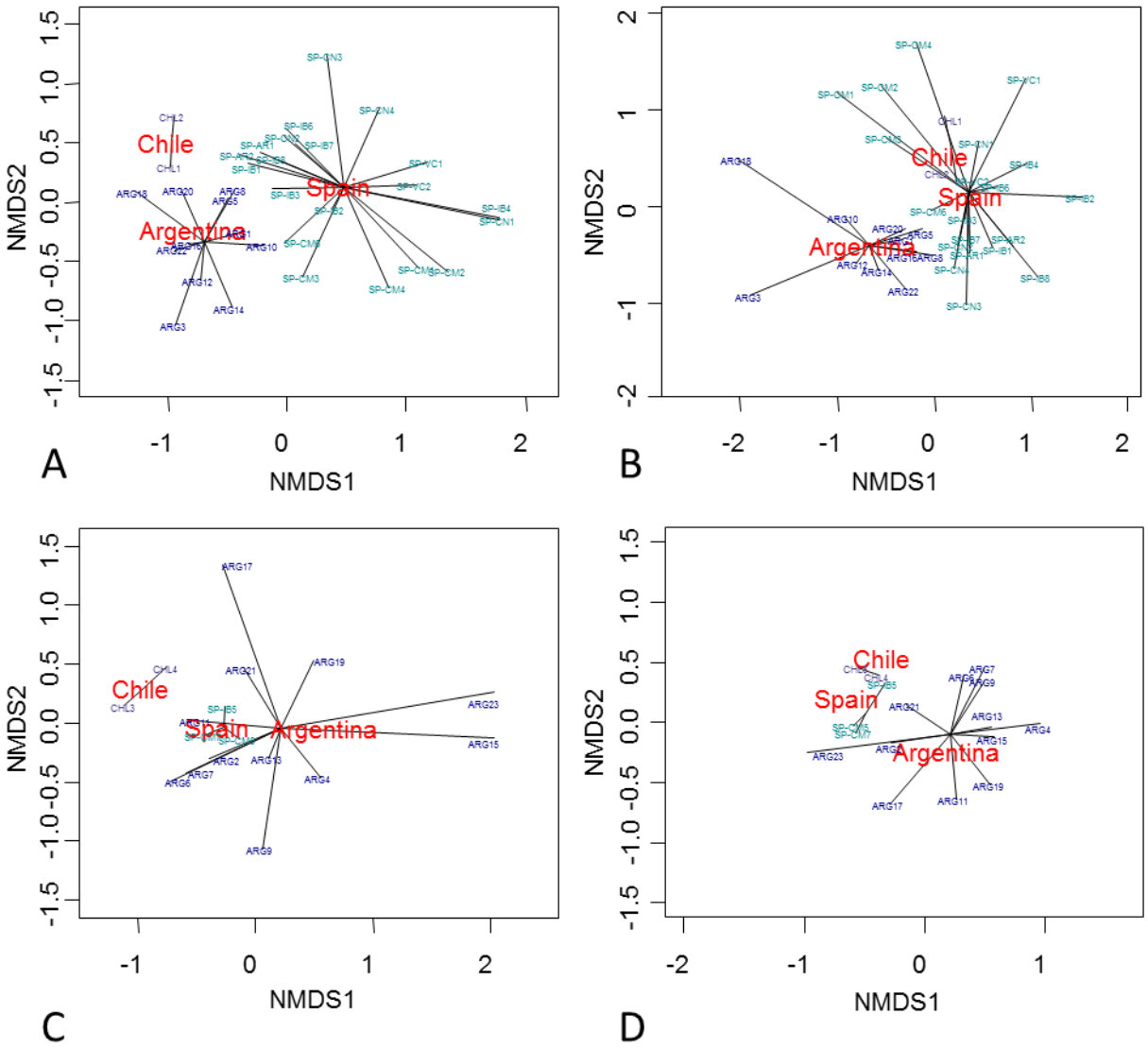

Figure 5.4 Two dimensional Non-metric multidimensional scaling (NMDS) of sediments (A-B) and brines (C-D) for *Bacteria* (A-C) and *Archaea* (B-D), based on the Operation Phylogenetic Units (OPUs) distribution. Stress value: A (0.18), B (0.18), C (0.12) and D (0.10).

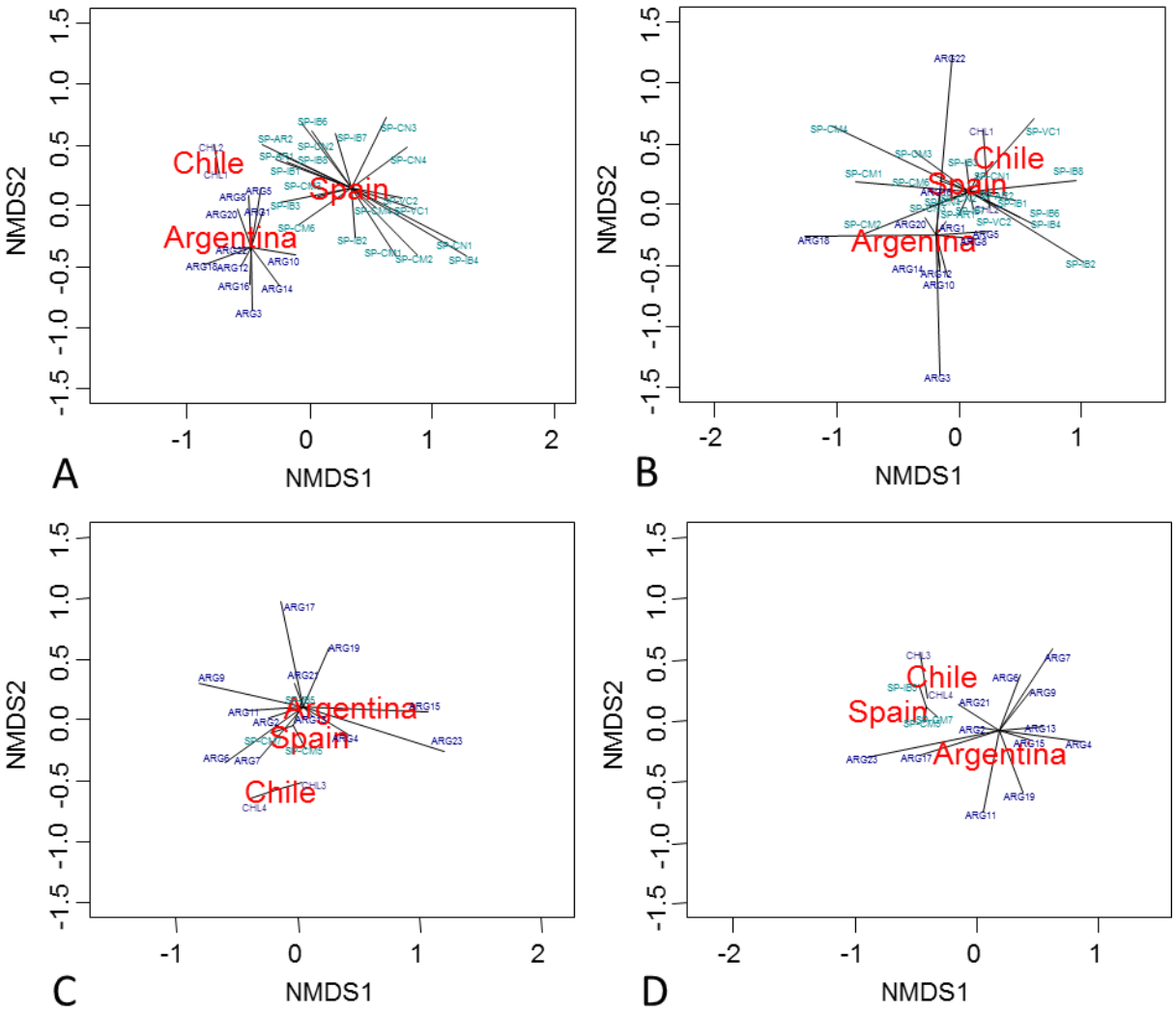

Figure 5.5 Two dimensional Non-metric multidimensional scaling (NMDS) of sediments (A-B) and brines (C-D) for *Bacteria* (A-C) and *Archaea* (B-D) based on the genus distribution. Stress value: A (0.19), B (0.18), C (0.16) and D (0.10).

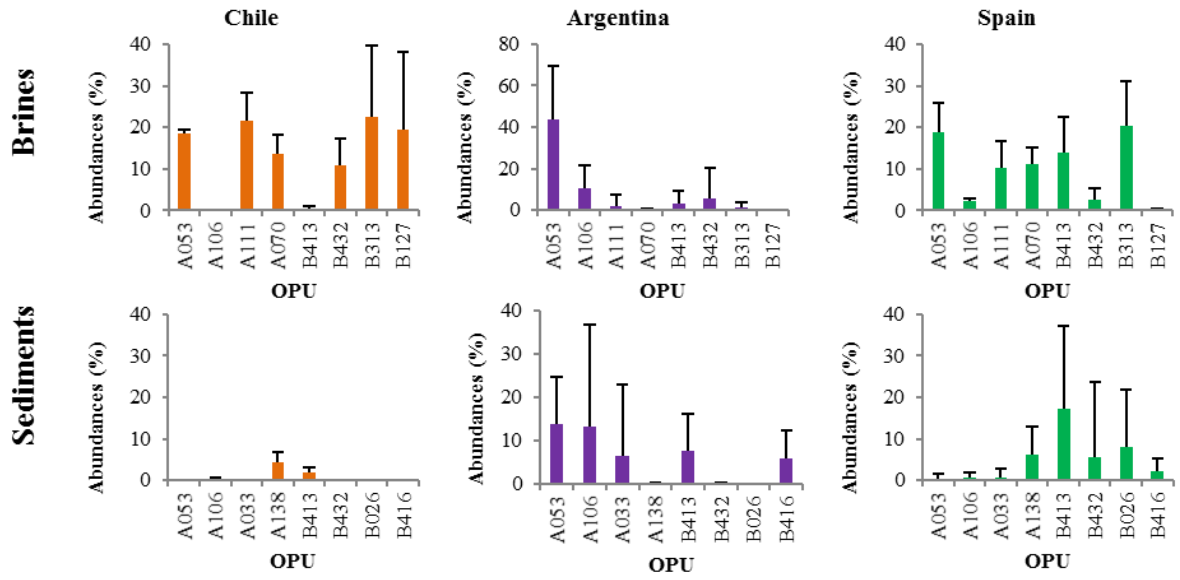

Figure 5.6 Relative abundances for the principal Operational Phylogenetic Units detected by Similarity Percentage analysis (SIMPER) analysis by type of sample (brines and sediments). *Bacteria*: B413 (uncultured *Halanaerobium*); B432 *(Halanaerobium lacunaru/H. salinarus*), B313 (uncultured *Sphingobacteriales*), B127 (uncultured *Rhodobacteraceae*), B026 (Uncultured *Acinetobacter*) and B416 (*Halanaerobium* sp.). *Archaea*: A053 (*Halorubrum* sp.), A106 (*Natronomonas* sp.), A111 (uncultured *Haloarcula*), A070 (uncultured *Halonotius*), A033 (uncultured *Halorhabdus*) and A138 (uncultured *Halorubrum*).

The SIMPER analysis for *Bacteria* showed more than 70% of the biogeographic segregation could be defined by the contribution of 44 OPUs and their relative abundances (Figure 5.6; Table A 5.7). For *Archaea*, the dissimilarity presented by country was explained at >70% by 45 OPUs (Table A 5.10). From them, the OPU053 (uncultured *Halorubrum*) was the most represented in brine samples independently of the location. But other OPUs were highly represented in one specific country, such as OPU033 *(*uncultured *Halorhabdus*) and OPU106 (uncultured *Natronomonas*) which were more abundant in Argentinean sediments, and OPU111 (uncultured *Haloarcula*) in Chilean brines (Figure 5.1; Table A 5.10).

As three different regions represented Spain, we performed an ordination analysis for Spanish sediments confirming that a biogeographic pattern can also be detected at regional level (Figure 5.2; Figure A 5.5; Figure A 5.7). Some OPUs responsible of this segregation were e.g. OPU013 and OPU014 (both uncultured *Halovenus*), more abundant in samples from Canary islands; OPU031 (uncultured *Halomicroarcula*), OPU032 (*Halorhabdus utahensis*), OPU077 (uncultured *Halobellus*), and OPU097 (*Halosimplex carlsbadense*) with a higher representation in the inland samples; and OPUs OPU125 (uncultured *Halobacteriaceae*), OPU131 (uncultured *Halococcus*), OPU148 (DSEG), and OPU253 (marine benthic group D and DHVEG-1), more abundant in Balearic samples.

Based on the bacterial diversity indices, the Spanish sediment samples SP-CN2 and SP-IB6 (Table A 5.11) were the most similar (the lower Whittaker value 0.34) and the maximum dissimilarity was observed between SP-CN1 and ARG20 (0.95). For brines, the highest similarity was found in CHL3-CHL4 (0.21) and the lowest in ARG4-ARG23 (0.77). For archaeal sediments, the highest Whittaker value (0.92) occurred in ARG3-SP-CM2 (Table A 5.12). Contrarily, SP-IB4 and SP-IB6 shared all OPUs (Whittaker value= 0).

For both domains, the beta diversity showed that the sample kind (brines or sediments) had lesser influence than the location (Figure A 5.8, Figure A 5.9), being most pronounced for *Bacteria* where the Argentinian samples seemed closer to the Spanish than to the Chilean samples, and the Balearic samples showed the strongest similarity. Differently, the Spanish samples remained grouped and near to the Chilean when analyzing *Archaea*. The simple and partial Mantel tests exhibited significant positive correlation with the geographic distance (Table 5.2). The PERMANOVA presented significant variations dependent on the country. This behaviour was shown for both domains, *Bacteria* (*F-statistic*=2.43, $p<0.001$; Fig. 4) and *Archaea* (*F-statistic*=4.19; $p=0.001$; Figure 5.5). The relationships between the community structure and environmental parameters indicated that for *Bacteria*, NO_2^- ($r^2=0.26$; $p=0.001$), Br^- ($r^2=0.17$; $p=0.01$) and salinity ($r^2=0.14$; $p=0.03$) were the environmental variables with higher effect; whereas for *Archaea*, the significant explanatory variables for the distribution of the samples were salinity ($r^2=0.28$; $p=0.001$), F^- ($r^2=0.18$; $p=0.009$), PO_4^{3-} ($r^2=0.17$; $p=0.009$), Mg^{2+} ($r^2=0.16$; $p=0.02$) and NO_2^- ($r^2=0.11$; $p=0.04$).

Table 5.2 Pearson correlation and *p*-values for simple and partial Mantel test in *Archaea* and *Bacteria* domains. Geo= geographical distance, Env= environmental factors.

	Bacteria				*Archaea*			
	Simple		Partial		Simple		Partial	
	Env	Geo	Env	Geo	Env	Geo	Env	Geo
r	0.21	0.13	0.13	0.18	0.13	0.36	0.08	0.36
p	0.007	0.0001	0.001	0.006	0.008	0.0001	0.05	0.0001

Discussion

In this study we analysed and compared the microbial composition of hypersaline sediments and brines of 22 different locations in Spain, including the Atlantic Ocean and Mediterranean Sea, and South America Altiplane and coast of the Pacific Ocean. We applied the OPU approach for partial 16S rRNA gene sequence analyses, where the supervised affiliation renders a much fine-tuned picture of the identity of the amplicons (Cortés-Lara *et al.* 2015; Mirete *et al.* 2015; Vidal *et al.* 2015) than the use of the conventional OTUs. With this analysis we detected a total of

1,026 OPUs that could be understood as putative species, varying in abundances and presence/absence in the different communities, showing a geographical segregation. This geographical discrimination was more diffuse, i.e. of lower resolution, when units with higher taxonomic rank (i.e. genera) were used. The results showed that distinct OPUs (or species) of the same genus might distinctly occur in geographically distant locations. On the other hand, we cannot discard that the individuals of coincident species (or OPUs) from different regions differ in their geno- and phenotypes as the 16S rRNA gene partial sequences lack enough resolution to guarantee the detection of genetic drifts (Hanson *et al.* 2012). In addition, even in a single site, distinct populations of the same species, with identical 16S rRNA gene sequence, may coexist expressing different metabolic profiles and exhibiting distinct genomic structures (Antón *et al.* 2013b). The regionalism observed was probably related to the environmental conditions (i.e. ionic composition or other site-dependent environmental parameters as altitude, rain and insolation regimes, origin of the solutes, etc.) rather than only geographical distance (Hanson *et al.* 2012), in where each OPU may have adapted to the specific environmental conditions of each site as demonstrated for different *S. ruber* strains (Rosselló-Mora *et al.* 2008b). However, and beyond the clear large-scale regionalism (South America - Europe -Africa), this geographic differentiation was also observed for the three Spanish zones sampled (Canary Islands, Mediterranean coast and inland). In fact, our results exhibited a relevant proportion of the variability in the structure of the community produced by the geographical distance (until 36% in Mantel test results) in accordance with a recent study performed in different salterns, where the authors found the distance effect exhibited a strong influence (25% of the variability) in protistan communities (Filker *et al.* 2017). Some OPUs were located in an unique specific location (13% of the total), and an important number summing a 40.6% of all OPUs detected in an specific coountry (13.5% for Spain, 24.8% for Argentina and 2.3% for Chile). These seemed to be regionally exclusive, but as none of them was present in a great part of the samples of the respective region, we could not treat them as regional endemism, the most evident demonstration of biogeography (Hanson *et al.* 2012).

Some community structure differences were correlated with distinct ion composition, such as Mg^{2+} for some *Archaea*, or Li^{+} between inland and coastal sites. In this regard, salinity and substrate type (sediments vs. brines) are considered the two most important factors structuring diversity (Lozupone and Knight 2007), and for this reason we expected marked dissimilarities between sediments and brines as previously reported in marine environments (Zinger *et al.* 2011). However, in our study, OPUs from brines were highly represented in the corresponding sediments suggesting a strong connection between both habitats, in a similar way to what had been observed for protists (Filker *et al.* 2017). However, protists were just studied in the oxic upper layers of the sediments, and in our case we pooled the first 20 cm of the sediments that were undoubtedly anoxic, evidenced by the grey to black coloration found

already in the first millimetres as an indication of high sulphide concentrations and anaerobic conditions (López-López *et al.* 2010). Despite brines are aerobic, eventual episodes of anoxia can happen when the respiration exceeds primary productivity (Javor 1989) and the high summer temperatures reduce the oxygen solubility (Tromans 1998), making the presence of facultative anaerobes feasible as part of their community. On the other hand, mainly aerobes were detected in important proportions in sediments suggesting an active presence and not just fallen from the water column. Some of them, as *Bacillus* sp. (Verbaendert *et al.* 2011) or *Haloferax* sp. (Philippot 2002; Cabello 2004), could potentially be denitrifiers, a fact that appears in accordance with NO_3^- influencing the community structures (both in *Bacteria* and *Archaea*) and the low concentration of NO_2^-. The higher presence of *Firmicutes* in inland samples had also been reported in some studies of athalassohaline lagoons, lakes (Jiang *et al.* 2006; Montoya *et al.* 2013) and sediments (Hollister *et al.* 2010). Despite the family *Halanaerobiaceae* (moderately halophilic bacteria) was expected to be the most abundant in sediments (Abdeljabbar *et al.* 2013; Oren and Hallsworth 2014), other families (*Moraxellaceae* and *Desulfohalobiaceae*) occurred also in high abundances in the Spanish locations. For example, *Acinetobacter* (*Moraxellaceae* family) was one of the most important bacterial genera found in this study and had been already reported in other brines (Satyanarayana, Raghukumar and Shivaji 2005; Cinar and Mutlu 2016), but poorly studied in hypersaline sediments (Foti *et al.* 2008). Also relevant was the candidate bacterial phylum Parcubacteria, observed in a wide range of anoxic environments (Harris, Kelley and Pace 2004), and here present in some brines and sediments. Its presence in brines is in accordance with the codification of genes related to aerobic metabolism (Nelson and Stegen 2015).

On the other hand, the high abundance of *Euryarchaeota* is well known in brines (Dillon *et al.* 2013; Podell *et al.* 2013a; Fernández *et al.* 2014; Vavourakis *et al.* 2016) and sediments (Hollister *et al.* 2010; López-López *et al.* 2010). Curiously, we detected *Nanohaloarcheota* in sediments, when these were reported as aerobes exclusively occurring in brines (Narasingarao *et al.* 2012; Podell *et al.* 2013a). To our knowledge, this is the first report of *Nanohaloarchaeota* in hypersaline sediments with a notable abundance. In addition, we especially detected the MCG group in the Spanish locations and poorly represented in the South American sites. This group has been hypothesized to be numerically and ecologically important in anoxic marine sediments (Biddle *et al.* 2006; Kubo *et al.* 2012; Meng *et al.* 2014; Parkes *et al.* 2014), and its abundance has been related with SO_4^{2-} (Kubo *et al.* 2012). In our case there was no correlation with SO_4^{2-}, but with other ions such Mg^{2+} that also correlated with the total archaeal community trends, especially with *Halorubellus* and *Halonotius.* We also detected representative sequences of MSBL1, an archaeal linage putative responsible of the methanogenesis processes in hypersaline anaerobic environments of the Mediterranean (Borin *et al.* 2009; López-López *et al.* 2010, 2013) and the Read Sea (Antunes, Ngugi and Stingl 2011).

MSBL1 was also detected in South American samples (e.g. CHL1) pointing to a possible global distribution of this group, as well as DHVEG-1 and MBGB which has been recently found with high abundances in Brava and Tebenquiche lakes of Atacama (Chile) (Fernández *et al.*, unpublished data). However, the most abundant known archaeal genera in sediments affiliated with *Halorubrum, Halobacterium, Halorhabdus,* and *Natronomonas* mostly known as aerobes, and some carrying facultative anaerobiosis (Grant 2015; Kamekura, Kamekura and Masahiro 2015; McGenity *et al.* 2015; Antunes, Ferrer and Yarza 2016). The presence of *Haloferax* (facultative fermenter) (Don, Chen and Chan 2006) was remarkably high in Argentinean sediments. It was also remarkable that *Halorubrum* dominated over *Haloquadratum* often reported as the major component of brines (Maturrano *et al.* 2006; Oh *et al.* 2010; Boujelben *et al.* 2012; Gomariz *et al.* 2014; Podell *et al.* 2014). However our results are similar to a few other studies detecting low abundance of *Haloquadratum* and a high abundance of *Halorubum* (Montoya *et al.* 2013; Williams *et al.* 2014).

In summary, brines and sediments studied here exhibited chemical and biological differences related with their geographical distribution. Although some environmental parameters seemed not to considerably affect the community structures, other such as salinity, NO_3^-, Br^-, F^-, PO_4^{3-} and Mg^{2+} might be playing an important role on the selection of some specific groups. It was remarkable that the geographical patterns were less clear at the genus than at the OPU (or species) level. This can be understood as a phylogenetic and metabolic redundancy at high taxa in where their different species acquired distinct advantages related to the regional physicochemical idiosyncrasy. In addition, the important OPU overlap between anoxic sediments and their overlaying brines, even for unexpected lineages such as *Nanohaloarchaeota* and Parcubacteria, point to a versatile metabolism of the pelagic organisms rather than just an accumulation due to particle sink.

Alternative uses of OPU approach: evidence in other environments

The diversity of microbial communities is a very elusive reality

Dr. Carlos Pedrós-Alió

Chapter 6. Exploring the diversity in other environments: an OPU approach.

Abstract

The new techniques of massive sequencing have increased several orders of magnitude the 16S rRNA gene sequences entries in public databases. However, due to the nature of the amplicon sequencing approach, such sequences are partial which implies a reduction of the gene resolution for identification purposes. The 454-sequencing, which produced sequences with minimum length of 300 bp in tandem with the affiliation process based on the OPUs has improved the accuracy in the identification at low taxonomic levels (genus and species). Although the focus of thesis is mainly the anlysis of diversity in hypersaline environments, we evaluated the use of OPUs in other fields. In this Chapter, we applied the approach in two specific cases: 1) The study of Mirete *et al.* (2015); in where the research was focused in the detection of novel salt resistance genes, and where our contribution was the description of the diversity from where the sample were originated; and 2) the study of Vidal *et al.* (2015), which can be considered the first application of OPUs in clinical microbiology, where the microbial diversity in patients with Crohn disease was analyzed. Our results show that the OPU strategy is a useful tool to evaluate diversity in other areas detecting ubiquitous and key species with a deeper taxonomic resolution.

Introduction

Molecular techniques has increased our knowledge of microbial diversity revealing that biodiversity is higher than that reported exclusively by cultures. This deeper exploration has been proportional to the increment of the use of 16S rRNA gene due to massive sequencing techniques (Ventosa and Arahal 2009; Yarza *et al.* 2014). Thanks to these tools, a huge amount of information (sequences) is constantly is being generated and needs to be properly analyzed. As general process, the sequences obtained by massive sequencing are clustered into OTUs (Rosselló-Mora and López-López 2008b), being the 97% the most common identity cutoff (Turnbaugh *et al.* 2009; Oh *et al.* 2010; Heidelberg *et al.* 2013; Birtel *et al.* 2015). However, this method presents some pitfalls derived from the length of the sequences produced and the excesive conservative cutoff threshold (Yarza *et al.* 2014). As shown in previous Chapters of this thesis using 454-pyrosequencing, the minimum sequences length permissible for our analysis is 300 bp with a maximum of 900 bp (Mora-Ruiz *et al.* 2015, 2016) which produce robust results (Birtel *et al.* 2015).

This Chapter extends the OPU approach to other systems not related with hypersaline environments; the studies of Mirete *et al.* (2015) and Vidal *et al.* (2015). The study of Mirete et al., (2015) shows that halophiles adapt to the presence of salt by employing different strategies to maintain the osmotic balance between the cytoplasm and the surrounding medium including the accumulation of ions and the generation of compatible solutes (Erwin A. 1995; Sleator and Hill 2002; Oren 2008). The OPU method was used to study the microbial diversity of the brines and the rhizosphere samples from the Es Trenc saltern (Mallorca, Spain) in where the DNA was isolated to clone and express in genetically engineered organisms. The study of Vidal et al., (2015) evaluates the microbiome associated to the epithelium of the Colon intestine in patients with Crohn disease (CD), a chronic disorder characterized by patchy inflammation of the gastrointestinal tract (Baumgart *et al.* 2007).

Material and methods

Sampling collection

Brine and rhizosphere samples used in the study of Mirete *et al.* (2015) were recovered from the Es Trenc saltern (Mallorca, Spain) in August 2012. The samples from patients with Crohn disease (CD) and control subjects (HC) were recruited between August 2011 and March 2012, the samples included biopsies and feces (more information in Vidal *et al.* 2015). Detailed explanation about the DNA extraction is given in each specefic publication.

PCR amplification and 454-pyrosequencing, clustering by Operational Taxonomic Unit (OTU) and phylogenetic affiliation using Operational Phylogenetic Unit (OPU)

The 16S rRNA gene amplifications were performed using bacterial primer pairs GM3 and 630R for *Bacteria* and 21F and 1492R for *Archaea* (Table A 6.1) and previously reported conditions (Lane et al. 1985). The amplicon preparation for the massive sequencing was performed as previously mentioned (Mora-Ruiz *et al.* 2016). The sequences obtained by 454-pyrosequening were trimmed **(**sequences with <300 bp were removed, and low-quality sequences were trimmed (Mora-Ruiz *et al.* 2016). The phylogenetic inference was performed by OPUs as described in (Mora-Ruiz *et al.* 2015, 2016; Vidal *et al.* 2015).

Ecological indexes and statistical analysis.

OPUs were used to calculate rarefaction curves and the Shannon-Wiener (H´), Chao 1, and Dominance (D) indexes per sample with PAST v 3.01 software (Hammer, Harper and Ryan 2001). The data analysis to explore the diversity in rhizospheric soil and brines (Mirete *et al.* 2015) and to test the hypothesis of differences between control patients and those with Crohn disease (Vidal *et al.* 2015) is detailed in each article.

Results

Briefly, in the next section we will describe the most relevant findings in both studies using the OPU approach. In both we used high quality 16S rRNA gene sequences (mean >500 pb) obtained by 454-pyrosequencing.

Microbial community structure of the brine and rhizosphere samples

16S rRNA gene sequences were clustered at an identity threshold 99%, resulting in a total of 970 OTUs (Table A 6.2) that after the phylogenetic inference produced a total of 226 OPUs, 200 for *Bacteria* and 26 for *Archaea* (Figure 6.1, Table A 6.3). Most bacterial OPUs (187 OPUs*)* were detected only in rizhosphere (RB), while brines (BB) contained just 13 OPUs, and only two were shared by both samples (OPUs 109 and 144). The sequences were distributed in 16 phyla. A total of 102 OPUs affiliated with the phylum *Proteobacteria*, (47 *Alpha-*, 8 *Beta-*, 30 *Gamma-* and 17 *Deltaproteobacteria);* 31 with *Actinobacteria*, 27 with *Bacteroidetes* and 17 with *Firmicutes*. The major OPUs in RB were OPU 120 (*Ardenticatenamaritima,* 5.0%), OPU 153 (*Cytophagales,* 3.6%), OPU125 (*Bacillus halosaccharovorans,* 3.3%), OPU172 (*Actinobacteria,* 3.0%), OPU 90 (*Sorangiineae,* 2.9%) and, OPU22 (*Rhodobacteraceae,* 2.4%). Any OPU exceeded 5.1% of the total sequences (Table A 6.3). On the other hand, the major OPUs in BB were OPU102 (uncultured GR-WP33-58, 43.38%, a *Deltaproteobacteria* close to

Myxobacteria), OPU 143 (uncultured *Chitinophagaceae,* 12.6%), and OPU34 (uncultured *Limimonas*, 12.6%). The OPU34 and the OPU109 (*Rhodopirellula*) were the unique OPUs present both in RB and BB (Table A 6.3).

Sequences affiliated with *Archaea* generated lower diversity yields with 26 OPUs, all of them in the *Euryarchaeota* phylum (Figure 6.1 B).). Most of the OPUs affiliated with *Halobacteriaceae* (90.8% for RA - rizhosphere *Archaea*) and 100% for BA - brines *Archaea*). *Methanosarcinaceae* and *Methanoregulaceae* were present only in RA with 3.9% and 5.3% respectively. The most representative in RA sample were OPUs 204 and 205 (*Haladaptatus* spp., 52.6%), OPUs 215 and 216 (*Halopelagicus* spp., 10.5%), OPUs 201-203 (*Halococcus* spp., 9.2%), OPU226 (*Methanolinea mesophila*, 5.3%), and OPU225 (*Methanosarcina* spp., 3.9%). While, sequences in sample BA were represented principally by OPUs 209-213 (*Halorubrum* spp., 61.2%), OPU220 (*Haloquadratum* spp., 16.7%), OPUs 221 and 222 (*Haloarcula* spp., 3.8%), OPU208 (*Halomarina oriensis*, 3.7 %), OPU223 (*Halonotius* spp., 3.7%), and OPU224 (*Halobacteriaceae,* 3.7%; Table A 6.3). *Bacteria*l diversity (H´) and richness (Chao-1) indexes were higher in RB (4.5 and 221.5 respectively) than in BB (1.8 and 12 respectively; Table A 6.2 However, the abundances were more homogeneously distributed in RB than in BB. In accordance Dominance index for RB was the lowest in comparison with all samples (Table A 6.2). *Archaea* presented similar values for diversity (2.0), richness (13) and dominance (0.2) in both samples.

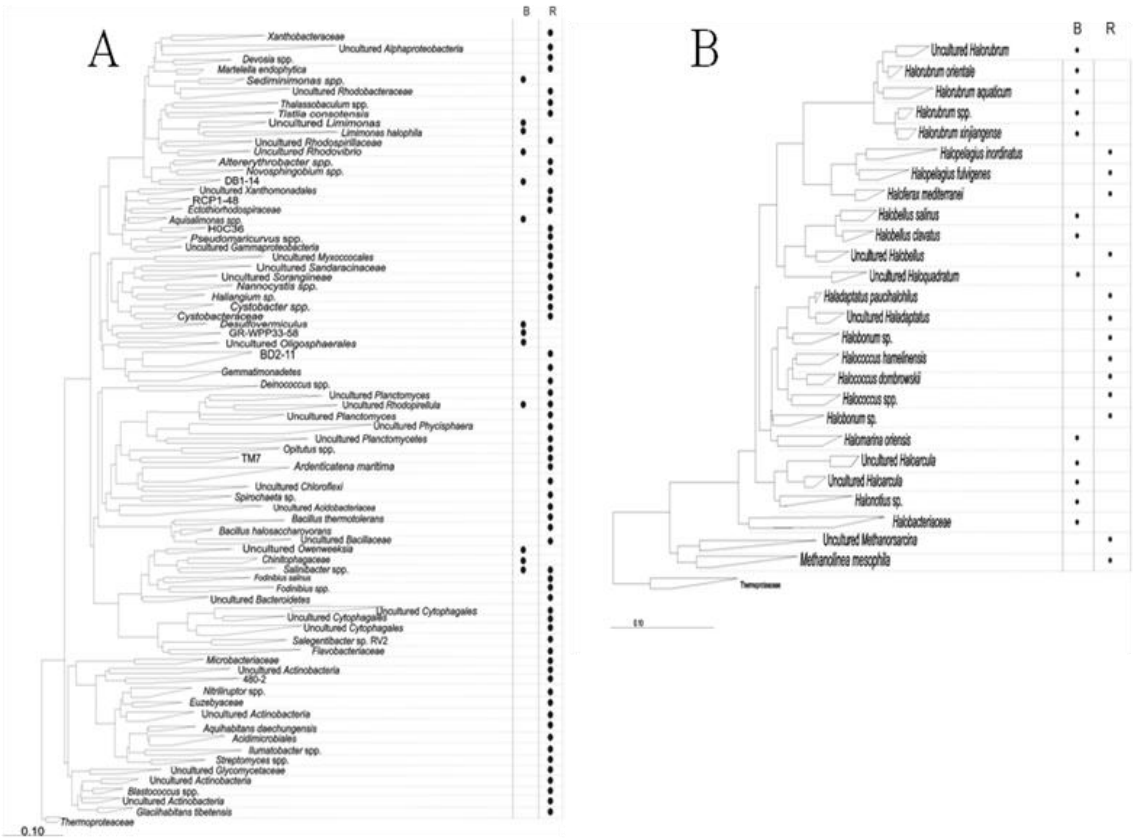

Figure 6.1 16S rRNA phylogenetic reconstruction for bacterial (A) and archaeal (B) sequences. The presence of OPUs with abundances >0.5% in each sample type (B = brines; R = rhizosphere) is indicated with a dot. Each OPU results from the phylogenetic inference resulting from the parsimony insertion of representatives of each sequence cluster at 99% identity, each representing independent OTUs.

Microbial diversity associated to Crohn disease

In this study, also with the clustering by OTU at 99%, the dataset was reduced to a mean of 216 (±55) OTUs in each sample (Table A 6.4). The phylogenetic inference produced a total of 338 distinct OPUs (mean 90 ±55). As in the study of Mirete *et al.* (2015), the most dominant phyla were *Proteobacteria, Bacteroidetes, Actinobacteria* and *Firmicutes* (Table A 6.5). In this case, the most relevant OPUs observed were OPU086 (*Bacteroides dorei* - *B. vulgatus*), and OPU098 (*B. fluxus*, - *B. helcogenes*, - *B. rodentium* – *B. uniformis*), OPU290 (*Faecalibacterium. prausnitzii*), and OPU215 (*Blautia wexlerae*) and OPU001 (*Escherichia* – *Shigella* sp.; Table A 6.5). Four OPUs were common to all samples including *Sutterella* (OPUs 046-1 and 046-2) and *Cupriavidus* (OPU028; Table A 6.5). The comparison of groups (CD *vs* HC) exhibited differences by number of OPUs (83 (±16) for CD and 101 (±19) for HC. Additionally, differences in the microbial structures between biopsies and feces of the same individual were observed, and that especially for HC, the analogous microbiomes were remarkably similar among themselves and significantly different between the sample kinds (*i.e.* feces versus

biopsies). On the other hand, with our approach was possible to detect four subsets into the CD samples (named in the study CD1 and CD2, CD3 and CD4), while the control patients formed a unique group (Table A 6.4).

Discussion

These pyrosequencing studies can be considered as of high quality because the sequences used were by far larger (>300 pb) than other former studies (Turnbaugh *et al.* 2009; Willing *et al.* 2009; Pilloni *et al.* 2012; França *et al.* 2014; Gobet, Boetius and Ramette 2014; Kambura *et al.* 2016; Yang *et al.* 2016), therefore with higher information content (Yarza *et al.* 2014), and also because the identification was made upon phylogenetic inference (Mora-Ruiz *et al.* 2015; Viver *et al.* 2015) rather than just identity matches (OTUs; Turnbaugh *et al.* 2009; Pilloni *et al.* 2012; França *et al.* 2014; Gobet, Boetius and Ramette 2014). In both cases, we observed the reduction of richness (from OTUs to OPUs) due the OPU clustering as expected (Mora-Ruiz *et al.* 2015, 2016).

Regarding the samples of rhizospheric soil and brines, both exhibited a microbial composition in accordance with the type of sample and high salinities (Mora-Ruiz *et al.* 2016, Mora-Ruiz et al., *in prep*.) The rhizosphere was very diverse in its bacterial composition with 187 distinct OPUs in accordance with the known complexity of the system (Philippot *et al.* 2013). Interestingly, as already stated in chapter 5, here is also detected the presence of the deltaproteobacterial GR-WP33-58, which are close relatives to *Myxobacteria*.

The archaeal composition was less complex with only representatives of the *Halobacteriaceae* family in accordance with the high salinity concentrations (Oren 2008), and representatives of the Rice Cluster I methanogens (*Methanosarcinales* and *Methanomicrobiales*; (Conrad, Erkel and Liesack 2006) also common in soils and widely distributed. The most remarkable observations were the high abundance (over 50% of the total archaeal diversity) of a close relative of the halobacterial genus *Haladaptatus,* originally isolated from low-salt and sulphide rich environments (Savage *et al.* 2007), the methanogenic species *M. mesophila* initially described in rice field soil (Sakai *et al.* 2012), and member of the Rice Cluster I (Conrad, Erkel and Liesack 2006). Altogether the results on the community structure of this soil agreed with the fact that the anaerobic hypersaline sediments below the salt crystallizers may be a source of methane and sulfide (López-López *et al.* 2010), and these may influence (by diffusion of ions and migration of microorganisms) the surrounding soils from which the plants were sampled.

The archaeal composition of the salt brines was remarkable. The archaeal community was only constituted by members of *Halobacteriaceae* and with the genera *Haloquadratum, Halorubrum* and *Haloarcula* as the most abundant. This structure was in accordance with the

known microbiota in brines (Oren 2008). However, the bacterial composition was remarkably different from what was expected. In general *Salinibacter* representatives have been found to be the major bacterial fraction in brines, in proportions that range from 5–30% (Antón *et al.* 2008). However, despite sequences of this lineage were found in the brines studied here, these constituted a minority (about 5% of the total bacterial diversity). The most represented bacterial lineage affiliated with representatives of the uncultured myxobacterial clade GR-WP33-58. Sequences of this deltaproteobacterial lineage were first detected in deep-sea Antarctic samples (Moreira, Rodríguez-Valera and López-García 2006). However, since its initial detection, similar sequences were retrieved mostly in marine samples (according to the identifiers in the entries from the NCBI). Some sequences of this clade had also been retrieved from hypersaline microbial mats (Harris *et al.* 2013) and saline soils (Castro-Silva *et al.* 2013), pointing to that its presence in brines may not be anomalous. The second most relevant proteobacterial group detected, and also in higher sequence abundances than *Salinibacter* were relatives of *Limimonas* (Amoozegar *et al.* 2013), an extremely halophilic member of *Rhodospirillaceae.* Finally, the third relevant group affiliated with relatives of the *Chitinophagaceae* lineage within *Bacteroidetes.* Similar sequences were detected in the hypersaline Lake Tyrrel in Australia (Podell *et al.* 2013b). Despite the sequences retrieved for the bacterial domain being in accordance with the hypersaline nature of the sample, the lower occurrence of *Salinibacter*, and the prevalence of representatives from the uncultured GR-WP33-58 clade need further investigation as such community structure has not been observed before.

Concerning to the study of Crohn disease, part of the treatment previous to the statistical analysis included the elimination the elimination of "rare" OPUs (those with <10 sequences; Vidal *et al.* (2015). This removing process is not uncommon, some authors suggest the removing of low represented sequences to avoid the Type II errors (Bokulich *et al.* 2014; Nelson *et al.* 2014; Krohn *et al.* 2016). On the other hand, in this study ubiquitous taxa were detected such as *Cupriavidus* (OPU028). Our approach allowed the detection of these ubiquitous elements; due the laxer clustering by OTUs, possibly those taxa would not have been detected as ubiquitous if we work using OTUs. Furthermore, a relevant finding here was the detection of differences between HC and CD. This differentiation was associated with variations in the abundances of specific groups such as some members of *Firmicutes*. We considered that OPU method plays an important role for the detection of key species in each group as already stated (Mora-Ruiz *et al.* 2016). All these results led us hypothesize the relevance of the OPU approach to detect key species producing part of the results in the global analysis shows in Annex I.

With the OPU strategy, some of the OTUs that would be identified as uncultured, could be identified up to true members of known genera and even species. This approach makes our findings different to other studies where an important proportion of the sequences were reported

as "uncultured" *Bacteria*/*Archaea* or only affiliating at the phylum rank (Redford *et al.* 2010; Xiong *et al.* 2012; Wanqiu *et al.* 2016). We can conclude that the OPU strategy is a powerfull tool for the analysis of other ecosystems such as those related to clinical microbiology (Vidal *et al.* 2015) opening new lines of research.

Chapter 7. Prokaryotic microbiota in the digestive cavity of the jellyfish *Cotylorhiza tuberculata*

Abstract

The microbiota associated to the gastric cavity of four exemplars of the jellyfish *Cotylorhiza tuberculata* has been studied by means of cultured-dependent and –independent methods. The pyrosequencing approach rendered a very reduced diversity of *Bacteria* with four major groups shared by the four exemplars that made up to 95% of the total diversity. The culturing approach recovered low abundant organisms and some of them also detected by the pyrosequencing approach. The major key organisms were related to the genera *Spiroplasma, Thalassospira, Tenacibaculum* (from the pyrosequencing data), and *Vibrio* (from the cultivable fraction). Altogether the results indicate that *C. tuberculata* harbors an associated microbiota of very reduced diversity. On the other hand, some of the major key players may be potential pathogens and the host may serve as dispersal mechanism.

Introduction

Cotylorhiza tuberculata is a scyphozoan of the phylum *Cnidaria*, often occurring in massive blooms in the Mediterranean waters, that follows an annual life-span cycle (Prieto *et al.* 2010). The medusae pelagic stage occurs at the late summer after abrupt temperature increases promoting blooms that may generate tons of biomass in relatively small seawater volumes. The life span of this species in the stage of pelagic jellyfish is one year (Kikinger 1992; Prieto *et al.* 2010). Practically, all the population of adult *C. tuberculata* disappears during late autumn - beginning of winter, a fact that cannot be explained just by mortality due to physical damage related to boats, or other human activities. The cause that triggers this mass mortality is unknown but has further consequences due to the release of organic and inorganic components (Pitt, Welsh and Condon 2009) and the activation of bacterial degradation and planktonic microbial community shifts (Tinta *et al.* 2010, 2012; Dinasquet, Granhag and Riemann 2012).

Outbreaks of jellyfishes as *C. tuberculata* among others, have important consequences on tourism and fisheries (Palmieri *et al.* 2014), as well as some of them have been reported as carriers of fish pathogens (Delannoy *et al.* 2011). Despite its relevance for human health, extensive aquiculture and touristic economies, not much is known on the lifestyle of jellyfishes, nor about the microbiota associated to them and its relevance. Some studies on molecular microbial ecology have been done on ctenophores (Daniels and Breitbart 2012; Hao *et al.* 2015), and on specific detection of fish pathogens in cnidarians (Ferguson *et al.* 2010; Delannoy *et al.* 2011; Fringuelli *et al.* 2012). However, exhaustive culture-dependent and -independent studies to reveal the associated microbiome, its diversity, relevance and potential use as indicators are yet to be reported.

The present study represents the first (to our knowledge) study on the microbiome of members of the class *Scyphozoa* by means of molecular microbial ecology tools independent and dependent of culture approaches. We focused the studies in the gastric cavity of the jellyfish *C. tuberculata*. This medusa feeds on microplanctonic organisms, which enter into the stomach through the numerous mouth arm openings, and remains of *Ciliata*, *Crustacea* and *Gastropoda* had been found among the particulate food items (Kikinger 1992). The gastric cavity is tightly connected with the surrounding waters, and thus a source of microorganisms' exchange that may constitute an effective dispersal mechanism, and a source of biological indicators. To this purpose, we combined high quality pyrosequencing (long nucleotide sequences and large amounts of reads; (Mora-Ruiz *et al.* 2015) with culturing of aerobic heterotrophs to reveal the microbial composition of the gastric cavity. The identification approach was based on the recognition of the Operational Phylogenetic Units (OPUs) that rely on phylogenetic inferences rather than sequence identity clustering, to better reflect the diversity observed (Mora-Ruiz *et al.* 2015; Viver *et al.* 2015).

Material and methods

Sampling and processing.

On September 2013 four exemplars of adults *C. tuberculata* (M1, M2, M3 and M4) were caught with a landing net. The sampling site was located in the Alcudia Bay, in the north of the Island of Mallorca, at about 0.5 miles from the shore (39º 45' 00''N; 13' 10'' E). The four exemplars were closely swimming in an area of about 10 m^2, and about 0.5 m below the surface. The sampling site is considered as one of the most pristine areas at the shore of Mallorca given the high water circulation rates (Suárez-Suárez *et al.* 2011). The four exemplars M1 (1.75 kg; male), M2 (1.5 kg; male), M3 (2 kg; female) and M4 (1 kg; male) were dissected onboard with sterile scalpel and the material of the digestive cavity was collected with sterile syringes and kept on ice in 50 mL sterile Falcon tubes. Samples were transferred to the laboratory (about 2 h later) and the material used for culturing purposes was mixed with 20% (v/v) glycerol (1:1) and stored at -80ºC.

Microbial DNA extraction, PCR amplification and pyrosequencing.

The DNA was obtained from either direct extraction of the gastric content (total biomass, BT, generating the samples M1BT, M2BT, M3BT and M4BT) by sizing with scalpel the gastric filaments, or from a previous separation of tissue material (BF, generating the samples M1BF, M2BF, M3BF and M4BF). The latter was prepared by mixing 300 µL of the gastric filaments with 500 µL of PBS 4X (548mM NaCl, 10.8mM KCl, 40mM Na_2HPO_4, 7.2mM KH_2PO_4,). The sample was gently broken up with a sterile mortar in a 2 mL microtube, followed by a centrifugation (10 min, 5400x g, 4˚C). The supernatant was stored in a sterile tube, and the pellet was washed 8x with 500 µL of PBS 4X, collecting the supernatants. Finally the pooled supernatants were centrifuged (10min, 15700x g, 4˚C) and the pellet was used for further DNA extraction. Both sorts of raw material were extracted according to previously published protocols (Urdiain *et al.* 2008). PCR reaction was performed in a volume of 50 µL using the Master Mix (5 PRIME GmbH, Deutschland) following the manufacturer's instructions. 16S rRNA genes were amplified using the specific primer pairs GM3 and S for *Bacteria* and 21F and 1492R *for Archaea* (Table A 6.1). PCR amplifications were performed as previously published using 30 cycles at 53°C (Melting temperature; Lane *et al.* 1986). A second short PCR (five cycles) was performed in a final volume of 25 µL in triplicate to incorporate tags and linker into the amplicon using 1:25 dilution of the original products as templates, and also using the same protocol that for the first PCR, primers GM3-PS and a variant of 907-PS (
Table A 7.1). The PCR products were purified and sequenced as previously published (Mora-Ruiz *et al.* 2015). The set of sequences has been deposited at the ENA sequence repository under the study project accession number PRJEB8518.

Sequence trimming, Operational Taxonomic Unit (OTU) clustering and OPU design.

Sequences with <300 bp were removed, and low-quality sequences were trimmed with a window size of 25 and average quality score of 25. No ambiguities and mismatches in reads with primer pairs and barcodes were allowed. Chimeras were removed with the application Chimera Uchime implemented in Mothur. The trimming process was performed using Mothur software (Schloss *et al.* 2009). The adequate selected sequences were clustered in OTUs at 99% using the UCLUST tool in QIIME (Caporaso *et al.* 2010). We consider one OTU each unique cluster of sequences with identities ≥99%. The longest read of each OTU was selected as representative, and was introduced to the non-redundant SILVA REF115 database using the ARB software package (Ludwig *et al.* 2004). Sequences were aligned with the SINA aligner (Pruesse, Peplies and Glöckner 2012), using LTPs115 database (Yarza *et al.* 2010). The OPU design by phylogenetic inference was performed as previously described (Mora-Ruiz *et al.* 2015; Viver *et al.* 2015).

Growth media, plating and isolation.

In all cases, a surface-spread plating method was used to isolate aerobic heterotrophic prokaryotes. One milliliter of homogenized gastric material was used to prepare the serial dilutions (until 10^{-4}) in PBS 4X. All samples and their respective dilutions were plated in duplicate in four different media: Seawater (SW; Rodriguez-Valera *et al.* 1985) at 5% and 10% salts, both with 0.05% yeast extract (YE, Cultimed, Scharlab); R2A agar (suplemented with NaCl 3.5% o 0.6M, Scharlab), and Marine Broth (0.5% peptone, 0.1% yeast extract, seawater and 1.6% agar). Plates were incubated at room temperature (22ºC), and monitored for at least two months. Colonies were selected taking into account different size, form and color to obtain the largest diversity possible making a mean of 48 ±7 for each sample and culture media. Selected colonies were brought to pure culture by re-streaking them on their respective solid media ensuring the recovery of a pure culture for each. For storage purposes, individual isolates were grown in their respective liquid medium, and the grown suspensions were mixed (1.5:1) with 50% (v/v) glycerol and stored at -80ºC.

WC MALDI-TOF MS analyses and identification by 16S rRNA gene sequencing.

The initial screening of the isolated strains was carried out with MALDI-TOF MS using whole cell biomass as previously published (Viver *et al.* 2015). Each single similarity cluster in the dendrogram was regarded as an Operational Taxonomic Unit (OTU), and was the basis for

further identification. The almost complete 16S rRNA gene sequences were obtained for one or two isolates of each OTU following the protocols previously published (Viver *et al.* 2015). The phylogenetic analysis was performed using the ARB software package (Ludwig *et al.* 2004). Newly added sequences were aligned using the LTPs115 database as template (Yarza *et al.* 2010), with SINA aligner (Pruesse, Peplies and Glöckner 2012), but the final alignment was manually improved. Tree reconstructions were performed using the Neighbor-Joining method with the Jukes-Cantor correction with the filter of 30%. For it, 750 high quality-supporting sequences from the LTP (Yarza *et al.* 2010) were incorporated to stabilize the tree topologies and obtain the final tree. OPUs were circumscribed based on the visual inspection of the tree (Viver *et al.* 2015). The sequences have been deposited at the EMBL repository under the accession numbers (LN812982-LN813005).

Statistical analyses.

Presence or absence of isolates detected for each OTU was coded as binary matrix and imported into the statistical program. The statistical significance of the differences among samples (for both culture dependent and independent approaches) was tested using a PERMANOVA analysis (*Permutational Multivariate Analysis of Variance Using Distance Matrices* Anderson 2001) using the Adonis function as implemented in the *Vegan* (Oksanen *et al.* 2016) package of R v 3.1.1 (www.r-project.org). Ecological indexes (Shannon, Dominance and Good´s coverage) and rarefaction curves were calculated using the PAST software version 3.01 (Hammer, Harper and Ryan 2001).

Results

The prokaryotic diversity thriving in the samples analyzed was amplified using bacterial and archaeal primers. No amplicon was produced with the archaeal primers in any of the samples studied. On the other hand, all samples amplified with the bacterial primers. The pyrosequencing approach generated a total of 59,117 sequences for a total of 7 samples, with a size mean of 568 ±159 bp, and with 12,694 sequences (25%) >700 bp. Unfortunately we did not get amplification from the M3BT biomass due to unknown reasons. However, as it is reported below, the diversity PERMANOVA analyses did not indicate that the two fractions (BT, total biomass; or BF, bacterial fraction collected by centrifugation and tissue discarding) were significantly different (p=0.5). The sequencing approach of all eight DNA samples of the four exemplars rendered between 18,533 (for M1BT) and 2,421 (for M2BF) sequences with a median value of 5,601 (for M4BF) sequences. After trimming, the number sequences for the study was reduced to about 78.6% to 88.3% in all samples (Table 7.1). The clustering approach into OTUs using the identity threshold of 99% sequence identity showed that among all samples

we could recognize a total of 882 OTUs, and these ranged between 93 to 344 for the BT samples and between 27 to 83 for the BF samples. Finally, the representative sequence of each OTU was inserted into a reference tree by phylogenetic inference and we could recognize a total of 143 distinct OPUs (Figure 7.1 and Figure A 7.1 and Table A 7.2) that made the complete diversity observed here. The number of OPUs, or putatively distinct species, observed was always higher for the BT samples (between 34 to 97) than for the BF samples (between 10 to 20). On the other hand, 88 OPUs were detected in single samples including 35 OPUs made of singletons and 18 OPUs made of doubletons.

Table 7.1 Total number of sequences obtained by pyrosequencing, after trimming and number of OTUs (clustered at 99% identity) and OPUs observed.

	Total	M1BT	M1BF	M2BT	M2BF	M3BF	M4BT	M4BF
Total seq.	59,117	18,533	7866	3241	2421	4035	17,420	5601
Trimmed seq.	50,779 (85.9%)	16,073 (86.7%)	6696 (85.1%)	2668 (82.3%)	1935 (79.9%)	3171 (78.6%)	15,287 (87.7%)	4949 (88.3%)
OTUs (99%)	882	344	83	93	68	27	202	65
OPUs	143	97	20	34	16	10	50	11

Ecological indexes (Table A 7.3) for the nonculturable data showed high dominance in most samples with values higher than 0.5 with exception of M2BT. The diversity index (H') ranged between 0.14 - 1.36. Richness estimation (Chao-1) and rarefaction curves (Figure A 7.3) showed that BT samples exhibited higher coverage reflected in a higher richness (Chao-1= 51.8 - 117.2) in comparison with BF (Chao-1= 14 - 27.2). On the other hand, culturable data presented similar diversity (0.9 - 1.4) as pyrosequencing, the dominance was slightly lower (0.4 - 0.7). In all cases, the jellyfish M4 showed the highest dominance and lower diversity values. Chao-1 in culturable fraction was considerably lower (10.6 - 31), and the rarefaction curves showed non-saturation.

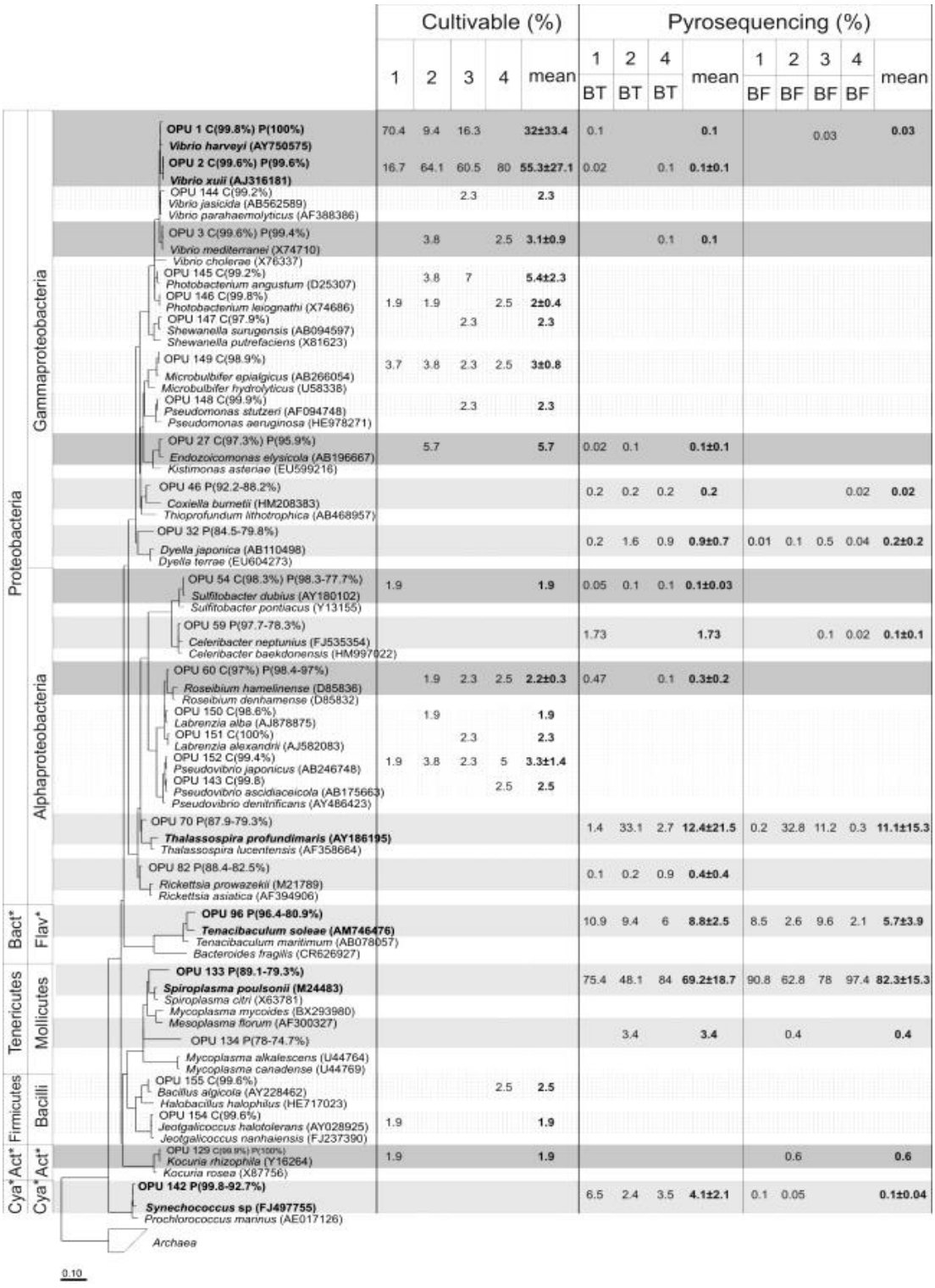

Figure 7.1 Compilation of the results observed regarding the major groups detected by the pyrosequencing and cultivation approaches. The left column shows the phylogenetic reconstruction of the representative sequences of each OPU detected and their affiliation with the closest relative type strains present in the LTP 119. Each OPU shows in brackets the identity value of the selected sequence and the closest relative reference sequence. Right columns indicate the occurrence (i.e. relative abundances in percentage of the total of each dataset) of each OPU in each of the different exemplars (1–4) studied here. In dark-gray background are indicated the OPUs occurring in both fractions (pyrosequences and cultures); in squared background those only detected by culture; in light-gray background the sequences only detected by the pyrotagging approach. In bold are highlighted the major groups detected by either pyrotagging or culturing.

Altogether the results pointed to a reduced diverse community of microorganisms in where four major groups of sequences made up to at least 95% of the total diversity. The major groups observed in decreasing abundances OPU 133 (affiliating with the genus *Spiroplasma;* summing about 69.2% ±18.8 for BT or 82.3% ±15.3 for BF), OPU 70 (affiliating with the genus *Thalassospira;* summing about 12.4% ±21.5 for BT or 11.1% ±15.3 for BF), OPU 96 (affiliating with *Tenacibaculum soleae;* summing about 8.8% ±2.5 for BT or 5.7% ±3.9 for BF), and OPU 142 mainly detected in the BT samples (affiliating with the genus *Synechococcus;* summing about 4.1% ±2.1 for BT or 0.1% ±0.04 for BF). Additionally, OPU 32 (*Dyella japonica*) was also present in all samples, but the abundances of this OPU were much lower (0.9±0.7 for BT or 0.2±0.2 for BF). The remaining OPUs (Figure A 7.1 and Table A 7.2) summed a maximum diversity of 2.5% and were very diverse. These low abundant OPUs affiliated mostly with the classes *Gammaproteobacteria* (OPUs 1-35; OPUs 45-53), *Betaproteobacteria* (OPUs 36-44), *Alphaproteobacteria* (OPUs 54-90); and with the phylum *Bacteroidetes* (OPUs 96-119). There were other minor representative taxa of *Planctomycetes*, *Delta-* and *Epsilonproteobacteria*, *Firmicutes* and *Actinobacteria*. From the low abundant taxa it is worth to mention a presence of several members of the *Vibrionaceae* (some also obtained in culture as indicated below), *Pseudoalteromonadaceae*, *Rhodobacteraceae* as well as representatives of the clade SAR116.

Samples were cultured onto four different media. All but SW 10% Salt, rendered colony forming units (CFUs) that were in the range between 1.3-6 x10^5 CFU/mL (Table 7.2). The highest counts were obtained for M4, but the samples M1-M3 rendered equivalent CFU yields for almost all media. A total of 190 valid profiles clustered in 19 distinct OTUs/OPUs (Table 7.2 and Figure A 7.2). Eight major OPUs were most relevant due to their presence and abundance in almost all samples (Table 7.3). The major group of isolates belonged to the *Vibrio* species *V. xuii* (101 isolates, OPU 2), *V. harveyi* (50 isolates; OPU 1) and *V. mediterranei* (3 isolates; OPU 3), which made up to 79% of the total set. Other relevant, but smaller groups of isolates belonged to OPU 145 (*Photobacterium angustum*; 5 isolates); OPU 146 (*Photobacterium leiognathi*; 3 isolates); OPU 149 (*Microbulbifer epialgicus*; 6 isolates); OPU 152 (*Pseudovibrio japonicus*; 6 isolates); and OPU 60 (*Roseibium hamelinense*; 3 isolates). The remaining OTUs (Table 7.3) occurred in very low numbers and just in one of the four samples, thus not treated as relevant.

The cultivable fraction made a very small percentage of the detected sequences by the pyrosequencing approach. However, seven of the cultured representatives (*V. harveyi*, *V. xuii*, *V. mediterranei*, *Endozoicomonas elysicola*, *Sulfitobacter dubius*, *R. hamelinense* and *Kocuria rhizophila*; Figure 7.1) were detected in the sequencing survey, but in very minor amounts (always < 0.3% of the total). The 11 additional cultured OPUs remained undetected by pyrosequencing.

Table 7.2 Data on the cultivable fraction of the samples.

Sample	SW 5% ($CFU \cdot 10^5 mL^{-1}$)	R2A ($CFU \cdot 10^5 mL^{-1}$)	Marine broth ($CFU \cdot 10^5 mL^{-1}$)	Nr. isolates	Nr. profiles	Nr. OTUs
M1	3.1 ± 0.6	3.8 ± 0.7	1.5 ± 0.7	76	54	8
M2	3.3 ± 0	3.2 ± 0	1.3 ± 0.4	69	53	10
M3	1.3 ± 0.6	3.9 ± 3.8	2.8 ± 1.7	48	43	10
M4	6 ± 0	5.5 ± 3.6	2.1 ± 1.6	46	40	8
Total				239	190	19

Table 7.3 OTUs detected in the MALDI-TOF MS dendrogram and their identification.

	M1	M2	M3	M4	SUM	OPU	Identification
OTU 1	1	0	0	0	1		*Sulfitobacter dubius* (AY180102)
OTU 2	1	0	0	0	1		*Kocuria rhizophila* (Y16264)
OTU 3	0	0	0	1	1		*Bacillus algicola* (AY228462)
OTU 4	0	0	1	0	1		*Shewanella surugensis* (AB094597)
OTU 5	0	0	1	0	1		*Vibrio jasicida* (AB562589)
OTU 6	38	5	7	0	50	1	*Vibrio harveyi* (AY750575)
OTU 7	0	2	3	0	5	145	*Photobacterium angustum* (D25307)
OTU 8	1	1	0	1	3	146	*Photobacterium leiognathi* (X74686)
OTU 9	0	2	0	1	3	3	*Vibrio mediterranei* (X74710)
OTU 10	9	34	26	32	101	2	*Vibrio xuii* (AJ316181)
OTU 11	0	3	0	0	3		*Endozoicomonas elysicola* (AB196667)
OTU 12	0	0	1	0	1		*Pseudomonas stutzeri* (AF094748)
OTU 13	2	2	1	1	6	149	*Microbulbifer epialgicus* (AB266054)
OTU 14	1	0	0	0	1		*Jeotgalicoccus nanhaiensis* (FJ237390)
OTU 15	0	0	1	0	1		*Labrenzia alexandrii* (AJ582083)
OTU 16	0	1	0	0	1		*Labrenzia alba* (AJ878875)
OTU 17	0	0	0	1	1		*Pseudovibrio ascidiaceicola* (AB175663)
OTU 18	1	2	1	2	6	152	*Pseudovibrio japonicas* (AB246748)
OTU 19	0	1	1	1	3	60	*Roseibium hamelinense* (D85836)
	54	53	43	40	190		

Discussion

This is the first report on the associated microbiota in the gastric cavity of the jellyfish *C. tuberculata*, member of the class *Scyphozoa* phylum *Cnidaria*. The very first study on gelatinous zooplankton based on molecular methods was performed studying the microbiota associated to *Mnemiopsis leidyi* and *Beroe ovata* of the phylum *Ctenophora* (Daniels and Breitbart 2012), using clone libraries and molecular fingerprints. However, studies on members of the class *Scyphozoa* have just been studied focusing on the specific detection of the

pathogenic bacterium *T. maritimum* (Ferguson *et al.* 2010; Delannoy *et al.* 2011; Fringuelli *et al.* 2012), and no report (to our knowledge) on the community structure of the associated microbiome has been published. The study is based on just 4 exemplars of the jellyfish in just one location and one season. We are aware that the number may be too low to make generalizations, but the very strong coincidence of the results among the exemplars make the analysis confident.

The high throughput survey revealed that the dominant diversity in this jellyfish cavity was very reduced as four different major OPUs were making at least 95% of the total diversity, and this dominance of few groups was in accordance with the observations made in ctenophores' tissues (Daniels and Breitbart 2012; Hao *et al.* 2015). The composition of the 139 minor OPUs detected, and mainly belonging to the *Gamma*- and *Alphaproteobacteria*, *Bacteroidetes* and *Planctomycetes* seem to be in accordance with the common microbial composition of seawater (Glöckner *et al.* 1999; Mary *et al.* 2006), and specifically west Mediterranean (Nogales *et al.* 2007; Díez-Vives, Gasol and Acinas 2014; Baltar *et al.* 2015). Due to the very low abundances of each of them, we cannot discard that these were not part of the *C. tuberculata* microbiome, but accompanied the seawater impregnating the tissues, or part of the ingested microplancton (or picoplancton in this case) through the moutharm openings (Kikinger 1992). On the other side, the four major OPUs detected were present in the four exemplars of jellyfish studied, and may respond to the associated microbiome of this species.

The most abundant sequences, making between 50% to 97% of the complete diversity in all cases, loosely affiliated with the members of the genus *Spiroplasma* (OPU 133), being *S. poulsonii* (Williamson *et al.* 1999) the closest relative with a maximum identity of 89.1%% in the sequenced stretch (Figure A 7.4). Despite the sequence is just partial, and then the observations may be taken with care, this *Spiroplasma* sequence may represent at least a new species given the low identity with the closest relative type strain (Yarza *et al.* 2014). *S. poulsonii* belongs to the family *Spiroplasmataceae* of the class *Mollicutes*. The members of this class are wall-less bacteria, generally of very small sized cells (1-2 µM diameter) and very small genomes (530-2220 Kb), and generally host-associated (Gasparich 2010). Specifically *Spiroplasma* has been reported to be a fastidious organism to be cultured, and generally associated to plant and insect diseases (Gasparich 2010). The reports of *Mollicutes* in *Scyphozoa* are very scarce. The first and unique report on the presence of a *Spiroplasma* in a jellyfish was published in the Ph-D thesis of O. Vega-Orellana (Vegan-Orellana 2014), in four exemplars of the luminescent jellyfish *Pelagia noctiluca* detected with a specific PCR amplification. Given the conspicuous abundance of these sequences in all four exemplars of *C. tuberculata* making >50% of the total diversity (69.2 ±18.7 for BT and 82.3 ±15.3 for BF), as well as the healthy aspect of the exemplars (at the sampling date the waters exhibited a bloom of this jellyfishes not showing any morphological anomaly) it is difficult to infer a pathogenic nature of this organism.

Actually, despite most of the *Mollicutes* are considered pathogens, some have been considered to establish symbiotic relations with *e.g.* coral species (*i.e.* the "*Candidatus* Mycoplasma corallicola" in the species *Lophelia pertusa*; Neulinger *et al.* 2009). Finally, also in the ctenophores studied previously (Daniels and Breitbart 2012), some *Mollicutes'* sequences were detected, but due to the short sequence generated, their accurate identification was not possible.

The second most abundant group of sequences affiliated with the genus *Thalassospira* (López-López *et al.* 2002), with identity values below 87.9% with the closest relative species that may be considered as a different species or even genus (Yarza *et al.* 2014). *Thalassospira* was first isolated from Mediterranean seawater and under heterotrophic aerobic conditions (López-López *et al.* 2002). There are not many reports on the occurrence of this organism in natural samples, and had been found in consortia degrading aromatic hydrocarbons (Gallego *et al.* 2014) as well as associated microbiota of *Annelida* in crude oil enrichments with potential production of biosurfactants (Rizzo *et al.* 2013). In addition, this organism was one of the most prominent in the ctenophores *M. leidyi* and *Beroe* sp. (Daniels and Breitbart 2012; Hao *et al.* 2015). Due to the scarce information on its physiology, we cannot hypothesize any relevant role in *C. tuberculata*.

The third most relevant sequence type due to its abundance affiliated with the fish pathogen genus *Tenacibaculum*, with the closest relative *T. soleae* (Pineiro-Vidal *et al.* 2008) with identity 96.4% that may indicate that this represents a different species (Yarza *et al.* 2014). All 20 species of *Tenacibaculum* hitherto classified (http://www.bacterio.net/tenacibaculum.html) have been isolated from marine environments. Most of them from water (five species) or sediment samples (four species), but others (*T. adriaticum and T. crassostreae*) associated to apparently healthy oysters (Lee *et al.* 2009), bryozoan (Heindl, Wiese and Imhoff 2008) or sponges and green algae (Suzuki *et al.* 2001). Only the three species *T. dicentrarchi, T. discolor* and *T. soleae* have been isolated from diseased marine fauna (Wang *et al.* 2008; Pineiro-Vidal *et al.* 2012). Especially *T. maritimum* (which actually belongs to a different phylogenetic clade; Figure A 7.5) is a known fish pathogen, for which a detection method based on specific PCR has been developed (Fringuelli *et al.* 2012). With this method, the presence of this organism has been reported in jellyfishes as *P. noctiluca* (Delannoy *et al.* 2011) and *P. quadrata* (Ferguson *et al.* 2010). However, we did not detect any similar sequence to this species in our survey. As most organisms have not been reported to be associated with disease, but naturally thriving in sediments, seawaters or saprophytes animals or plants, we cannot rule out that in *C. tuberculata* this organism may also be a naturally occurring as commensal or symbiotic microbiota. Some of the isolates had been shown to exhibit properties as synthesis of algaecides (Li *et al.* 2013) that could be relevant for the host.

The fourth major group observed was OPU 142 affiliating with the genus *Synechococcus*. Nearly all the sequences of this OPU were detected in the BT fraction, and nearly insignificant amounts were detected in the BF samples. These results indicated that the cells of *Synechcococcus* should have been pelleted with the tissue material after being disaggregated and centrifuged. *Synechococcus*, together with *Prochlorococcus* are the major microbial primary producers in the oceans (Ting *et al.* 2002) and globally distributed. Moreover, it has been hypothesized that this organism can be actively ingested by the dinoflagellate *Symbiodinium*, a widely spread symbiotic organism associated to *Cnidaria*, serving as primary source of nitrogen in oligotrophic waters (Jeong *et al.* 2012). *Symbiodinium* in jellyfishes seems to be generally associated to the tentacles and oral endodermic tissue (Muscatine 1974), and this would be in accordance with the *Synechococcus* sequences to be just detected in the non-fractionated biomass. Contrarily, the other three major groups (*Spiroplasma*, *Thalassospira* and *Tenacibaculum*), appear with similar amounts in both fractions may be extracellular or not strongly bound to the tissues, thus probably part of the digestive microbiome.

The culturable fraction made a very small proportion <0.6% of the total diversity detected by the pyrosequencing approach in accordance with the low cultivability in natural ecosystems (Amann, Ludwig and Schleifer 1995). This fraction showed also very a low diversity trend, and was strongly dominated by few groups. The most relevant were members of the family *Vibrionaceae*, and in special *V. xuii* and *V. harveyi* that made nearly 80% of the total culturable fraction and were present in all samples. *V. xuii* was initially isolated from shrimp culture waters as well as associated to invertebrates (Thompson *et al.* 2003), and yet a pathogenic nature of this organism has not been reported. On the other hand, *V. harveyi* is considered a serious pathogen for marine fish and invertebrates (Austin and Zhang 2006), promoting diseases as vasculitis, gastro-enteritis and eye lesions.

In summary, *C. tuberculata* seems to have an associated microbiome in its gastric cavity that is of very low diversity with at least three major prokaryotic taxa (*i.e. Spiroplasma*, *Thalassospira* and *Tenacibaculum*) representing over 95% of the total sequence retrieval in our survey, and specially *Spiroplasma* that summed over 69 to 82%. But none of the three major taxa had been reported to be abundant free living in plankton, nor in among the emerging taxa associated to jellyfish biomass degradation after blooms (Tinta *et al.* 2010, 2012; Dinasquet, Granhag and Riemann 2012). Contrarily, the low abundant and very diverse taxa detected were more reminiscent of the planktonic marine communities (Glöckner *et al.* 1999; Mary *et al.* 2006; Nogales *et al.* 2007; Díez-Vives, Gasol and Acinas 2014; Baltar *et al.* 2015). It is remarkable that the three major taxa had been already detected in association with the two ctenophores *M. leidyi* (M.L) and *B. ovata* (B.O; Figure A 7.6; Daniels and Breitbart 2012), and thus as hypothesized for corals these should be rather specifically associated to the medusa than

the occupation by opportunistic bacteria ingested during the feeding process (Agostini *et al.* 2012). These organisms may be just saprophytic colonizers of the digestive organ of this jellyfish. However, due to the fact that at least spiroplasmas may establish symbiotic relations with their hosts (Neulinger *et al.* 2009), it seems plausible that *C. tuberculata* could benefit from the interaction with the hosted microorganisms. Moreover, we cannot discard that such abundant microorganisms may interact with the host releasing substances that may be of very much relevance to induce changes in their life-cycle as occurs in other cnidarian (Tebben *et al.* 2011), or may perhaps be responsible for the annual life cycle that ends with the mortality of the pelagic forms (Prieto *et al.* 2010). On the other hand, some members of *Spiroplasma*, *Tenacibaculum* and *Vibrio* are potential pathogenic colonizers, and in this case, *C. tuberculata* could operate as dispersive mechanism in a similar way to what it has been reported for *P. noctiluca* carrying *T. maritimum* (Delannoy *et al.* 2011). In any case, the pathogenic nature of the organisms detected, excepting *V. harveyi*, needs still to be proven. Further investigation on the microbiomes of such pelagic organisms, thriving in all coasts and experiencing seasonal blooms, is necessary to overcome the lack of knowledge about the relevance of the associated microbiota and understand the threatens of disseminating potential pathogens.

Section V. General discussion

Life Uh, finds a way

Dr. Ian Malcom

Discussion of the main findings

The study of the complexity of microbial diversity in different saline and hypersaline environments and their driving factors at different spatial and temporal scales is a crucial aspect for the understanding of such environment's microbiology. This thesis contributes to this field with some novel advances, as the OPU approach. Along the different chapters, we have answered different questions related to diverse saline and hypersaline environments as plants, sediments, brines and animals. In every chapter we provided a detailed discussion on each specific issue; therefore here we provide an integrative view of the general findings of this thesis.

Microbial diversity and the relevance of environmental factors

Most studies on hypersaline environments have been focused on the description of the microbial richness (Jiang *et al.* 2006; Maturrano *et al.* 2006; Ventosa 2006;); some other studies have been directed to reveal the existence of relationships between community structure and environmental factors (Crump *et al.* 2004; Chapters 2 and 5), or spatial distance (Ma *et al.* 2013; see Chapter 5). Finally, others have analysed the main variables affecting specific taxa when large set of environmental variables were conjointly collected (Ramette 2007; Chapter 2).

Although salterns have been the focus of attention and the model hypersaline environment, here we have also explored new saline environments as jellyfish and halophytes. We found that the jellyfish cavity presents a high microbial dominance principally represented by *Spiroplasma* and *Tenacibaculum.* In general *Spiroplasma* members have been reported as a fastidious organism associated to plants and insects (Gasparich 2010), while some *Tenacibaculum* species (*T. adriaticum* and *T. crassostreae)* are associated with marine and healthy animals, such as oysters (Lee *et al.* 2009), bryozoan (Heindl, Wiese and Imhoff 2008) or sponges (Suzuki *et al.* 2001). Although *Spiroplasma* was found as the most abundant group in the jellyfish cavity, there are some interesting recent results by metagenomics which evidence the high abundance of other taxa in *C. tuberculata*: Candidatus Syngnamydia medusae and Candidatus Medusoplasma mediterranei (Viver *et al.* 2017). The differences between our results and those from Viver et al. (2017) could be related with PCR amplification bias in the 454-pyrosequencing data.

Regarding the exploration of the microbiota associated to halophytes, we initially studied the epiphytic and endophytic communities (Chapter 1). Also this thesis explore the endophytic communities since they are less influenced by factors such as rainfall, wind and sea spray (Reinhold-Hurek and Hurek 2011; Natalia V. Malfanova 2013). In the culture-dependent exploration of the microbial communities associated to the endosphere, the cultivation of both

moderate halophiles and mesophiles, produced a recovery of ~70% of the total microbial diversity detected by 454-pyrosequencing, far away from the paradigm that considers that culture-based techniques recover only a 1% or less of the total prokaryotic diversity (as in Chapter 7; Amann, Ludwig and Schleifer 1995). Our results also showed that most of the microbial communities were halophiles (Chapter 1, 2 and 6). However, we detected both by culture-dependent and -independent methods an important proportion of mesophilic microorganisms (Chapter 1, 2 and 3). Due to the internal compartmentation of the ions in the internal part of halophytes (Parida and Das 2005; Grigore, Ivanescu and Toma 2014), it would be common to found hypersaline and non-saline microenvironments in the plant. In our case, both microbial communities (halophiles and mesophiles) were coexisting in the endosphere. The exploration of the microbial diversity in the endosphere of halophytes also exhibited a bacterial diversity pattern, with higher values in the rhizosphere which decreased towards the more mature aerial parts. A higher diversity in the rhizosphere has been already reported (Bodenhausen, Horton and Bergelson 2013) reinforcing the hypothesis of a microbial rhizospheric origin (Taghavi *et al.* 2010). For *Archaea*, this pattern was inverted, suggesting that bacterial and archaeal habitats are poorly overlapped.

The efficiency of the OPUs approach to detect patterns is not only restricted to a small spatial scale (centimeters in the endosphere of halophytes). In Chapters 2 and 5, we showed that halophilic microbiota is strongly dependent on the origin of the location at a middle (Chapter 2) and large-scale (Chapter 5). Although the microbial paradigm of "everything is everywhere" remains, previous studies have showed evidence of the conditioning of biogeography on microbial communities (Sibuet and Olu 1998; Martiny *et al.* 2006; Ward and Bora 2006; Cetecioğlu *et al.* 2009; Zinger *et al.* 2011; Livermore and Jones 2015) as well as this thesis. Even though a part of the differences found between locations can be explained by the physicochemical differences of each site (Fierer and Jackson 2006; Bulgarelli *et al.* 2012), a remnant proportion is associated to the geographical distance (*e.g.* Chapter 5; 36% in Mantel test). Our results are in accordance with previous studies who found that the distance effect produces a strong influence on microbial communities (Foissner 2006; Ward and Bora 2006; Gray *et al.* 2007; Cetecioğlu *et al.* 2009; Filker *et al*., 2017). Additionally, although some biogeographic patterns were observed at genus level, those patterns were diffused, being clearer at the OPU level (Chapter 5).

The use of OPUs does not only allow the detection of biogeographical patterns, but also made possible to recognize a novel diversity associated to the different environments. Putative new species were found not only by culture-dependent analysis (Chapter 3) but also by culture-independent (Chapter 1 and 2). The detection of putative new taxa is not uncommon if we consider that part of this thesis is focused in poorly explored environments (*e.g.* jellyfishes and halophytes). In fact Yarza *et al.* (2014) predicted that with the current sequencing efforts, most

taxa will be discovered before the end of this decade. Additionally, this thesis remarks that, although we detected novel diversity, our approach contrasted with other culture-independent studies in where a significant percentage of sequences remained unclassified (Vaz-Moreira *et al.* 2011; Bodenhausen, Horton and Bergelson 2013). In fact, the OPU approach by means of phylogenetic inferences produced more accurate identifications (*e.g.* 92.8% of the OPUs could be affiliated at the genus level in Chapter 2). In this work, we detailed some interesting groups such as the *Deltaproteobacteria* GR-WP33-58, conspicuously present in different works (Chapters 2, 5, 6 and 6), suggesting its relevance in hypersaline sediments and brines. The relative recent detection of this taxa in different habitats (Moreira, Rodríguez-Valera and López-García 2006; Li *et al.* 2015; Kambura *et al.* 2016) as well as its presence in the different samples used in this work led us considering it of especial interest and a target for future research.

Beyond the novel diversity detected in this thesis, we also observed relevant variations in abundances of specific OPUs associated to the changes in some environmental factors. In Chapter 5, we observed anaerobic taxa, such as *Halanaerobium* and *Halanerobacter*, which were incremented in high salinity environments suggesting the importance of anaerobic taxa in brines close to salt saturation. The presence of those anaerobic groups could be related with the episodes of anoxia principally produced by reduction of the oxygen solubility due the high temperature (Javor 1989; Tromans 1998). Contrarily, aerobic groups such as *Salinibacter* and members of *Nanohalarchaeota* were detected in sediments; their abundances could be evidencing that there is an active fraction of aerobic groups which live in sediments and that not only come from a deposition process from the water column.

Although salinity has been considered as the main driving factor in the microbial communities of hypersaline environments (Rodriguez-Valera *et al.* 1985b; Casamayor *et al.* 2002; Dillon *et al.* 2013; Canfora *et al.* 2014; Mani *et al.* 2015), we found other natural drivers strongly affecting bacterial and archaeal communities, such as organic matter, Mg^{2+} and K^{+} (Chapter 2, 5 and 5) according with the results of Gomariz *et al.* (2015) . The relevance of the two last ions is associated with the microbial resistance to the osmotic pressure: while K^{+} is used as osmotic solute to maintain the osmotic equilibrium in several representative members of hyperhalophilic *Archaea* and *Bacteria*, Mg^{2+} is considered as the most abundant divalent cation in halophilic microorganisms. The accumulation of Mg^{2+} has been shown to play important roles in stabilizing halophilic enzymes (Madern, Ebel and Zaccai 2000; Becker et al. 2014). For example, evidence of the relevance of magnesium ions involved in the posttranslational modification of the S-layer glycoprotein makes this ion necessary for the growth of species such as *Haloferax volcanii* (de Medicis 1986; Eichler 2001; Oren 2008).

This thesis also includes the screening of genes related to salt resistance by functional metagenomics. This experimental approach has been applied for studying gene function in

different environments such as humans and soils (Allen *et al.* 2009; Angelov *et al.* 2009; Culligan *et al.* 2012, 2013; Cecchini *et al.* 2013). We identified diverse genes conferring salt resistance, as those related to osmoadaptation, in *E. coli* such as a glycerol permease, a proton pump, DNA repair enzymes and hypothetical proteins. Due to the high environmental pressures in extreme habitats (i.e. solar radiation), microorganisms inhabiting them require an effective DNA repair system. This is in agreement with previous studies who linked DNA repair genes with ionic radiation, dehydration and UV radiation in extremophilic microoganisms such as *Deinococcus radiodurans* and *Halobacterium salinarum* (Mattimore & Battista, 1996, Kish & DiRuggiero, 2012). Finally, the hypothetical proteins identified could involve novel mechanisms of osmoadaptation which require a deeper study in a future.

Comparison of OPU and OTU approaches

In general, the clustering method has been considered as a bottleneck in the analysis of sequences from complex microbial communities (Preheim *et al.* 2013). However, several approximations have been generated to improve the efficiency of the sequencing analysis. Blaxter *et al.* (2005) developed the idea of MOTUs (Molecular Operational Taxonomic Units) based on the groups identified using a DNA barcode. Later, Sharpton *et al.* (2011) pointed that an alternative to sequence similarity-based clustering is a phylogeny-based OTU clustering, taking into account a model of sequence evolution which might lead to more accurate clusters. However, OTU is still the most extended method used with constant modifications of the pipelines for the sequences analysis.

In this thesis, we obtained different datasets to evaluate the OPU approach.. As already stated, several advantages in the use of OPUs versus OTU have been exposed along the different chapters of this Thesis. However, a more formal approach is necessary to quantify the differences. Therefore, part of this discussion is oriented to provide a global analysis of the obtained databases. We have compared the OPU and OTU approaches including different cut-offs of OTUs in order to evaluate the robutstness of the method. At this point, we want to remark that part of this analysis is still under progress and it will be addressed in a future work (see Annex).

For the global analysis we used different techniques, including network statistics, to determine the importance of microorganisms in co-occurrence networks (*e.g.*, degree, betweenness, measures of centrality), by identifying key species within an given ecosystem (Bauer, Jordán and Podani 2010; Steele *et al.* 2011; Eiler, Heinrich and Bertilsson 2012). method, together with multivariate test and ecological metrics, can enhance interpretation increasing our knowledge related to microbial communities (Williams, Howe and Hofmockel 2014).

Regarding the diversity metrics, the comparison between the richness and diversity indices showed that OPU values were lower than those recovered by OTUs. Apparently, OPU is able to minimize one of the most noticeable problems of OTUs, the overestimation of both richness and diversity (Nelson *et al.* 2014; Flynn *et al.* 2015; Krohn *et al.* 2016). For instance, Nelson *et al.* (2014) detected an overestimation between 25-125 times higher than the expected. In this way, Krohn *et al.* (2016) already suggested the use of phylogenetic metrics to diminish the effects of complications associated with systematically-inflated OTU diversity. We found that the OPU approach minimizes these overestimations since it can recognize as a same unit sequences with different lengths and/or slightly sequence dissimilarities that are usually grouped within different OTUs (Vidal *et al.* 2015)

Although alpha diversity estimations are more sensitive to the effects of systematic errors (such as those produced in the OTU approach) than beta diversity calculations (Krohn *et al.* 2016), we also evaluated the effect of the beta diversity. We tested the main hypotheses previously proposed in each Chapter but in this case we tested them not only on OPU but also on OTU databases (Table N 1.1). As we mentioned in the previous section, differences by location in the microbial communities were detected using OPU approach in Chapters 2 and 5. This biogeography pattern was also detected at all OTUs levels (see databases from Chapters 2 and 5 in Table N 1.1). reinforcing the conclusions previously obtained in each Chapter.

Due to the results obtained in Plant_Bac (Chapter 2), where we did not detect differences in the total structure of the community from different internal areas of the plant, we expected the same behavior with Arch database (Chapter 4). This hypothesis was corroborated in our results (Annex 1). Although all the levels were congruent with this conclusion, it is interesting to remark that the results based on OTUs at 97% were marginally significant. This could be related to the evidence that spurious OTUs may provide artificial support to the statistical separation of experimental treatments. Furthermore, the artificial noise of these OTUs makes difficult to detect differences (Schirmer *et al.* 2015; Krohn *et al.* 2016). In the opposite case, for other databases where we found differences by location, this difference was detected also in all levels; however, the amount of variance explained was generally higher using OPU (r^2) as, for example, in *e.g.* Sed_sed_bac Chapter 5 and Time_Bac Chapter 6.

An alternative of finding the keystone species is the use of the IndVal analysis (De Caceres and Jansen 2015); which showed that, in most cases, OPU approach detected a higher number of indicator species with the exception of brines (Chapter 5 and Chapter 6; Table N 1.1) suggesting that OPU is a more determinant method.

Regarding the exploration of our data using co-occurrence networks, this technique has been successfully applied in different environments (Newman 2006; Ruan *et al.* 2006; Freilich *et al.* 2010; Barberán *et al.* 2011). Previous studies have evidenced patterns of co-occurrence for microorganisms and macroorganisms suggesting that non-random community assembly may be

a general characteristic across all life domains (Horner-Devine *et al.* 2007). The non-random distribution of the microbial communities has been explained in Chapter 2 and 5, where a geographic pattern was detected; thus we decided to deeply explore our results using co-occurrence techniques. The co-occurrence network analysis showed also differences by level, being OPU the most different according with our multivariate analysis, finding also that OTU 97% seemed to be more similar to OPU. As expected, sediments samples were more complex, with higher number of nodes than other environments (Table N 1.2). Sediments have been considered as one of the most heterogeneous environments harboring highly diverse prokaryotic communities (Jiang *et al.* 2006; López-López *et al.* 2010; Kubo *et al.* 2012; Rasuk *et al.* 2016). The topological structure of the networks also showed that in most cases OPUs produced networks with modular structures (modularity >0.4; Newman 2003; Newman and Reynolds 2005). An interesting case is Soil *Bacteria* database (Chapter 2; Table N 1.2) that despite modularity was expected (Barberán *et al.* 2011), where although modular structure was only found using OPU approach as the basic unit. Lower density was observed using OPUs principally by the effect of lower richness.

On the other hand, nodes were more connected (mean degree; Table N 1.2) in OTU 99% and 98.7% databases and the activity of the nodes in OPU was lower and more similar to 97% (Faust and Stanley Wasserman et Kathrine Faust 1994). Our results coincided with the behavior of non simulated networks, where most nodes have a relatively small degree, but a few nodes will have very large degree as already suggested by Restrepo, Ott and Hunt (2006, 2007). The marked differences of degree between databases is not strange due the previous evidences of non simulated networks that have very different number of connections of nodes (degree distributions; (Restrepo, Ott and Hunt 2006).

Although we evaluated and compared the general properties of the networks, the individual role of the OTUs/OPUs as well as the distribution of generalist/cosmopolitan taxa in the network is an ongoing work that we expected will help to detect deep differences between both methods.

We have seen now some objective evidences on the benefits of OPU approach against different levels of OTU. However, there are other reasons that are discussed to reinforce this argument. In the approach based on OPUs, we have a higher control in comparison with other approaches (Kirchman, Cottrell and Lovejoy 2010; Redford *et al.* 2010; Lin *et al.* 2012; Krohn *et al.* 2016). In general we used sequences >500 bp to avoid artificial sequences which can greatly overestimate the total number of OTUs (Preheim *et al.* 2013). Additionally, the problem for obtaining OTUs with different algorithms increase when the clustering cutoff decrease (Preheim *et al.* 2013). Our approach minimize this problem by clustering the sequences at 99%. Being so strict in the clustering identity, the problems associated to sequencing errors are reduced at the same time as the dataset decreases in number of single sequences. It is also

interesting that different identifications may vary depending on the selection of the representative sequence (Nguyen *et al.* 2016). In our approach, we always selected the longest sequences as these where the ones containing the highest phylogenetic signal of each OTU (Yarza *et al.* 2010).

A common option used for the identification of the best matching sequences in a reference database is the BLAST algorithm (usually BLASTn) (Altschul *et al.* 1997). Doing so it is possible to assign the identifier of the best match to the specimen if the similarity is judged to be good enough (Blaxter *et al.* 2005). However, this method has many pitfalls, 1) its reliance on well-populated (and correctly named) databases; 2) the lack of close relatives in the database can make this approach less-than-rewarding; 3) although, the alignment is a turning point for the identification processes, the alignment cannot be reviewed or even improved (Blaxter *et al.* 2005). In the OPU approach we tried to avoid those problems, first using an accurate database (LTP) supplemented with the SILVA-ref database in case to not having any close relatives. Secondly, the alignment was visually inspected and additionally the OPU approach ensures the monophyly of each group, which is an absolute premise to guarantee that the organisms in the same biological unit have a common evolutionary history (Rosselló-Móra and Amann 2015). The use of this approach based on phylogenetic inference results in a more accurate identification of taxa and therefore, it is also possible to examine their relationships with other groups. We reinforce the idea of Nguyen *et al.* (2016) who, based on the discrepancies observed in the use of OTUs, concluded that OTUs are an artificial artifact that makes the bioinformatics analyses easier. However, easiness in running the analyses does not mean accuracy. Despite the benefits of the approach, since the quality of the estimated richness is very high, there is a clear limitation in the OPU approach: it is time-consuming. Finally, OPUs do not need to be exclusively used for 16S rRNA gene sequences, as it can be used with other genes that can give a better resolution for other lineages. It is important to remark that the OPU approach does not rely on a cutoff, but the structure of the phylogenetic inference determines what will be considered as a unit.

The global analysis of the comparison of OTUs and OPUs showed that the sensibility of the OPU allows the obtaining of conclusions that are not reached with OTUs. Despite the OPU most similar cut-off was 97%, some notable differences could be observed. Finally, we provide evidence of the efficiency of OPU clustering methods and this thesis offers a substantiated framework to guide microbiologists in the use of OPUs for microbial datasets.

Section VI. Conclusions

- OPU approach is an appropriated method to study microbial communities; the use of this method is not restricted to saline or hypersaline environments, but can be used in other habitats as it offers the opportunity to obtain a more realistic biological unit of study for fundamental questions about microbial biodiversity, environmental driving factors and biogeography.
- The microbiota associated to halophytes is principally dominated by moderate halophiles, however near to 5% of the richness can be considered as mesophilic. With our large-scale culturing approach is possible to recover ~70% of the total microbial endophytic diversity detected by 454-pyrosequencing, and despite most of the diversity seems to be of the *Bacteria* domain, members of *Archaea* seem capable of colonizing the endosphere of halophiles.
- The diversity of endophitic communities in halophytes decrease from the soil to the distant mature red shoots. The microbiota in the endosphere is strongly influenced by the microbial composition of the soils in where these plants grow, and the soils themselves by their environmental physicochemical parameters, reinforcing the idea of the soil as the principal source of endophytes
- The high abundance and the constant presence of *Chromohalobacter canadensis* and *Psychrobacter* sp. suggest the relevance of those taxa inhabiting the endosphere of halophytes. Other species not previously reported as endophytes including *Alkalibacillus salilacus, Rudaea celullosilytica* and *Salinisphaera dokdonensis* seemed to be also relevant in the endophytic community.
- Endophytic strains affiliating with *Paenibacillus borealis, Staphylococcus equorum, Salinicola halophilus* and *Marinococcus tarijensis* seem to be capable to increment the weight and root length in *Arabidopsis thaliana* both under saline and non-saline conditions. The growth promoting activity exhibited in these strains suggests their potential helping in agriculture as biofertilizers.
- Microbial communities inhabiting brines and sediments of salterns are strongly influenced by their origin location and environmental parameters principally total salinity, Mg^{2+}, Na^{+} and Cl^{-}
- The comparison of approaches shows an overestimation of richness and diversity when using the OTU method. The results obtained by both methods using the same dataset produced different conclusions, principally related with the identification of key species, being the most sensitive method OPU.
- No clustering rendered as satisfactory results as that of the OPU. The cutoff threshold that produced the most similar yields was at 97% sequence identity. However, both approaches cannot be taken as equivalent as clustering OTUs at 97% identity can easily produce false positives.

Section VII. References

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Annex

Comparison of OPU and OTU approaches using databases of saline and hypersaline environments

Introduction

Recent advances in high-throughput DNA sequencing provide an unprecedented opportunity to characterize the composition of microbial communities in environmental samples (Chan *et al.* 2011). However, their analysis require the determination of a biological unit. Currently, this unit is the Operational Taxonomic Unit (OTU) in most studies. The OTU is obtained clustering in a same group elements under a similarity cutoff. However, new evidence mentioned many problems in the use of OTU such as the richness overestimation (Nguyen *et al.* 2016) and the non consensus in the use of a OTU cutoff in the scientific community.

Here we performed a comparison of OPU methods with different OTU similarity percentage: 94.5%, 97%, 98.7% and 99%. The 99% was used because this cutoff allows to reduce briefly the dimension of the databases but avoiding the clustering of sequences into incorrect OTUs, we use 99% as a first step in the OPU approach. Both 94.5% as 98.7% were used based on the Stackebrandt and Ebers (2006) and Yarza *et al.* (2014) thresholds proposed for genera and species respectively. Finally 97% is the most common threshold used in analysis of NGS data (Turnbaugh *et al.* 2009; Pilloni *et al.* 2012; Gobet, Boetius and Ramette 2014; Birtel *et al.* 2015)

For the comparison, we used different methods including diversity estimators because as we mentioned previously studies reports several problems with a overestimation of richness and consecutively of diversity due to the OTU use. We tested our hypothesis also using multivariate analysis and co-occurrence networks.

Co-occurrence relationships are ecologically important because those patterns can reflect niche processes that drive coexistence and diversity maintenance within biological communities (Hillerislambers *et al.* 2012; Williams, Howe and Hofmockel 2014). The analysis of microbial co-occurrence patterns has been developed to aboard question in different environments (Horner-Devine *et al.* 2007; Freilich *et al.* 2010; Eiler, Heinrich and Bertilsson 2012; Faust *et al.* 2012) that can allow researchers to understand microbial coexistence at multiple levels of biological organization (Williams, Howe and Hofmockel 2014). The analysis of co-occurrence patterns among microbial communities has ranged from simple pairwise comparisons between all community members to direct hypothesis testing between focal species

with the potential to reveal ecological processes (Berry and Widder 2014; Williams, Howe and Hofmockel 2014). Applying co-occurrence analyses to microbial systems can provide valuable information for characterizing the biogeography, functional distribution or ecological interactions of microbes at the community scale or for identifying ecological traits of taxa that co-occur with well-characterized microorganisms (Williams, Howe and Hofmockel 2014).

With the objective to have a more real biological unit, this thesis has used the Operational Phylogenetic Unit (OPU). However, with the aim to prove if the conclusions obtained using OPU and OTU are different we performed a global analysis of our databases. We developed the comparison using diversity metrics, multivariate tests and co-occurrence networks to compare and contrast different OTU level and also the OPU approach to show that OPU is advantageous over previous methods and provides a better resolution of the communities.

Materials and methods

Databases analyzed, sequence trimming, OTU grouping and OPU databases.

The databases analyzed in this section were those obtained along the thesis and additionally one produced in the exploration of the microbiota associated to the nostrils of *Puffinus mauretanicus* (Urdiain et al., *in prep*; Table N 1.1). In the databases from the Chapter 6 (named Time; Table N 1.1), the pond SH was removed from the analysis, because the microbial community structures exhibited notable differences with other ponds. Sequence data were processed using Mothur (Schloss *et al.* 2009) software to trim and remove chimeras. The trimming process was the same as previously described in our chapters along this thesis. Chimeras were removed using Chimera Uchime (Mothur) and the sequences were clustered in OTUs at 94.5%, 97%, 98.7% and 99% using the UCLUST tool included in QIIME (Caporaso *et al.* 2010). The longest read of each OTU was selected as representative and using SILVA NGS the sequences belonging to the domain not analyzed were removed. The OPU databases were obtained from the previous chapters.

Main hypothesis evaluation and sensibility of the methods

In order to compare the results from the use of different cutoff levels and OPU on the reduced taxa datasets (one per "level of cutoff" and experiment) a permutational multivariate analysis of variance (PERMANOVA) was carried out to account for the potential effect of the main hypothesis evaluated in each of the experiments. This main hypothesis ranged from "Location of the samples", "Part of the plant or jellyfish", or "time of collection" (Table N 1.1, Table N

1.2). The main hyphotesis for Plant_Bac (C (Chapter).2), Soil_Bac (C.2), Sed_brines_bac (C.5), Sed_sed_bac (C. 5), Sed_brines_arch (C.5), Sed_sed_arch (C. 5) were the difference in the microbial community by location. For Plant_Arch (C.4) and Jellyfish_Bac (C. 7) the difference in the microbial community by structure in the host and Time_Arch and Time_Bac the hyphotesis was focussed to variations in the microbial community along the time. As each dataset had an initial variable main hypothesis, this analysis was performed to evaluate the sensitivity of a commonly used in ecology multivariate permutational method. PERMANOVA is robust to assumption violations as normality and has been widely used in ecology and microbiology to assess direct influence on the variability in the abundance matrices of different set of variables. PERMANOVA is based on the F-statistic, which is a multivariate equivalent of the Fisher's F-ratio, and the p values were calculated based on data permutations (Anderson 2001).

A Multilevel Indicator species analysis was used to identify the association between most important species (or indicator species) within and among each of the identified groups of samples defined by clustering methods (De Cáceres and Legendre 2009; Cáceres, Legendre and Moretti 2010). In this case, the clustering groups were *a priori* selected by the main hypothesis being tested, since the main objective was to see the sensibility of ISA to detect indicator species when different cutoff levels were selected. Therefore, a metric of performance of ISA was calculated as the % of the input taxa being selected as indicator species. All the statistical analyses were carried out using the packages vegan and indicspecies (Cáceres, Legendre and Moretti 2010; Oksanen 2011) on R software (www.r-project.org,2015)

Co-ocurrence analysis, construction of networks and evaluation of network properties

The co-existence network was determined using Spearman correlations between the taxa from different places. Before calculating correlation coefficients, all OPUs and OTUs with abundances lower than 0.01% were removed in order to avoid Type II errors. For each correlation, we obtained a correlation coefficient (rho) and a *p*-value. Thus, we define as co-existing taxa to those who presented a correlation $> 0.7 < -0.7$, and a $p < 0.05$, (Barberán *et al.* 2011). Networks were created based on the co-occurrences obtained. For visualization of the networks all nodes with eigenvector centrality = 0 were eliminated.

The evaluation among the different networks was performed based on the topological properties suggested by Berry and Widder (2014) and Faust *et al.* (2012) potentially relevant for community roles and functioning: (i) Mean degree and (ii) Degree distribution: metrics of the number of connections of nodes; (iii) Average shortest path length: the average number of steps along the shortest paths for all possible pairs of network nodes; (iv) Mean clustering coefficient: measure of the degree to which nodes in a graph tend to cluster together; (v) Betweenness

centrality: measure of centrality in a graph based on shortest paths; (vi) Closeness centrality: measure of centrality in a network, calculated as the sum of the length of the shortest paths between the node and all other nodes in the graph; (vii) Modularity: measure of the structure of networks or graphs. This metric is calculated as the strength of division of a network into modules (also called groups, clusters or communities); (viii) Diameter lenght of the networ and (ix) Connex component: subgraph in which any two vertices are connected to each other by paths, and which is connected to no additional vertices in the supergraph. Afterwards a multivariate linear regression via redundancy analysis (RDA) was performed on this output (log transformed to avoid high difference of variances among variables) considering the topological properties of each network as a response dependent of "Level of cutoff" after removing via conditioning the variance explained by the different experiments (in this way the inherent variability explained by having a different amount of starting sequences was removed). RDA analysis was carried out based on vegan package from R software as well.

Diversity indices

The diversity index Jost q=0 (richness) and q=1 (diversity) indices calculation, a re-sampling was conducted by Monte-Carlo method with 1,000 simulations (Mora-Ruiz *et al.* 2016). A Generalized Linear Model (GLM) was applied on the distribution of q=0 (richness) and q=1 (diversity) of all data. In the model, we included the "level of cutoff", the "Domain" as fixed effect and the different "experiment" as random effect.

Results

Alpha diversity

The global analysis here performed included a total of 1,757,124 sequences distributed in eight databases (Table N 1.1, Table N 1.3). Both for richness and diversity, lower values were found in OPU. The ANOVA applied on the GLM exhibited significant differences among OPU values and OTUs in richness (*Level*: $F_{4,970}$: 226.145; p<0.001; *Domain* $F_{1,970}$: 0.567; p= 0.466) and diversity (*Level*: $F_{4,970}$: 144.087; p<0.001; *Domain* $F_{1,970}$: 0.096; p= 0.7616; Figure N 1.1).

Beta diversity

The results of the beta diversity in the databases of different OTU cutoffs and OPUs exhibited that although most of the general conclusions were similar independently of the unit circumscription approach or OTU cutoff, the proportion of the variance explained was different (Table N 1.1 Databases in the global analysis including the name of the databases (Study) and the Chapter number (C) in this thesis; the númer of taxa in the complete (Total) and reduced (Red) databases; the different unit circumscription category of analysis (Level); the hypothesis

tested (Hyphothesis previously mentioned); the PERMANOVA results: degree of freedom (Df) and values of F model, R2 and significance value (Pr(>F)); the number (and percentage= Total%) of indicator species (INDVAL) results with the number of groups formed, the group (G) correspond to those mentioned in the hypothesis (e.g. for Plant_Bac, G1= Alcúdia and G2= Campos).*For OTU the number is a percentage of similarity.). For example, the Sed_Sed_Bac (Chapter C. 5) was significantly different by location, however the value of variation explained by the location was higher when OPUs were used. (14.2%). Taking in account the R^2 and the *p-values*, no OTU cutoff seemed to have a similar behavior to that of the OPUs. While in some databases such as Plant_Arch (C. 4) is 98.7% in other as Sed_Sed_Bac (C. 5) is 97%. Additionally, the analysis of indicator species (INDVAL) was different also depending the cutoff. In this case for five databases, OPU seemed to provide a higher proportion of indicator species. In general the OTU cutoff at 97% was the one producing the most similar results to that of the OPUs.

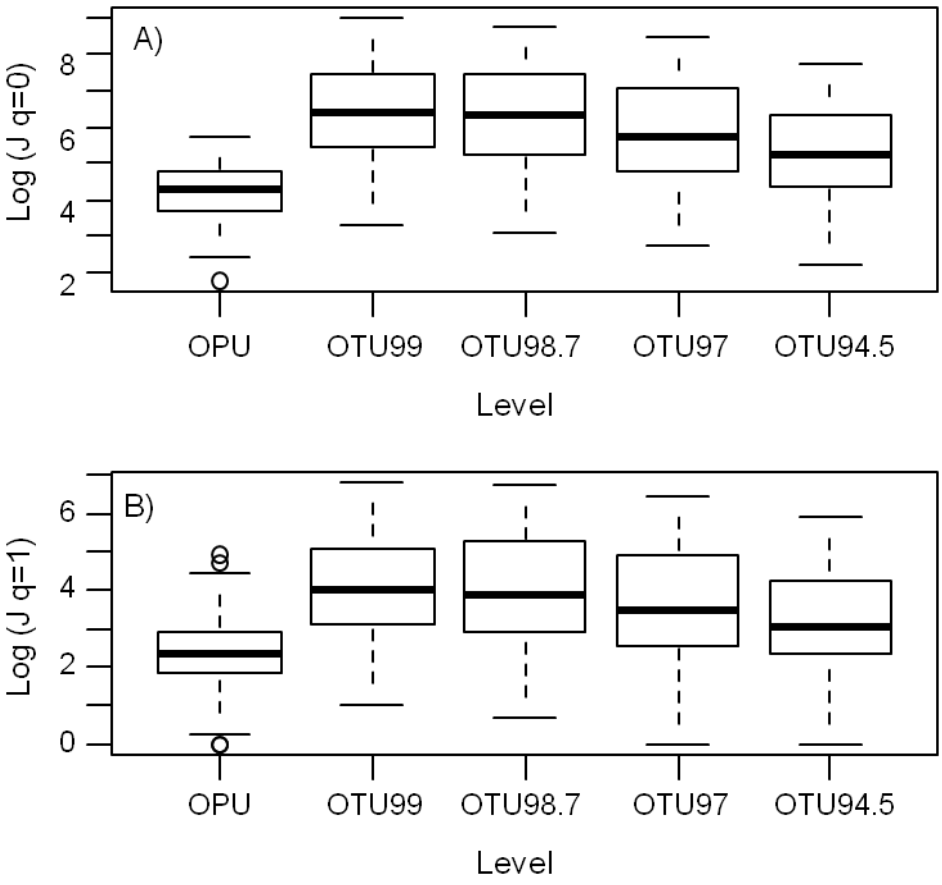

Figure N 1.1 Boxplots of Alpha diversity indices Jost q=0 (A, richness) and Jost q=1 (B, diversity) including all the databases considered in this analysis. Boxes represent the lower and upper quartiles (25 and 75%) of the values, the horizontal line the median (50%) and the vertical lines the minimum and maximum.

Table N 1.1 Databases in the global analysis including the name of the databases (Study) and the Chapter number (C) in this thesis; the número of taxa in the complete (Total) and reduced (Red) databases; the different unit circumscription category of analysis (Level); the hypothesis tested (Hyphothesis previously mentioned); the PERMANOVA results: degree of freedom (Df) and values of F model, R2 and significance value (Pr(>F)); the number (and percentage= Total%) of indicator species (INDVAL) results with the number of groups formed, the group (G) correspond to those mentioned in the hypothesis (e.g. for Plant_Bac, G1= Alcúdia and G2= Campos).*For OTU the number is a percentage of similarity.

General information						PERMANOVA RESULTS				INDVAL						
Study	Reads	Number of taxa		Level*	Hypothesis	Df	F.Model	R^2	Pr(>F)	Nº Indicator species	Total %	% per groups				
		Total	Red									G 1	G 2	G 3	G 4	G 5
Plant_Bac (C. 2)	50,013	249	185	OPU	Alcúdia Campos	1	2.133	0.107	0.029	34	18.37	5	29			
		1876	1334	99%		1	4.772	0.206	0.001	72	5.40	12	60			
		1363	995	98.7		1	4.942	0.210	0.001	69	6.94	10	59			
		852	646	97		1	5.212	0.218	0.001	69	10.68	13	56			
		595	503	94.5		1	5.586	0.226	0.001	53	10.54	14	39			
r	6 3	56	46	OPU	Green	2	2.786	0.527	0.092	4	8.70	4				

		1054	252	99	Red Root	2	1.544	0.382	0.157	2	0.79			2		
		650	211	98.7		2	2.694	0.519	0.099	3	1.42	3				
		195	87	97		2	2.851	0.533	0.066	3	3.45	3				
		92	54	94.5		2	3.251	0.565	0.070	1	1.85	1				
Sed_Brines_Bac (C. 5)	204,497	480	480	OPU	Argentina Chile Spain	2	1.012	0.142	0.477	41	8.54		8	33		
		38442	383	99		2	1.165	0.152	0.048	59	16.16		22	37		
		37176	365	98.7		2	1.210	0.155	0.022	65	16.97		23	42		
		35811	408	97		2	1.077	0.139	0.224	76	18.63		18	58		
		8978	1406	94.5		2	1.175	0.153	0.200	236	16.79	1	40	195		
Sed_Sed_Bac (C. 5)	304,654	596	597	OPU	Argentina Chile Spain	2	2.784	0.140	0.001	132	22.11	78	41	13		
		78892	2132	99		2	1.678	0.094	0.001	94	4.41	18	74	2		
		59454	1212	98.7		2	1.800	0.101	0.001	33	2.72	4	29			
		51560	4000	97		2	2.188	0.117	0.001	332	8.30	96	178	58		
		22877	4215	94.5		2	1.687	0.100	0.003	296	7.02	281	2	13		
Sed_Brines_Arch (C. 5)	285,069	315	141	OPU	Argentina Chile Spain	2	2.620	0.258	0.009	19	13.48	6	5	8		
		22291	1009	99		2	1.150	0.150	0.099	211	20.91		131	80		
		18055	1493	98.7		2	1.269	0.161	0.040	326	21.84		188	138		
		60484	1550	97		2	1.385	0.178	0.033	316	20.39		185	131		
		2545	948	94.5		2	8.367	0.253	0.032	130	13.71	1	72	57		
Sed_Sed_Arch (C. 5)	478,460	140	316	OPU	Argentina Chile Spain	2	1.408	0.079	0.019	23	7.28	14	8	1		
		44752	2258	99		2	1.507	0.087	0.001	53	2.35	21	23	9		
		38987	2829	98.7		2	1.608	0.093	0.001	98	3.46	71	5	22		
		19221	4138	97		2	1.705	0.098	0.001	337	8.14	275	6	56		
		7560	2398	94.5		2	1.698	0.098	0.010	313	13.05	237		76		
Time_Bac (C. 6)	196,608	416	349	OPU	T00 T15 T22 T34 T78	4	10.987	0.553	0.001	52	14.90	17	2	8	16	9
		5884	1630	99		4	6.869	0.523	0.001	447	27.42	43	111	122	5	166
		4765	1339	98.7		4	7.051	0.527	0.001	359	26.81	38	86	106	1	128
		2831	867	97		4	8.114	0.540	0.001	225	25.95	26	47	58	2	92
		1923	1425	94.5		4	8.685	0.543	0.001	173	12.14	18	36	44	2	73
Time_Arch	145,216	173	119	OPU	T00 T15 T22 T34 T78	4	5.759	0.289	0.001	12	10.08	3		2	2	5
		8275	1645	99		4	7.582	0.396	0.001	383	23.28	6	118	18	73	168
		6534	1359	98.7		4	8.206	0.396	0.001	336	24.72	13	77	20	80	146
		2554	1587	97		4	9.141	0.395	0.001	163	10.27	9	48	15	12	79
		807	571	94.5		4	10.962	0.406	0.001	80	14.01	2	17	11	1	49
Jellyfish_Bac (C. 7)	50,780	143	57	OPU	Filaments Total	1	0.771	0.134	0.526	8	14.04		8			
		740	280	99		1	0.438	0.081	0.640	12	4.29	1	11			
		444	153	98.7		1	0.733	0.128	0.501	15	9.80	2	13			
		250	83	97		1	0.720	0.126	0.507	8	9.64	8				
		209	173	94.5		1	0.730	0.127	0.487	6	3.47	6				
a c	2 4	449	323	OPU	Alcúdia	1	4.573	0.533	0.100	0	0.00					

		4238	1677	99	Campos	1	2.482	0.383	0.100	0	0.00					
		3664	1351	98.7		1	2.552	0.390	0.100	0	0.00					
		2719	1008	97		1	2.510	0.386	0.100	0	0.00					
		2059	851	94.5		1	2.514	0.386	0.100	0	0.00					

Comparison of the network properties for the OTU and OPU approaches

The analysis of the general properties of the networks obtained was performed using a total of 113 networks (databases x treatments). In order to assess non-random co-occurrence pattern, we restricted the analysis to those with a *p*-value <0.05. The highest number of nodes was detected in sediments samples (Sed_Sed C. 5) and the lowest number in Birds (*Archaea*; Table N 1.2). The connection (mean degree) of those nodes was observed higher in the databases of Time (T00, C. 6) being always lower in OPU than OTU 99% and 98.7%. Although the closeness was most elevated in OPU, it was more similar to 94.7 AND 97% is all the databases (Table N 1.2).

Previously to the ordination analysis we observed a increment of the Degree of distribution when the Average shortest path length increases. Basedo on this high correlation (r^2= 0.86 Table3), the degree distribution was removed to facilitate the ordination analysis. The results of RDA showed a noticeable segregation of the OPUs co-occurrence datasets (Figure N 1.2 Ordination analysis for the principal properties of the networks for the databases in this work., model adjusted r^2= 14%). The RDA axis 1 was significant (Explained variance: 0.86534; *p*<0.001***) in the separation of the data distribution with Betweenness, Mean degree and Conex component as principal explanatory variables (Level $F_{4,96}$: 6.7851; *p*< 0.001 ***). While OTU 99% seemed to have a higher dissimilarity with OPU; OTU 94.5% and 97% had a likely behavior with our approach. Due to the high correlation of the Conex components with the sampling size (nodes; r^2= 0.79) we decided to remove this variable to control the effect of the sampling size in the ordination analysis. However, the results the general pattern being OPU more different to OTU was still maintained. (data no shown)

The analysis of the different modules as well as the individual analysis of the key species in the community is until in prep with the aim of detecting if the role of the species in each of the different levels studied is similar.

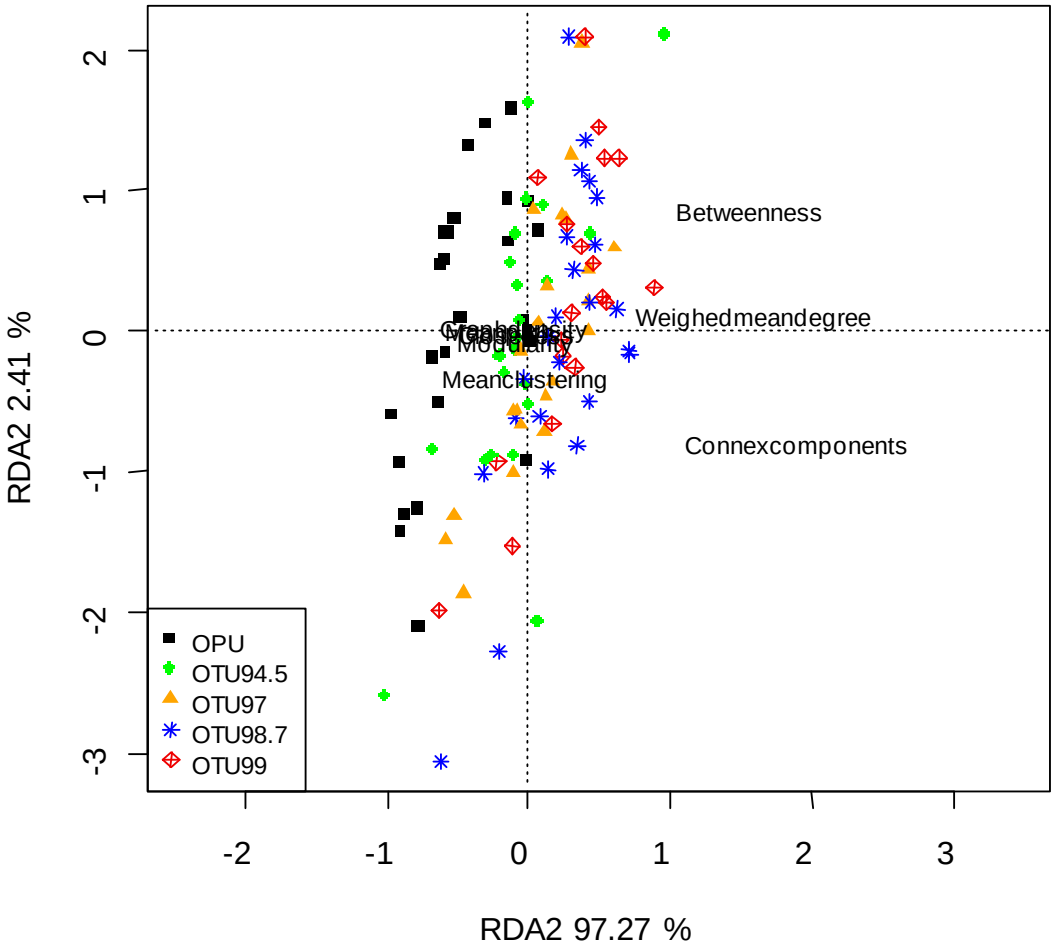

Figure N 1.2 Ordination analysis for the principal properties of the networks for the databases in this work.

Table N 1.2 Co-ocurrence network values in the databases used for the comparative analysis of OTU and OPU approaches including the databases (Study) and the number of Chapter (C) in this thesis; the different levels of analysis (Level); the hypothesis tested (Hypothesis previously mentioned).

Study	Domain	Level	Hyphothesis	Nodes	Edges	Mean degree	Graph density	Diameter	Mean path	Modularity	Connex components	Mean clustering	Closeness ($x\ 10^5$)	Betweenness
Plant (C. 2)	*Bacteria*	OPU	Campos	256	407	2.6	0.01	6.97	3.29	0.52	129	0.12	2.26	71.7
		99		1689	84889	96.64	0.06	4.99	2.44	0.53	219	0.16	0.24	924.81
		98.7		1364	44162	61.94	0.05	5	2.56	0.56	227	0.14	0.28	738.99
		97		1011	18663	35.15	0.04	4.72	2.67	0.55	236	0.09	0.34	498.27
		94.5		819	9780	22.39	0.03	5.65	2.59	0.56	270	0.27	0.35	292.66
		OPU	Alcudia	256	332	2.26	0.01	6.64	3.43	0.52	156	0.18	1.9	47.03
		99		1689	6054	6.78	0	7.85	3.36	0.62	1161	0.04	0.04	195.21
		98.7		1364	3891	5.35	0	10.2	3.65	0.62	933	0.06	0.06	179.98
		97		1011	1525	2.77	0	9.57	4.32	0.72	702	0.03	0.11	155.2
		94.5		819	714	1.59	0	9.55	4.91	0.72	619	0.23	0.16	83.52
Soil (C.2)		OPU	Campo	256	154	1.2	0	8	3.13	0.44	209	0.01	1.6	9.41
		99		1689	59283	70.2	0.04	5	2.23	0.18	957	0.29	0.05	194.67
		98.7		1364	34033	49.9	0.04	6	2.33	0.19	791	0.28	0.07	160.08
		97		1011	17334	34.29	0.03	6	2.28	0.19	605	0.23	0.12	104.25
		94.5		819	8924	21.79	0.03	5	2.13	0.18	541	0.37	0.18	53.34
		OPU	Alcudia	256	72	0.56	0	8	3.46	0.53	224	0.25	1.56	5.08
		99		1689	26129	30.94	0.02	6	2.29	0.18	1190	0.14	0.04	95.59
		98.7		1364	18023	26.43	0.02	6	2.29	0.19	947	0.13	0.06	82.33
		97		1011	8858	17.52	0.02	6	2.33	0.2	716	0.22	0.11	57.57
		94.5		819	5908	14.43	0.02	6	2.34	0.17	600	0.43	0.16	39.42
t (C	*Archaea*	OPU	Green	55	58	2.11	0.04	8	3.6	0.58	23	0.12	54.23	22.13

		99		1052	21098	40.11	0.04	7	2.68	0.67	504	0.92	0.14	239.32
		98.7		648	6580	20.31	0.03	6	2.18	0.76	360	0.99	0.26	26.05
		97		193	697	7.22	0.04	8	3.4	0.68	64	0.45	6.23	104.19
		94.5		90	149	3.31	0.04	5	1.99	0.81	43	0.87	13.6	3.39
Sed_Brines (C. 5)	*Bacteria*	OPU	Spain	478	3	0.01	0	3	1.67	0.17	475	0	0.44	0.01
		99		364	416	2.29	0.01	1	1	0.27	324	1	0.76	0
		98.7		382	739	3.87	0.01	4	1.79	0.42	304	0.63	0.71	3.68
		97		407	1611	7.92	0.02	3	1.02	0.64	310	0.99	0.62	0.08
		94.5		1405	12807	18.23	0.01	3	1.12	0.63	1117	0.96	0.05	1.21
		OPU	Argentina	478	455	1.55	0	8.03	4.69	0.68	313	0.16	0.51	94.62
		99		364	6356	31.09	0.1	8.16	3.49	0.42	73	0.84	2.35	251.61
		98.7		382	6617	30.74	0.09	4.48	2.34	0.34	56	0.56	4.06	186.97
		97		407	5131	21.47	0.06	7.89	3.47	0.45	88	0.78	1.9	279.86
		94.5		1405	37874	45.09	0.04	5.58	3.06	0.67	308	0.64	0.19	885.09
	Archaea	OPU	Spain	140	292	4.17	0.03	6	2.54	0.1	86	0.06	6.38	16.29
		99		1008	8992	17.84	0.02	5	2.04	0.26	757	0.76	0.11	32.63
		98.7		1492	18097	24.26	0.02	3	1.02	0.22	1220	0.99	0.05	0.26
		97		1549	33187	42.85	0.03	5	2.07	0.27	1119	0.91	0.05	64.26
		94.5		947	19264	40.68	0.04	5	1.93	0.37	587	0.79	0.14	63.49
		OPU	Argentina	140	427	4.91	0.04	6.2	3.04	0.49	17	0.12	33.59	109.27
		99		1008	14202	24.61	0.03	5.06	2.7	0.61	281	0.49	0.28	447.5
		98.7		1492	31871	37.27	0.03	8.73	3.72	0.68	515	0.75	0.1	870.06
		97		1549	40404	44.02	0.03	5.22	2.67	0.65	378	0.57	0.14	739.36
		94.5		947	17344	30.27	0.04	5.15	2.76	0.58	145	0.49		597.34
Sed_Sed (C. 5)	*Bacteria*	OPU	Spain	596	1673	4.55	0.01	8.93	4.21	0.67	146	0.07	0.63	545.19
		99		1211	16379	24.61	0.02	8.55	3.13	0.69	206	0.54	0.33	874.83
		98.7		1690	36426	38.42	0.03	7.83	3.48	0.75	254	0.72	0.19	1484.63
		OPU	Argentina	596	4876	13.21	0.03	4.58	2.99	0.54	31	0.11	4.97	533.02
		99		1211	2650	4.12	0	8.02	3.6	0.75	913	0.64	0.07	76.09
		98.7		1690	2531	2.73	0	7.03	3.62	0.69	1463	0.86	0.04	32.96
	Archaea	OPU	Argentina	277	1386	8.18	0.04	5.45	3.01	0.51	38	0.16	8.19	208.37
		OPU	Spain	277	1295	7.31	0.03	5.21	3.05	0.4	30	0.15	10.19	224.7
Time (C. 6)	*Bacteria*	OPU	T00	227	274	2.32	0.01	9.52	3.09	0.79	146	0.81	2.03	8.63
		99		1629	38948	47.71	0.03	6.87	2.89	0.24	746	0.24	0.06	453.25
		98.7		1338	24383	36.36	0.03	7.97	2.93	0.25	612	0.17	0.09	380.57
		97		866	11138	25.67	0.03	9.49	3.02	0.21	507	0.82	0.17	142.78
		94.5		661	7293	22.02	0.03	8	2.73	0.18	385	0.78	0.28	85.18
		OPU	T15	227	393	3.38	0.02	9	2.74	0.78	130	0.82	2.05	9.26
		99		1629	6573	7.88	0	11	3.95	0.77	859	0.08	0.05	535.57
		98.7		1338	4122	6.01	0	12.79	4.24	0.77	729	0.03	0.08	447.2
		97		866	1948	4.39	0.01	13.41	4.74	0.8	592	0.74	0.15	143.48
		94.5		661	1268	3.74	0.01	16.96	6.8	0.81	430	0.71	0.27	234.99
		OPU	T22	227	460	3.91	0.02	12.58	4.73	0.86	86	0.73	2.25	47.98
		99		1629	10454	12.35	0.01	6.6	3.47	0.72	588	0.03	0.08	823.92
		98.7		1338	7304	10.51	0.01	6.79	3.62	0.72	463	0	0.13	749.64
		97		866	3145	7.01	0.01	10.58	4.47	0.83	456	0.7	0.19	322.85
		94.5		661	1632	4.77	0.01	14.2	5.66	0.85	363	0.69	0.3	281.83
		OPU	T34	227	982	8.37	0.04	13.98	4.91	0.75	37	0.77	8.87	302.49
		94.5		661	13686	41.28	0.06	6	2.54	0.69	120	0.65	1.07	341.2
		97		866	23066	53.09	0.06	5.84	2.45	0.7	170	0.73	0.56	401.8
		98.7		1338	49239	73.41	0.06	6	2.5	0.53	157	0.14	0.42	780.6
		99		1629	74834	91.69	0.06	6	2.5	0.53	171	0.14	0.32	980.71
		OPU	T78	227	503	4.43	0.02	8	2.58	0.62	146	0.83	2.1	11.06
		99		1629	11496	14.11	0.01	8	3.28	0.45	985	0.09	0.05	289.29
		98.7		1338	7334	10.96	0.01	10	3.49	0.54	798	0.1	0.07	263.3
		97		866	3045	7.03	0.01	13	4.68	0.64	611	0.73	0.15	114.09
		94.5		661	1708	5.17	0.01	10	3.46	0.61	461	0.56	0.25	60.26
	Archaea	OPU	T00	118	927	15.61	0.13	4.71	2.34	0.24	24	0.42	27.67	49.54
		98.7		1358	81931	120.57	0.09	7.71	1.94	0.13	659	0.81	0.08	162.79
		97		957	76692	160.22	0.17	7	1.9	0.12	255	0.65	0.33	231.41
		94.5		296	10731	72.45	0.25	7.81	1.99	0.13	46	0.77	5.89	102.18
		OPU	T22	118	246	4.13	0.04	7.76	3.22	0.48	42	0.3	14.27	50.76
		98.7		1358	12463	18.28	0.01	8.6	3.16	0.27	921	0.75	0.06	145.55
		97		957	12613	26.29	0.03	8.58	2.83	0.3	457	0.47	0.17	239.04
		94.5		296	2643	17.81	0.06	8	2.9	0.22	117	0.72	2.04	94.62
		OPU	T34	118	343	5.51	0.05	6.6	3.05	0.45	33	0.38	19.45	62.03
		98.7		1358	38859	55.31	0.04	6.71	2.26	0.6	585	0.65	0.09	271.44
		97		957	14874	29.95	0.03	5.92	2.54	0.56	358	0.44	0.22	289.89
		94.5		296	3102	19.91	0.07	5.81	2.53	0.47	80	0.54	3.37	120.97
		OPU	T15	118	397	6.5	0.06	8.74	3.11	0.43	34	0.26	18.23	60.97
		98.7		1358	85243	123.48	0.09	7.55	1.97	0.1	683	0.81	0.08	162.68
		97		957	17150	34.83	0.04	6.84	2.49	0.25	483	0.5	0.16	169.04
		94.5		296	1760	11.46	0.04	7.61	2.63	0.37	154	0.58	1.59	51.26
		OPU	T78	118	131	2.22	0.02	7	2.35	0.35	72	0.34	8.03	6.54
		98.7		1358	3007	4.43	0	15	4.51	0.76	1079	0.73	0.06	85.88
		97		957	2826	5.91	0.01	9	3.98	0.63	649	0.35	0.12	116.96
		94.5		296	130	0.88	0	6	1.71	0.61	256	0.78	1.15	0.53
Jellyfish (C. 8)	*Bacteria*	OPU	Filaments	56	181	6.46	0.12	4	2.09	0.24	18	0.29	77.4	14.36
		99		279	1994	14.29	0.05	5	2.05	0.46	153	0.72	1.77	30.16
		98.7		152	371	4.88	0.03	5	1.78	0.69	82	0.8	4.66	3.52
		97		82	82	2	0.02	3	1.07	0.64	57	0.99	15.48	0.07
		94.5		172	242	2.81	0.02	6	2.08	0.68	120	0.82	3.54	3.24
		OPU	Total	56	217	7.75	0.14	6	2.6	0.46	10	0.57	127.31	29.52
		99		279	2347	16.82	0.06	7	2.72	0.67	86	0.74	2.92	105.06
		98.7		152	136	1.79	0.01	8	2.98	0.54	115	0.48	4.70	9.16
		97		82	34	0.83	0.01	1	1	0.52	70	1	15.22	0
		94.5		172	1710	19.88	0.12	5	2.13	0.32	72	0.84	6.13	33.06

Birds	*Archaea*	OPU	Birds	24	33	2.75	0.12	5	2.46	0.42	9	0.5	352.06	7.29
		99		162	2256	27.85	0.17	6	1.66	0.53	35	0.97	5.43	13.59
		97		114	694	12.18	0.11	6	1.37	0.54	37	0.99	9.1	2.85
		98.7		169	1975	23.37	0.14	8	2.32	0.45	37	0.9	10.5	60.69
		94.5		56	96	3.43	0.06	7	3	0.45	18	0.17	71.13	25.13

Table N 1.3 Pearson correlation of the networks properties evaluated in this thesis.

	Nodes	Edges	Mean degree	Graph density	Diameter	Mean path	Modularity	Connex components	Mean clustering	Closenness
Edges	0.62									
Mean degree	0.47	0.93								
Graph density	-0.29	0.22	0.47							
Diameter	0.09	-0.15	-0.16	-0.20						
Mean path	0.11	-0.22	-0.29	-0.34	**0.86**					
Modularity	-0.02	-0.28	-0.38	-0.37	0.42	0.52				
Connex components	**0.79**	0.23	0.10	-0.42	0.15	0.10	-0.11			
Mean clustering	-0.20	-0.03	0.07	0.24	-0.05	-0.29	0.10	-0.07		
Closenness	-0.30	-0.15	-0.14	0.33	-0.12	-0.07	-0.07	-0.24	-0.04	
Betweenness	0.57	0.51	0.39	-0.06	0.07	0.23	0.27	-0.01	-0.26	-0.15

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Glossary

ACC deaminase: 1-Aminocyclopropane-1-Carboxylate deaminase
ANI: Average Nucleotide Identity
Ap: Ampicillin
BMU: Best-Maching Unit
BetA: Choline dehydrogenase
BetB: Glycine betaine aldehyde dehydrogenase
BF: *Bacteria*l fraction
BM: Culture media Burk's
BMS: Culture media Burk's salino
BrEt: Ethidium bromide
bsf: below sea floor
BT: Total biomass
CARD-FISH: Catalyzed reporter deposition Fluorescence In Situ Hybridization
CCA: Canonical correspondence analysis
CD: Crohn diagnosed patients
CFU: Colony Forming Units
CO32-: Carbonates
CsCl: Caesium chloride
CTAB: Cetyl Trimethylammonium Bromide
CVA: Canonical variate analysis.
D: Dominance
DAPI : 4',6-diamidino-2-phenylindole
DGGE: Denaturing gradient gel electrophoresis
DNA: Deoxyribonucleic Acid
DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen
EDTA: Ethylenediaminetetraacetic Acid
EMBL: European Molecular Biology Laboratory
ENA: European Nucleotide Archive
EtOH: Ethyl Alcohol
FISH: Fluorescence in situ hybridization
ICR-FT/MS: Fourier Transform Ion Cyclotron Resonance Mass Spectrometry
h: hours
H´: Shannon-Wiener index
H+-Ppase: Proton pumping membrane-bound pyrophosphatase
H_2O_2: Hydrogen peroxide
HCl: Hydrochloric Acid
HRP: Horseradish Peroxidase
IAA: Indoleacetic Acid
IBD: Inflammatory Bowel Diseases
ICP-MS: Inductively Coupled Plasma Spectroscopy-Mass Spectrometry
IPTG: Isopropyl β-D-1-thiogalactopyranoside
IR: Ionizing Radiation
ISA: Indicator species analysis
K^+: Potassium
KCl: Potassium chloride
KH_2PO_4: Monopotassium Phosphate
L: Litter
LB: Luria-Bertani
LTP: Living Tree Project
M523: Culture media 523
MALDI-TOF/MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
masl: meters above sea level
matK: Maturase K gen
MCM: Modified Competence Medium
MDS: Multidimensional Scaling

mg: miligrams
Mg2+: magnesium
min: minutes
mL: mililiters
mM: milimolar
MS: Murashihe-Skoog Culture media
MSP : Main Spectrum Profile
N: Nitrogen
Na+: Sodium
Na2HPO4: Sodium phosphate dibasic
NaCl: Sodium chloride
NaClO: Sodium hypochlorite
NaHSO3: sodium hydrogen sulfite
NGS: New Generation Sequencing
NMDS: Non Metric Multidimensional Scaling
NP: Culture media LGT
nth: genes gncoding the endonuclease
O.M.: Oxidizable Organic Matter
ºC: Centigrade grades
OD: Optical Density
OPU: Operational Phylogenetic Unit
ORFs: Open Reading Frames
OTU: Operational Taxonomic Unit
P: Phosphorus
pb: pare base
PBS: Phosphate Saline Buffer
PBS : Phosphate Buffer Solution
PCA: Principal Component Analysis
PCoA: Principal Coordinate Analysis
PCR: Polymerase Chain Reaction
PERMANOVA: Permutational Multivariate Analysis of Variance
PGPA: Plant Growth Promoting Activity
PGPB : Plant Growth Promoting *Bacteria*
PPi: Inorganic Pyrophosphate
pSKII+: pBluescript SKII (+) plasmid
PVP: Polyvinylpyrrolidone
R2A: Culture media Reasoner's 2A
RAPD: Random amplified polymorphic DNA
RAxML: Randomized Axelerated Maximum Likelihood)
RDA: Redundancy analysis
rhlE: RNA helicase
RMR: Culture media Rennie
rRNA: Ribosomal Ribonucleic Acid
RT: Room temperature
SDS: Sodium dodecyl sulphate
SOM: Self-Organizing Maps
SEM: Scanning electron microscopy
SINA: SILVA Incremental Aligner
Sp: Spectinomycin
SSCP: Single-Strans Conformation Polymorphism
STCC: Spanish Type Culture Collection
SW: Culture media Sea Water
TAE: Tris-acetate-EDTA
TGGE: Temperature Gradient Gel Electrophoresis
TH: Culture media of Thioglicolate
T-RFLP: Terminal Restriction Frafment Length Polymorphism
UV: Ultra Violet
v: volume
VPL: Virus-like Particles
w: weight
WC MALDI-TOF MS: Whole-Cell Matrix Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry
xg: times gravity
YE: Yeast extract

Appendices

1. Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily *Salicornioideae (Amaranthaceae*)

Table A 1.1 Primer pair sequences used for molecular identification.

Cultures and first amplification for pyrosequencing

	Sequence (5' → 3')
GM3 (B)*	AGAGTTTGATCMTGGC
S (B)	GGTACCTTGTTACGACTT
21F (A)**	TTCCGGTTGATCCTGCCGGA
1492R (A)	TACGGYTACCTTGTTACG

Second amplification for pyrosequencing

	Sequence (5' → 3')			
	Adaptor	Key	MID	Primer
GM3-PS	CCTATCCCCTGTGTGCCT TGGCAGTC	TCAG	-	AGAGTTTGATCMT GGC
907-PS	CCATCTCATCCCTGCGTG TCTCCGAC	TCAG	-	CCGTCAATTCMTT TGAGTTT
Sample				
PI			TAGTATCAGC	
LV			ACGAGTGCGTTGAT	
AL			ACGAGTGCGTGACT	
CA			ACGAGTGCGTCGTA	
SF			TCTCTATGCG	

**Bacteria* and ***Archaea* primers

Table A 1.2 Identification of plant samples using *matK* gene. Sequences were aligned with representative *matK* genes of different plant species. The values shown are based on the partial gene sequence of about 306 nucleotides. The values indicate the identity percentages for the plants studied with the closest representative species in the database. Data for Chilean samples are in yellow and Mediterranean in green.

	PI	**LV**	**AL**	**CA**	**SF**	***Allenrolfea vaginata***	***Arthrocnemum macrostachyum***	***Salicornia ramossisima***
PI	100							
LV	97.7	100						
AL	100	97.7	100					
CA	98.4	97.6	98.4	100				
SF	99.9	97.6	100	100	100			
Allenrolfea vaginata	98	100	97.9	96.7	98	100		
Arthrocnemum macrostachyum	99.7	97.3	99.9	100	100	97.6	100	
Salicornia ramossisima	97.7	96.5	97.6	97.4	97.4	95.2	97.4	100

Table A 1.3 Colony forming units of culturable epiphytes ($1x10^3$) and endophytes ($1x10^6$) per gram per location and salt concentration in the culture media. PI=Pichidangui, LV=Lo Valdivia, AL=Alicante, CA=Campos, SF=Ses Fontanelles, P=epiphytes and N= endophytes. X^2 and *p*-values for Kruskal-Wallis analyzes by location. PI: 13.9 and $<2x10^{-4}$; LV: 2.5 and 0.11; AL: 18.2 and <1 $x10^{-4}$; CA: 17.3 and <3 $x10^{-5}$; SF: 44.36 and <3 $x10^{-11}$.

Epiphytes

% salt	**SF**		**CA**		**AL**		**LV**		**PI**	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
5	7.6	1.7	7.0	0.9	16.4	1.8	78.4	5.2	2738.5	111.3
15	2.5	0.2	0.2	0.06	12.3	1.4	56.7	3.7	288.6	6.7
20	1.6	0.2	0.3	0.09	11.8	1.5	12.5	1.4	1351.5	6.2
25	0.04	0	0	0	3.7	0.3	5.4	0.8	574.8	6.8
30	0	0	0	0	0	0	0	0	0	0

Endophytes

	SF		**CA**		**AL**		**LV**		**PI**	
5	25.1	3.2	27.2	2.8	2.5	0.3	0.01	0.006	8.9	0.6
15	2.2	0.2	3.9	0.6	2.8	0.3	0.02	0.002	6.3	0.09
20	2.6	0.9	0.023	0.01	0.3	0.08	0.009	0.004	3.6	0.5
25	2.4	0.1	0.005	.002	0.2	.005	0.008	0.005	1.9	0.3
30	0.01	1.6	0	0	0	0	0	0	0	0

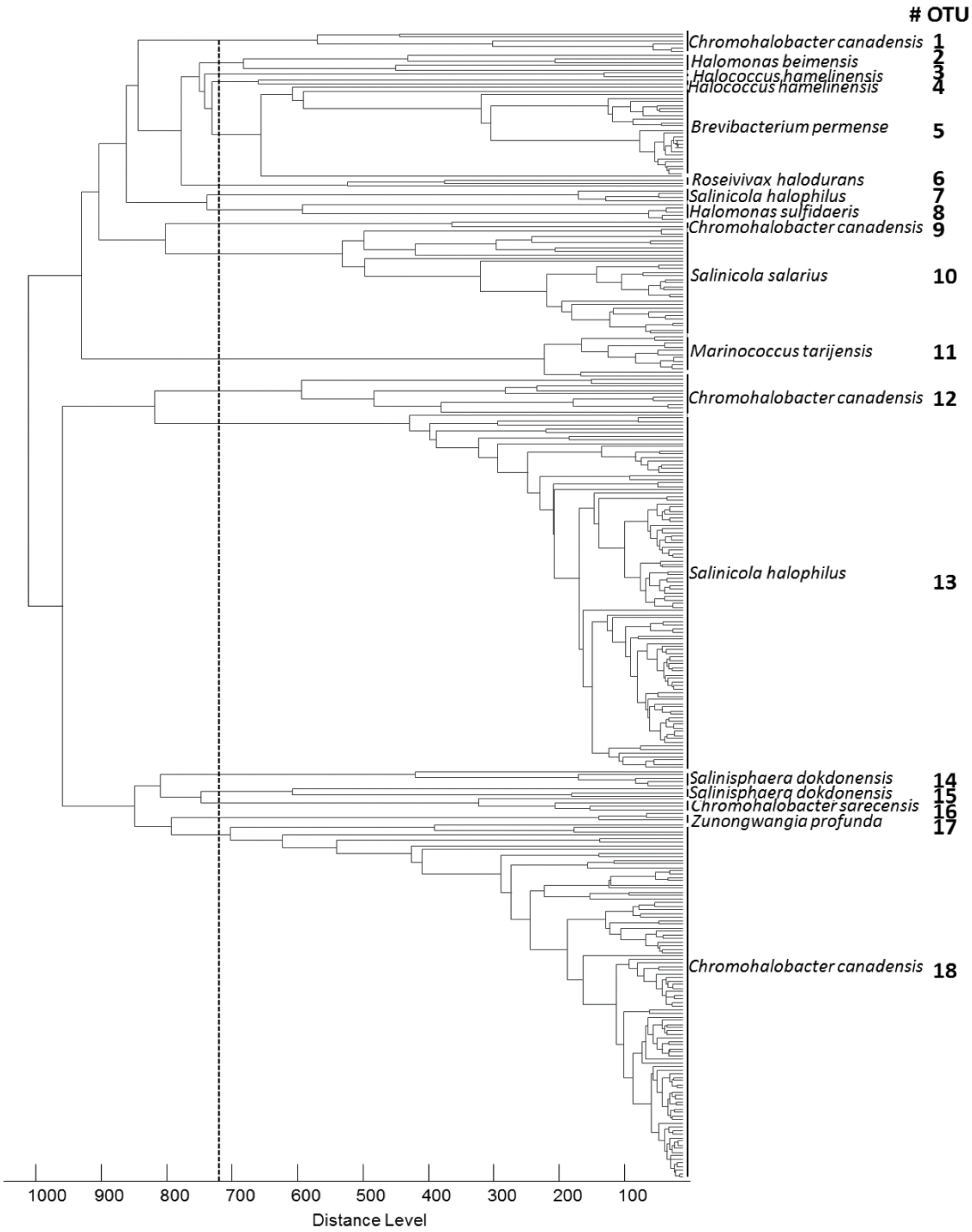


Figure A 1.1 Dendrogram of MALDI-TOF MS profiles from 318 epiphytic isolates generated by MALDI Biotyper *v* 3.21 software. The distance measure was set at correlation and average. 18 OTUs were distinguished with a cut-off distance level of 720.

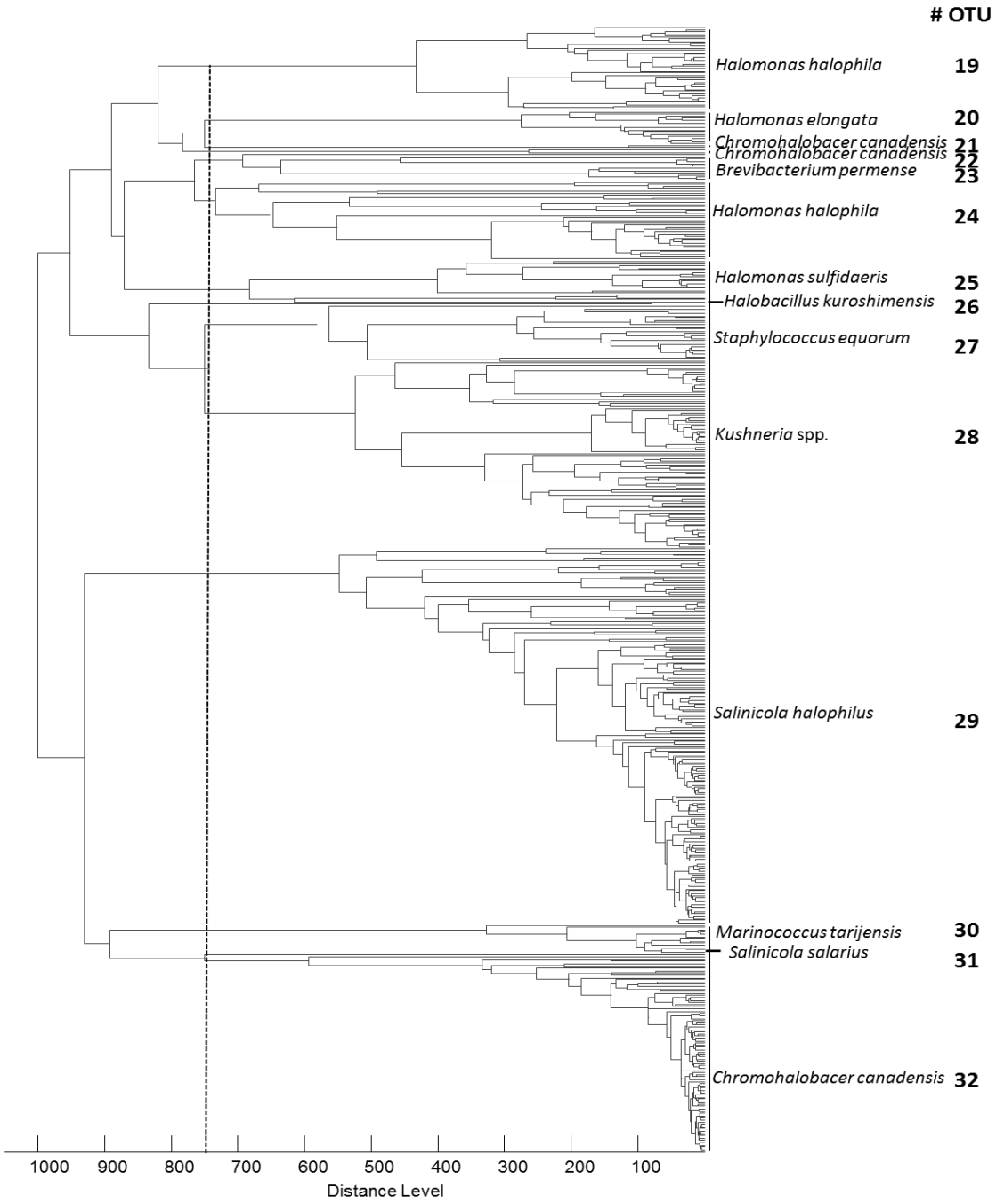


Figure A 1.2 Dendrogram of MALDI-TOF MS profiles from 495 endophytic isolated generated by MALDI Biotyper v 3.21 software. The distance measure was set at correlation and average. 14 OTUs were distinguished with a cut-off distance level of 750.

Table A 1.4 Relative abundances of minor OPUs referenced to total sequences for each sample and their taxonomic correspondence with the closest relative sequence. First column (from left to right): OPUs number; second: OPUs affiliation in phylum, class and family (first line), and genus, species (second line). The information about access number, type species and the similitude percentage is also specified in the same line. The rest of the columns contain the percentage of the relative abundances of each OPU referenced to the total sequences for each sample (PI=Pichidangui, LV=Lo Valdivia, AL=Alicante, CA=Campos and SF=Ses Fontanelles).

		PI	LV	AL	CA	SF
OPU 39	*Proteobacteria. Gammaproteobacteria. Halomonadaceae* *Halomonas boliviensis* (AY245449). >90%	0.00	0.51	0.40	0.73	0.00
OPU 40	*Proteobacteria. Gammaproteobacteria. Pseudomonadaceae* *Pseudomonas geniculata* (AB021404). >97%	0.13	0.06	0.00	0.36	0.40
OPU 41	*Proteobacteria. Gammaproteobacteria. Moraxellaceae* *Acinetobacter puyangensis* (JN664255). >96%	0.00	0.00	0.00	0.27	0.16
OPU 42	*Proteobacteria. Gammaproteobacteria. Moraxellaceae* *Psychrobacter pacificensis* (AB016057). >94%	0.00	0.03	0.00	0.00	0.08
OPU 43	*Proteobacteria. Gammaproteobacteria. Orbaceae* *Frischella perrara* (JX878306). >84%	0.00	0.00	0.06	0.00	0.08
OPU 44	*Proteobacteria. Gammaproteobacteria. Alteromonadaceae* *Marinobacter litoralis* (AF479689). >90%	0.00	0.00	0.00	0.00	0.08
OPU 45	*Proteobacteria. Gammaproteobacteria. Alteromonadaceae* *Aestuariibacter litoralis* (AB473549). >85%	0.00	0.03	0.11	0.46	0.16
OPU 46	*Proteobacteria. Gammaproteobacteria. Alteromonadaceae* *Marinobacter coralli* (GU183820). >95%	0.00	0.00	0.00	0.00	0.08
OPU 47	*Proteobacteria. Gammaproteobacteria. Xanthomonadaceae* *Luteimonas aestuarii* (EF660758). >97%	0.00	0.00	0.00	0.18	0.00
OPU 48	*Proteobacteria. Gammaproteobacteria. Xanthomonadaceae* *Thermomonas koreensis* (HQ315827). >99%	0.00	0.00	0.11	0.00	0.00
OPU 49	*Proteobacteria. Gammaproteobacteria. Xanthomonadaceae* *Stenotrophomonas maltophilia* (KC986351). >98%	0.00	0.00	0.00	0.18	0.00
OPU 50	*Proteobacteria. Gammaproteobacteria. Piscirickettsiaceae* *Methylophaga marina* (X95459). >84%	0.00	0.00	0.00	0.00	0.08
OPU 51	*Proteobacteria. Gammaproteobacteria. Enterobacteriaceae* *Cronobacter sakazakii* (EF088379). >99%	0.00	0.00	0.06	0.00	0.00
OPU 52	*Proteobacteria. Gammaproteobacteria. Enterobacteriaceae* *Enterobacter cloacae* (CP006580). >83%	0.13	0.10	0.11	0.46	0.00
OPU 53	*Proteobacteria. Gammaproteobacteria. Enterobacteriaceae* *Lelliottia nimipressuralis* (Z96077). >99%	0.00	0.03	0.00	0.00	0.00
OPU 54	*Proteobacteria. Gammaproteobacteria. Pasteurellaceae* *Haemophilus parainfluenzae* (AY362908). >96%	0.13	0.00	0.00	0.00	0.00
OPU 55	*Proteobacteria. Gammaproteobacteria. Pasteurellaceae* *Pasteurella langaaensis* (AY362922). >95%	0.00	0.00	0.17	0.00	0.16
OPU 56	*Proteobacteria. Gammaproteobacteria. Nevskiaceae* *Nevskia aquatilis* (JQ710440). >90%	0.04	0.00	0.17	0.00	0.00
OPU 57	*Proteobacteria. Betaproteobacteria. Oxalobacteraceae* *Massilia alkalitolerans* (AY679161). >99%	0.00	0.00	0.00	0.27	0.08
OPU 58	*Proteobacteria. Betaproteobacteria. Burkholderiaceae* *Cupriavidus pauculus* (AF085226). >99%	0.00	0.03	0.23	0.00	0.00
OPU 59	*Proteobacteria. Betaproteobacteria. Burkholderiaceae* *Cupriavidus pinatubonensis* (AB121221). >94%	0.00	0.00	0.00	0.36	0.24
OPU 60	*Proteobacteria. Alphaproteobacteria. Methylobacteriaceae* *Mehtylobacterium soli* (EU860984). >87%	0.00	0.00	0.00	0.00	0.40

OPU 61	*Proteobacteria. Alphaproteobacteria. Methylobacteriaceae Methylobacterium trifolii* (FR847848). >96%	0.00	0.00	0.63	0.09	0.00
OPU 62	*Proteobacteria. Betaproteobacteria. Hyphomicrobiaceae Aquabacterium parvum* (AF035052). >98%	0.13	0.00	0.00	0.27	0.00
OPU 63	*Proteobacteria. Alphaproteobacteria. Hyphomicrobiaceae Devosia submarina* (AB712348). >98%	0.00	0.00	0.06	0.00	0.00
OPU 64	*Proteobacteria. Alphaproteobacteria. Hyphomicrobiaceae Aquabacterium limnoticum* (GU319965). >96%	0.00	0.00	0.00	0.09	0.00
OPU 65	*Proteobacteria. Alphaproteobacteria. Acetobacteraceae Roseomonas aerilata* (EF661571). >97%	0.00	0.00	0.00	0.18	0.00
OPU 66	*Proteobacteria. Alphaproteobacteria. Sphingomonadaceae Sphingobium aromaticiconvertens* (AM181012). >97%	0.04	0.00	0.00	0.00	0.00
OPU 67	*Proteobacteria. Alphaproteobacteria. Anaplasmataceae Heliimonas saccharivorans* (JX458466). >98%	0.00	0.00	0.06	0.00	0.00
OPU 68	*Proteobacteria. Alphaproteobacteria. Rhodobacteraceae Paracoccus aestuarii* (EF660757). >99%	0.04	0.03	0.00	0.09	0.16
OPU 69	*Proteobacteria. Alphaproteobacteria. Rhodobacteraceae Ruegeria intermedia* (FR832879). >97%	0.00	0.00	0.00	0.09	0.00
OPU 70	*Proteobacteria. Alphaproteobacteria. * Reyranella soli* (JX260424). >96%	0.09	0.00	0.00	0.00	0.00
OPU 71	*Proteobacteria. Alphaproteobacteria. Rhodospirillaceae Rhodovibrio salinarium* (D14432). >99%	0.00	0.00	0.06	0.00	0.00
OPU 72	*Proteobacteria. Alphaproteobacteria. Caulobacteraceae Caulobacter profundus* (KF360052). >95%	0.09	0.03	0.00	0.18	0.08
OPU 73	*Proteobacteria. Alphaproteobacteria. Caulobacteraceae Brevundimonas mediterranea* (AJ227801). >99%	0.00	0.00	0.00	0.09	0.00
OPU 74	*Proteobacteria. Deltaproteobacteria. Geobacteraceae Geobacter metallireducens* (L07834). >94%	0.00	0.00	0.40	0.00	0.00
OPU 75	*Proteobacteria. Deltaproteobacteria. Desulfuromonadaceae Desulfuromusa kysingii* (X79414). >93%	0.00	0.00	0.17	0.00	0.00
OPU 76	*Proteobacteria. Deltaproteobacteria. Desulfuromonadaceae Pelobacter seleniigenes* (DQ991946). >99%	0.00	0.03	0.00	0.00	0.00
OPU 77	*Proteobacteria. Deltaproteobacteria. Polyangiaceae Byssovorax cruenta* (AJ833647). >96%	0.04	0.00	0.00	0.00	0.16
OPU 78	*Proteobacteria. Deltaproteobacteria. Bacteriovoracaceae Peredibacter starrii* (AF084852). >79%	0.00	0.00	0.06	0.00	0.00
OPU 79	*Bacteroidetes. Flavobacteriia. Flavobacteriaceae Gelidibacter mesophilus* (AJ344133). >92%	0.00	0.00	0.00	0.00	0.40
OPU 80	*Bacteroidetes. Flavobacteriia. Flavobacteriaceae Snuella lapsa* (Hhm475133). >96%	0.00	0.03	0.00	0.00	0.00
OPU 81	*Bacteroidetes. Flavobacteriia. Flavobacteriaceae Tenacibaculum crassostreae* (EU428783). >96%	0.09	0.06	0.06	0.18	0.08
OPU 82	*Bacteroidetes. Sphingobacteriia. Chitinophagaceae Niastella populi* (EU877262). >93%	0.00	0.00	0.00	0.09	0.00
OPU 83	*Bacteroidetes. Sphingobacteriia. Sphingobacteriaceae Mucilaginibacter herbaticus* (JN695632). >83%	0.00	0.00	0.00	0.46	0.00
OPU 84	*Acidobacteria. Acidobacteria. Acidobacteriaceae Vibrionimonas magnilacihabitans* (FJ816610). >90%	0.00	0.00	0.06	0.00	0.00
OPU 85	*Acidobacteria. Acidobacteria. Acidobacteriaceae Alsobacter metallidurans* (AB231946). >94%	0.04	0.00	0.00	0.00	0.00
OPU 86	*Acidobacteria. Acidobacteria. * Bryobacter aggregattus* (AM162405). >82%	0.00	0.03	0.11	0.64	0.08

OPU 87	*Chlamydiae. Chlamydiae. Chlamydiaceae* *Chlamydophila psittaci* (U68447). >99%	0.00	0.03	0.11	0.18	0.08
OPU 88	*Deinococcus-Thermus. Deinococci. Deinococcaceae* *Deinococcus piscis* (DQ683348). >84%	0.00	0.00	0.00	0.00	0.32
OPU 89	*Firmicutes. Clostridia. Clostridiaceae* *Clostridium saccharoperbutylacetonicum* (U16122). >99%	0.00	0.03	0.00	0.27	0.00
OPU 90	*Firmicutes. Clostridia. Peptostreptococcaceae* *Peptostreptococcus canis* (HE687281). >94%	0.00	0.00	0.00	0.18	0.00
OPU 91	*Firmicutes. Clostridia. Family XI** *Anaerococcus octavius* (Y07841). >99%	0.04	0.00	0.00	0.00	0.00
OPU 92	*Firmicutes. Bacilli. Planococcaceae* *Sporosarcina saromensis* (AB682135). >99%	0.00	0.00	0.00	0.00	0.08
OPU 93	*Firmicutes. Bacilli. Planococcaceae* *Sporosarcina luteola* (AB473560). >99%	0.00	0.00	0.00	0.00	0.08
OPU 94	*Firmicutes. Bacilli. Planococcaceae* *Sporosarcina newyorkensis* (GU994085). >97%	0.00	0.00	0.00	0.00	0.08
OPU 95	*Firmicutes. Bacilli. Planococcaceae* *Sporosarcina koreensis* (DQ073393). >98%	0.04	0.06	0.06	0.09	0.40
OPU 96	*Firmicutes. Bacilli. Planococcaceae* *Paenisporosarcina indica* (FN397659). >94%	0.00	0.00	0.00	0.00	0.16
OPU 97	*Firmicutes. Bacilli. Planococcaceae* *Planomicrobium glaciei* (EU036220). >98%	0.00	0.00	0.00	0.00	0.08
OPU 98	*Firmicutes. Bacilli. Bacillaceae* *Thalassobacillus devorans* (AJ717299). >80%	0.00	0.00	0.11	0.00	0.16
OPU 99	*Firmicutes. Bacilli. Streptococcaceae* *Streptococcus troglodytidis* (JF414111). >94%	0.04	0.06	0.23	0.36	0.63
OPU 100	*Firmicutes. Bacilli. Streptococcaceae* *Streptococcus rubneri* (JX861483). >94%	0.00	0.00	0.00	0.09	0.00
OPU 101	*Firmicutes. Bacilli. Lactobacillaceae* *Lactobacillus acetotolerans* (M58801). >91%	0.00	0.00	0.00	0.18	0.00
OPU 102	*Firmicutes. Bacilli. Lactobacillaceae* *Lactobacillus delbrueckii subsp. sunkii* (AB641833). >98%	0.00	0.00	0.00	0.00	0.40
OPU 103	*Armatimonadetes. Fimbriimonadia. Fimbriimonadaceae* *Fimbriimonas ginsengisoli* (GQ339893). >90%	0.04	0.10	0.23	0.64	0.40
OPU 104	*Armatimonadetes. Fimbriimonadia. Fimbriimonadaceae* *Fimbriimonas ginsengisoli* (GQ339893). >79%	0.04	0.00	0.00	0.00	0.00
OPU 105	*Actinobacteria. Actinobacteria. Corynebacteriaceae* *Corynebacterium diphtheriae* (Xx84248). >97%	0.00	0.00	0.00	0.09	0.00
OPU 106	*Actinobacteria. Actinobacteria. Mycobacteriaceae* *Mycobacterium llatzerense* (AJ746070). >99%	0.00	0.00	0.17	0.09	0.00
OPU 107	*Actinobacteria. Actinobacteria. Mycobacteriaceae* *Mycobacterium moriokaense* (AJ429044). >98%	0.04	0.00	0.00	0.00	0.00
OPU 108	*Actinobacteria. Actinobacteria. Micrococcaceae* *Arthrobacter soli* (EF660748). >97%	0.00	0.00	0.00	0.00	0.16
OPU 109	*Actinobacteria. Actinobacteria. Micromonosporaceae* *Micromonospora kangleipakensis* (JN560152). >95%	0.00	0.00	0.00	0.09	0.00
OPU 110	*Actinobacteria. Actinobacteria. Microbacteriaceae* *Microbacterium lacticum* (X77441). >98%	0.00	0.00	0.11	0.18	0.00

* No more information about taxonomic hierarchy has been found.

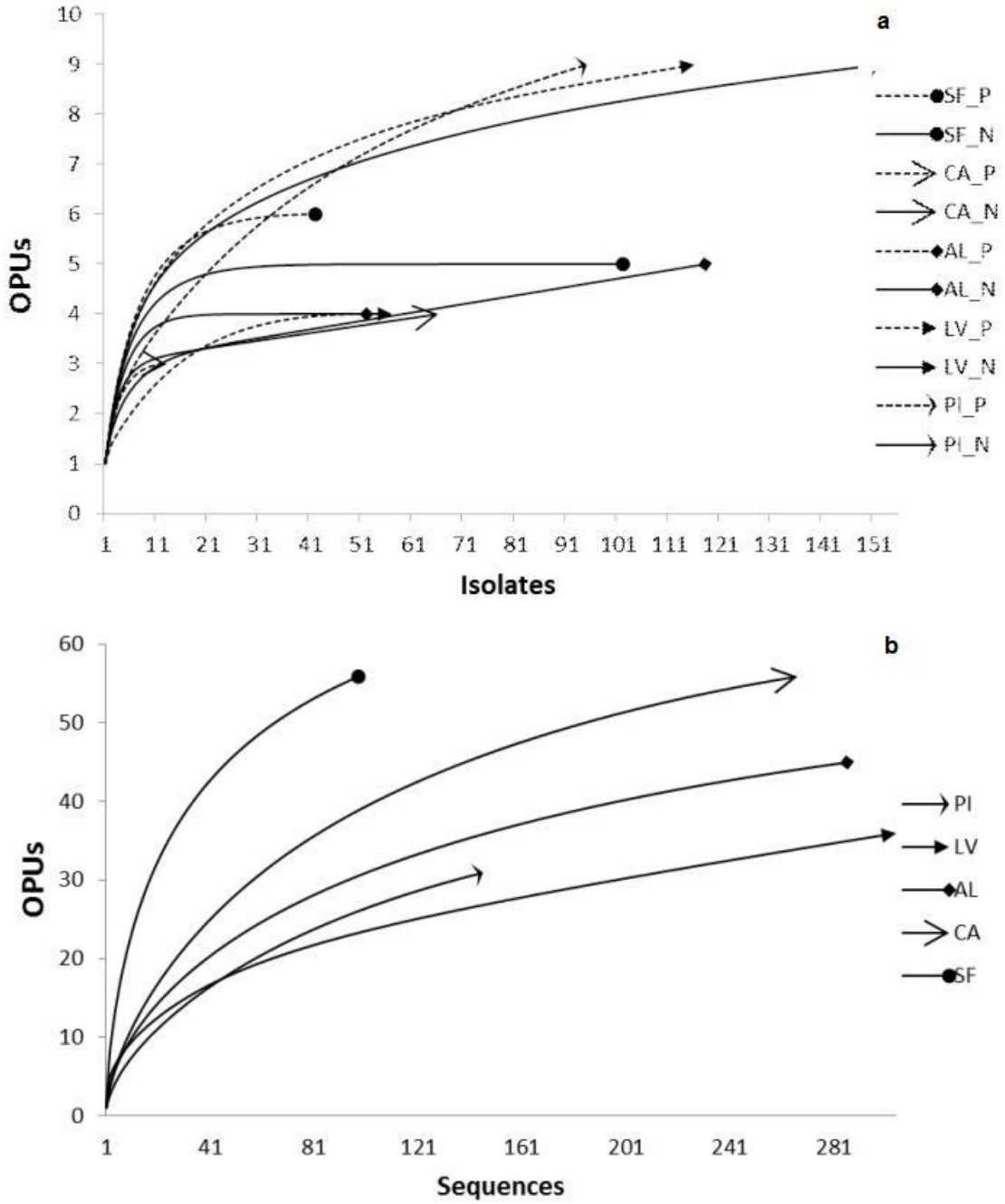


Figure A 1.3 Rarefaction curves of culturable (a) and pyrosequencing (b) OPU-based diversity.

Table A 1.5 Percentage of clonality for endophytic and epiphytic isolates analyzed by RAPD. In brackets it is indicated the number of OTU according Supporting Information Fig. S1 and Fig. S2.

Species (# OTU)	SF		CA		AL		LV		PI	
	N	P	N	P	N	P	N	P	N	P
B. permense (5, 23)	-	-	-	-	-	-	100	100	-	0
C. canadensis (18, 32)	100	100	-	0	100	100	100	50	100	100
C. canadensis (22)	-	-	-	-	-	-	0	-	-	-
C. canadensis (21)	-	-	-	-	-	-	-	-	100	-
C. canadensis (12)	-	0	-	0	-	0	-	-	-	-
C. canadensis (1)	-	-	-	-	-	-	-	0	-	0
C. canadensis (9)	-	0	-	-	-	-	-	-	-	0
C. sarecensis (16)	-	-	-	-	-	-	-	0	-	-
H. kuroshimensis (26)	-	-	-	-	-	-	-	-	0	-
H.beimenensis (2)	-	-	-	-	-	-	-	-	-	100
H. elongata (20)	-	-	-	-	-	-	-	-	0	-
H. halophila (19)	100	-	-	-	-	-	-	-	0	-
H. elongata (24)	100	-	100	-	-	-	-	-	100	-
H. hamelinensis (3)	-	100	-	-	-	-	-	-	-	-
H. hamelinensis (4)	-	-	-	-	-	-	-	0	-	0
H. sulfidaeris (8, 25)	-	-	100	0	0	-	-	0	0	-
Kushneria spp.(28)	0	-	0	-	0	-	0	-	0	-
M. tarijensis (11, 30)	0	0	-	-	0	0	-	0	0	0
S. halophilus (13, 29)	75	57	-	-	0	61	0	0	88	50
S. halophilus (7)	-	-	-	-	-	-	-	-	-	100
S. salarius (10)	-	100	-	0	-	0	-	100	-	0
S. salarius (31)	-	-	-	-	-	-	-	-	0	-
S. dokdonensis (14)	-	0	-	-	-	-	-	-	-	-
S. dokdonensis (15)	-	-	-	-	-	-	-	-	-	0
S. equorum (27)	-	-	0	-	-	-	-	-	-	-
R. halodurans (6)	-	-	-	-	-	-	-	100	-	-
Z. profunda (17)	-	-	-	-	-	-	-	-	-	100

0 = no similar pattern in RAPD found.

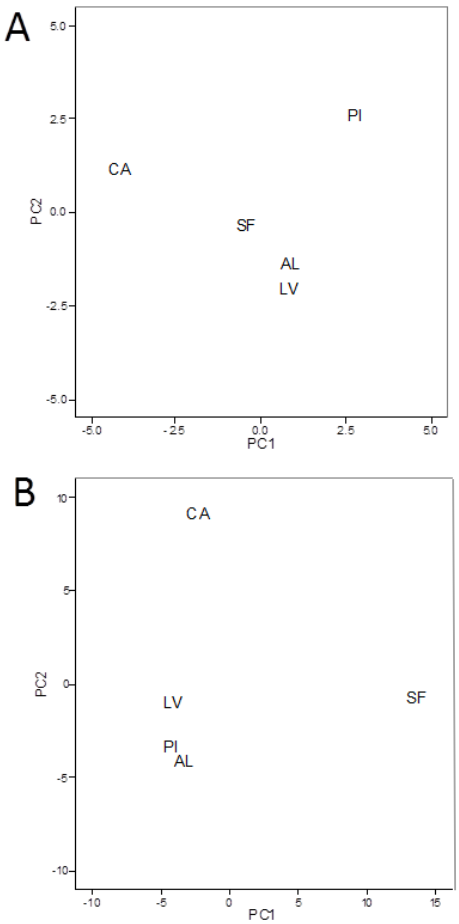


Figure A 1.4 PCA by location (PI=Pichidangui, LV=Lo Valdivia, AL=Alicante, CA=Campos and SF=Ses Fontanelles) utilizing OPUs detected by culture (A) and pyrosequencing (B).

Table A 1.6 Classification of halophilic microorganisms isolated from halophytes phyllosphere.

OPU	Affiliation	Halophilic	# isolates
1	*Chromohalobacter canadensis*	Moderate	204
2	*Chromohalobacter sarecensis*	Moderate	4
3	*Salinicola halophilus*	Moderate	269
4	*Salinicola salarius*	Moderate	31
5	*Halomonas halophila*	Moderate	72
6	*Halomonas elongata*	Extreme	15
7	*Halomonas beimensis*	Moderate	5
8	*Halomonas sulfidaeris*	Moderate	24
9	*Kushneria spp.*	Moderate	82
10	*Salinisphaera dokdonensis*	Moderate	8
11	*Roseivivax halodurans*	Moderate	3
12	*Marinococcus tarijensis*	Moderate	25
13	*Halobacillus kuroshimensis*	Moderate	1
14	*Staphylococcus equorum*	Halotolerant	25
15	*Brevibacterium permense*	Moderate	38
16	*Zunongwangia profunda*	Moderate	3
17	*Halococcus hamelinensis*	Moderate	4

2. Endophytic microbial diversity of the halophyte *Arthrocnemum macrostachyum* across plant compartments

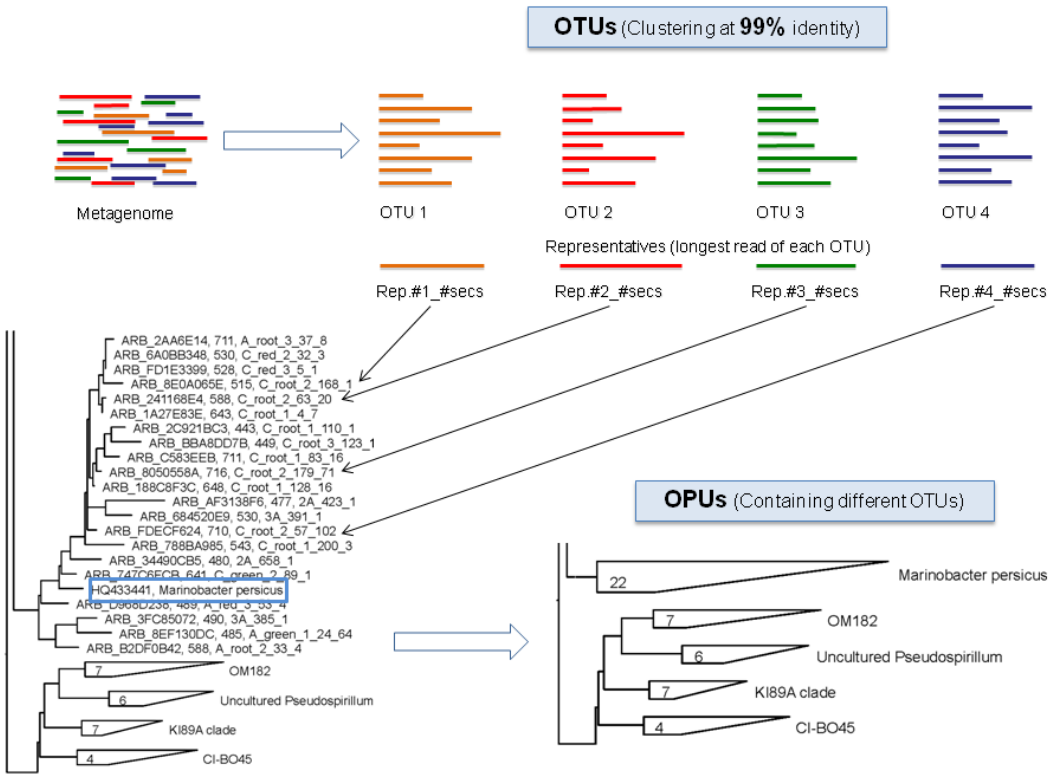


Figure A 2.1 Rationale to circumscribe the Operational Phylogenetic Units (OPUs) for representative sequences of OTUs. An OPU is the smallest monophyletic group of sequences containing OTU representatives together with the closest reference sequence, including the sequence of a type strain when possible. Briefly, the pyrosequencing data, after being trimmed, is clustered into OTUs at 99% identity. The longest representative of each OTU is selected for a parsimony insertion using a pre-reconstructed and optimized tree containing the representative type strain sequences and additional relevant sequences. After insertion, the tree is manually inspected and the OPUs circumscribed according to their phylogenetic uniqueness.

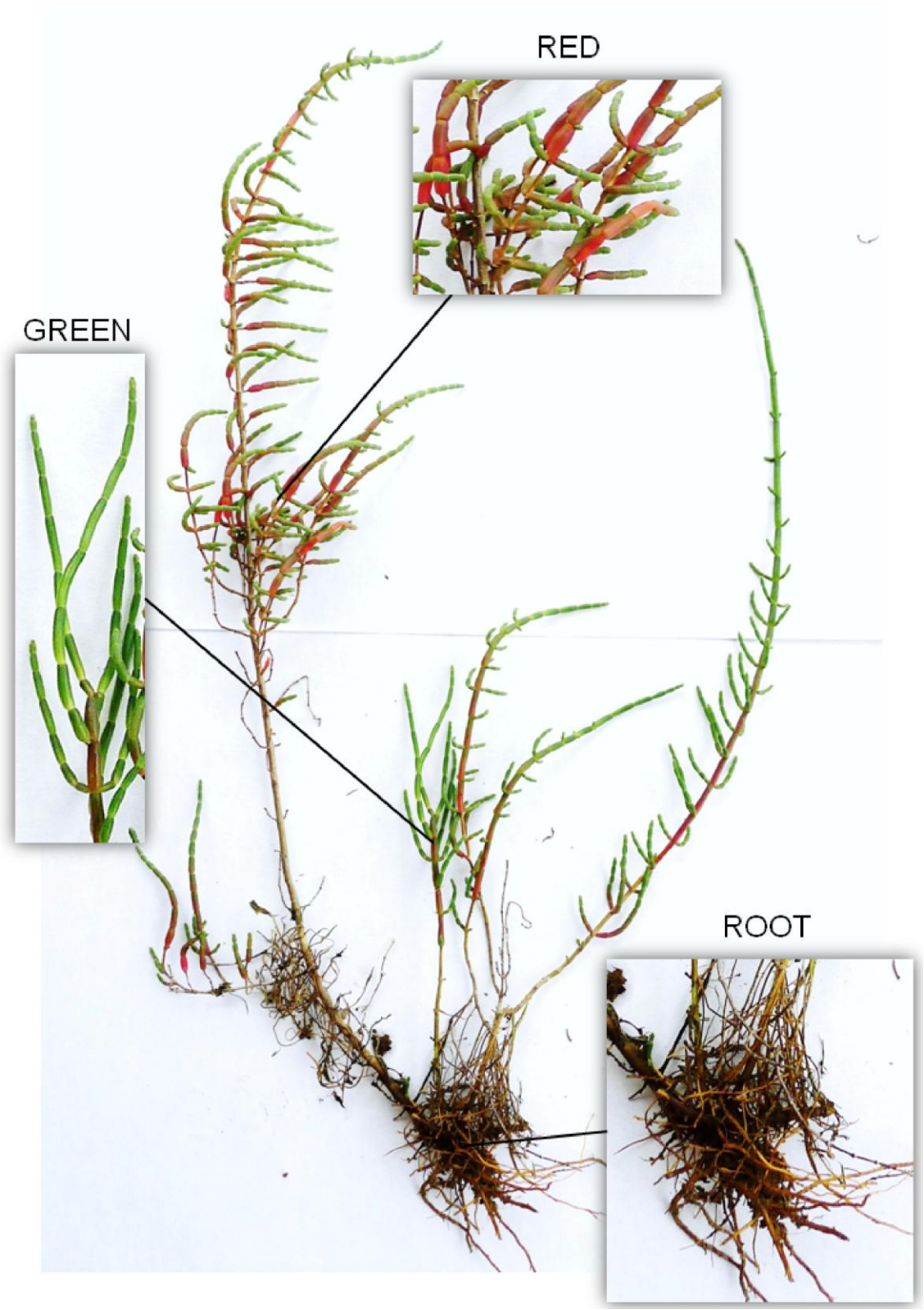


Figure A 2.2 Images of green (G), red (RE) and root (RO) compartments of *Arthrocnemum macrostachyum*.

Table A 2.1 Primer pair sequences used for molecular analyses.

First amplification for pyrosequencing		
		Sequence (5' → 3')
Bacteria primers	GM3	AGAGTTTGATCMTGGC
	S	GGTACCTTGTTACGACTT

Second amplification for pyrosequencing					
		Sequence (5' → 3')			
		Adaptor	Key	MID**	Primer
GM3-PS		CCTATCCCCT GTGTGCCTTG GCAGTC	TCA G	-	AGAGTTTGATCMTGGC
907-PS		CCATCTCATC CCTGCGTGTC TCCGAC	TCA G	-	CCGTCAATTCMTTTGAGTTT
Loc*	Sam*				
ALCÚDIA	Green			ACGAGTGCGT	
	Green			AGACGCACTC	
	Green			ATATCGCGAG	
	Red			TAGTATCAGC	
	Red			AGCACTGTAG	
	Red			CGTGTCTCTA	
	Root			ACGCTCGACA	
	Root			ATCAGACACG	
	Root			CTCGCGTGTC	
CAMPOS	Green			ATCAGACACG	
	Green			TACTGAGCTA	
	Green			CATAGTAGTG	
	Red			TGATACGTCT	
	Red			TACTGAGCTA	
	Red			CATAGTAGTG	
	Root			CTCGCGTGTC	
	Root			ACGAGTGCGTACGT	
	Root			ACGAGTGCGTTACG	

*Loc: Location, Sam: Sample

**MID: Multiplex identifier

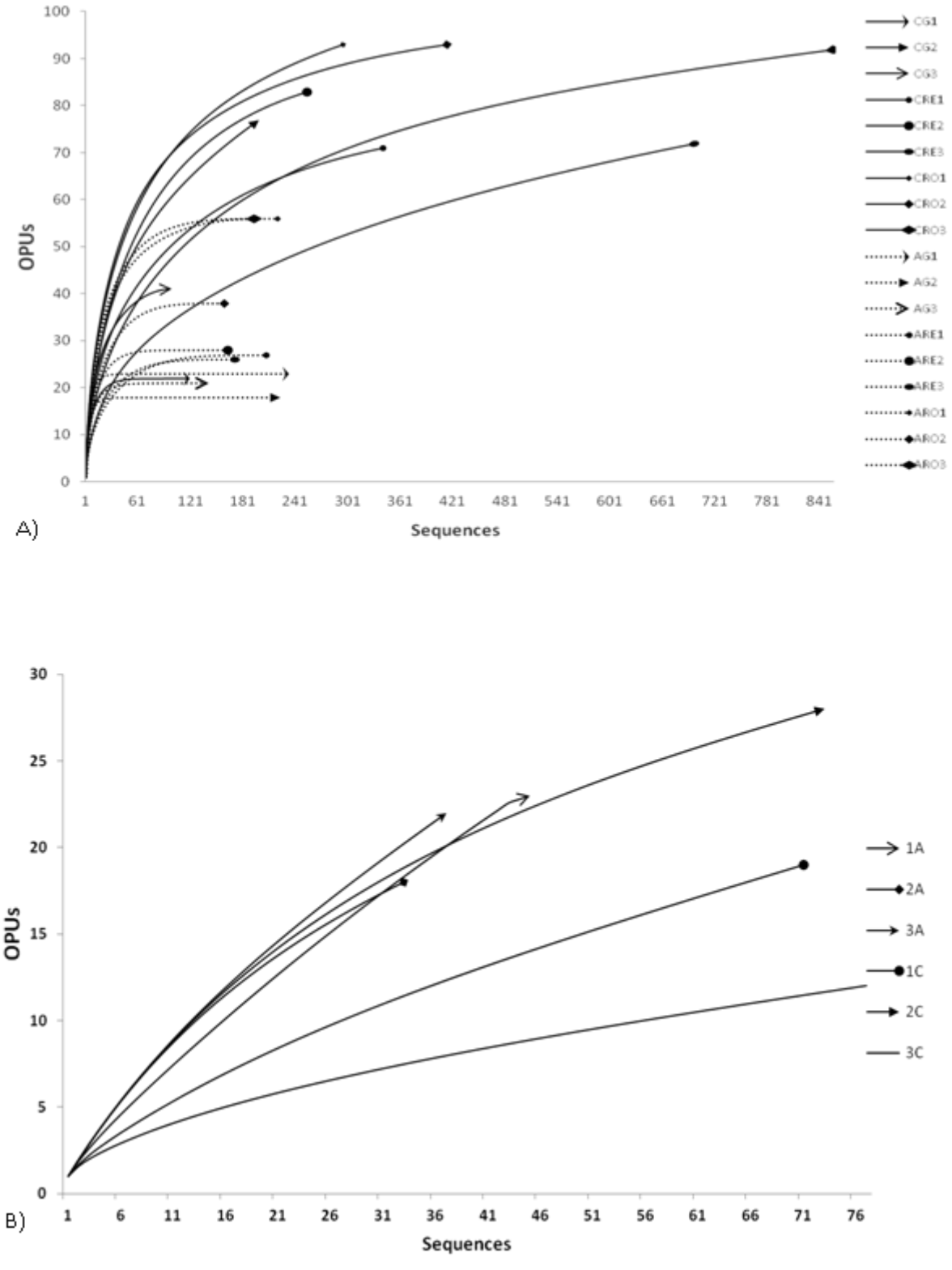


Figure A 2.3 Rarefaction curves of pyrosequencing data, OPU-based diversity for endosphere (A) and rhizosphere (B).

Table A 2.2 PERMANOVA p values for the comparisons between biological repetitions, compartment and location.

Comparison	Samples compared	*n*	*p* value
Compartment in Alcúdia	green $_{1,2 \text{ and } 3}$ - red $_{1,2 \text{ and } 3}$ -root $_{1,2 \text{ and } 3}$	9	0.413
Compartment in Campos	green $_{1,2 \text{ and } 3}$ - red $_{1,2 \text{ and } 3}$ -root $_{1,2 \text{ and } 3}$	9	0.049
Location	All endophytic samples	18	0.001
Location	$_{1, 2 \text{ and } 3}$ Alcúdia - $_{1, 2 \text{ and } 3}$ Campos	6	0.001

Table A 2.3 Endophytic indicator species analysis (ISA) results based on the hierarchical cluster obtained. IndValij is the indicator value for the species in parts per unit. * significant *p*-value; ** highly significant *p*-value.

Location	OPU	Identity	$IndVal_{ij}$	*p*	
Campos	8	*Acinetobacter bouvetii*	0.816	0.02	*
	9	*Acinetobacter baylyi*	0.816	0.02	*
	10	*Acinetobacter* sp.	0.745	0.04	*
	14	*Salinicola halophilus*	0.87	0.02	*
	21	Uncultured *Steroidobacter*	0.866	0.03	*
	22	*Nevskia aquatilis*	1	0.01	**
	23	Uncultured *Solimonadaceae*	0.745	0.03	*
	27	NKB5	0.816	0.02	*
	28	*Coxiella* spp.	0.816	0.02	*
	78	*Cupriavidus* sp.	0.861	0.01	**
	79	*Ralstonia* sp.	0.79	0.02	*
	82	*Comamonas koreensis*	0.81	0.01	**
	85	*Variovorax* sp.	0.796	0.03	*
	109	*Bradyrhizobium* sp.	0.845	0.04	*
	122	*Methylobacterium* sp.	0.745	0.03	*
	125	*Caulobacter leidyia*	0.905	0.01	**
	127	*Sphingomonas* sp.	0.882	0.01	**
	128	*Sphingomonas rhizogenes*	0.938	0.01	**
	143	*Reyranella* sp.	0.882	0.01	**
	148	Uncultured *Rickettsiaceae*	0.943	0.01	**
	175	*Leuconostoc* sp.	0.745	0.03	*
	177	*Streptococcus termophilus* MN-ZLW-002	0.865	0.01	**
	216	*Sediminibacterium goheungense*	0.943	0.01	**
	218	Uncultured *Chitinophaga*	0.745	0.04	*
	226	Uncultured *Acidobacteria*	0.9	0.01	**
	242	Uncultured *Propionibacterium*	1	0.01	**
	244	*Fimbriimonas gingsengisoli*	0.882	0.01	**
Alcúdia	4	*Lysobacter deserti*	0.812	0.03	*
	164	*Planomicrobium* sp.	0.916	0.01	**
	204	Uncultured *Flavobacteriaceae*	0.864	0.01	**
	246	*Deinococcus* sp.	0.867	0.01	**

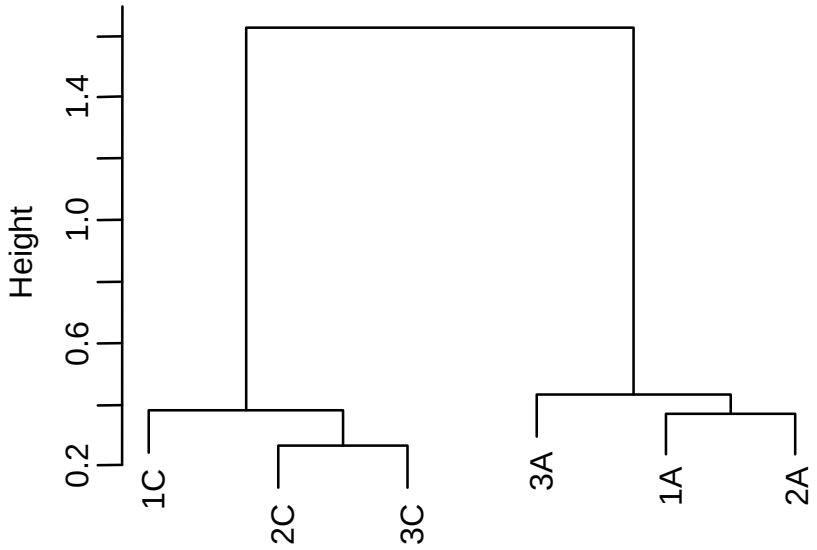


Figure A 2.4 Hierarchical cluster analysis of the total rhizospheric communities in Campos (C) and Alcúdia (A) locations.

3. Non-halophilic endophytes associated to the euhalophyte *Arthrocnemum macrostachyum* and their potential plant growth promoting activity

Table A 3.1. *p*-values of post-hoc pairwise permutational group comparison.

	M523	TH	R2A	NP	BM	EX	RMR
M523	-	-	-	-	-	-	-
TH	0.74	-	-	-	-	-	-
R2A	0.77	0.94	-	-	-	-	-
NP	0.21	0.31	0.27	-	-	-	-
BM	0.001	0.001	0.001	0.006	-	-	-
EX	0.001	0.001	0.001	0.001	0.06	-	-
RMR	0.001	0.001	0.001	0.001	0.001	0.003	-

Table A 3.2 Isolates belonging to each OPU, including the abundances per culture media and plant (P1, P2 and P3). The isolation method where the OPU was detected is also indicated: random (R), selected by morphology (S) and both (B).

OPU	OTU	Culture media	P1	P2	P3	Isolates per culture media	Total	Affiliation	Isolation method
1	1 14 29 34	BM	4	8	1	13	232	*Kushneria indalinina* *Kushneria marisflavi*	B
		EX	66	39	17	122			
		M523	18	8	6	32			
		NP	16	10	1	27			
		R2A	4	-	2	6			
		RMR	-	-	4	4			
		TH	4	22	2	28			
2	4 26	BM	14	4	7	25	144	*Providencia rettgeri*	B
		M523	26	4	-	30			
		NP	10	3	1	14			
		R2A	36	7	13	56			
		TH	14	3	2	19			
3	15 16 18 31	M523	4	6	7	17	74	*Pantoea eucrina*	B
		NP	1	2	11	14			
		R2A	5	11	1	17			
		RMR	1	-	8	9			
		TH	-	4	13	17			
4	3 24	NP	-	-	2	2	7	*Pseudomonas psychrotolerans*	B
		R2A	5	-	-	5			
5	6 12 16 28	BM	2	13	7	22	126	*Pseudomonas zhaodongensis*	B
		EX	-	-	3	3			
		M523	-	1	40	41			
		NP	2	-	15	17			
		R2A	1	4	24	29			
		TH	-	2	12	14			
6	5	BM	-	1	-	1	75	*Pseudomonas graminis*	B
		M523	-	3	-	3			
		NP	-	3	-	3			
		R2A	-	7	-	7			
		RMR	-	52	-	52			
		TH	-	9	-	9			
7	2	RMR	56	-	-	56	56	*Pseudomonas cichorii*	B
8	33	TH	-	-	1	1	1	*Acinetobacter johnsonii*	M
9	32	BM	-	-	1	1	2	*Sphingomonas desicabilis*	B
		NP	1	-	-	1			
10	20	EX	-	-	1	1	1	*Paracoccus chinensis*	R
11	25	EX	-	4	3	7	8	*Sanguibacter*	B

		NP	1	-	-	1		*kedieii*	
12	-	BM	1	-	-	1	1	*Janibacter sanguinis*	B
13	17	M523	-	-	1	1	6	*Curtobacterium flaccumfaciens pv. flaccumfaciens*	B
		NP	-	1	-	1			
		R2A	-	2	-	2			
		TH	-	2	-	2			
14	21	EX	1	-	-	1	1	*Nesterenkonia halotolerans*	R
15	7 8 35 36	BM	-	-	1	1	142	*Bacillus safensis*	B
		EX	6	2	-	8			
		M523	5	38	5	48			
		NP	23	6	14	43			
		R2A	2	17	4	23			
		RMR	-	-	1	1			
		TH	-	18	-	18			
16	27	EX	1	-	-	1	1	*Bacillus pumilus*	R
17	22	M523	-	-	1	1	56	*Staphylococcus saprophyticcus subs. saprophyticcus*	B
		NP	1	-	-	1			
		R2A	-	1	1	2			
		TH	34	-	18	52			
18	29	M523	-	-	1	1	19	*Staphylococcus equorum*	B
		NP	1	-	-	1			
		R2A	8	-	7	15			
		RMR	2	-	-	2			
19	11 21	EX	-	2	1	3	29	*Marinilactibacillus piezotolerans*	B
		R2A	-	5	21	26			
20	9	BM	-	-	28	28	34	*Paenibacillus borealis*	B
		NP	-	6	-	6			
21	10	NP	1	1	-	2	7	*Paenibacillus tundrae*	B
		R2A	2	1	2	5			
22	13	BM	-	1	-	1	3	*Paenibacillus taichungensis*	R
		NP	-	1	-	1			
		R2A	-	1	-	1			
		TH	-	-	9	9			

Table A 3.3 Minimum sampling number of isolates per culture media.

Culture media	Minimum sampling number
EX	23
M523	32
NP	32
R2A	35
RMR	28
TH	29

Table A 3.4 Similarity values of Morisita index of the isolates obtained by culture media. The highest value is marked in blue.

Media	R2A	M523	EX	RMR	NP	TH	BM
R2A	1	-	-	-	-	-	-
M523	0.791	1	-	-	-	-	-
EX	0.103	0.398	1	-	-	-	-
RMR	0.099	0.076	0.051	1	-	-	-
NP	0.658	0.945	0.445	0.092	1	-	-
TH	0.507	0.602	0.363	0.132	0.593	1	-
BM	0.648	0.589	0.269	0.029	0.488	0.371	1

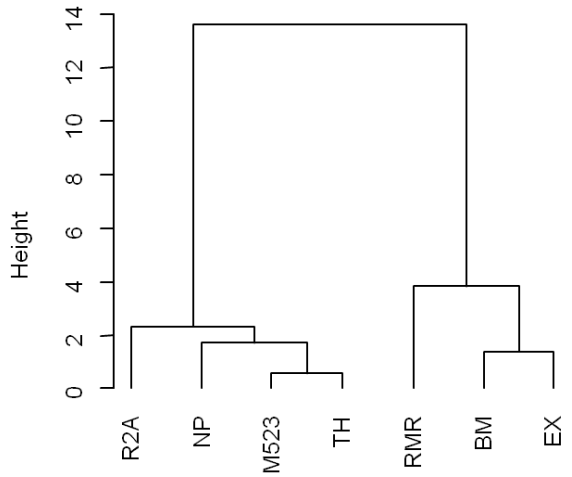


Figure A 3.1 Hierarchical clustering of total isolate abundances per culture media.

Table A 3.5 Jost index (q=0: number of the Operational Phylogenetic Units (OPU)s) and q=1

Culture medium	q=0		q= 1	
	Mean	SD	Mean	SD
RMR	1.67	1.15	1.44	0.72
EX	4.33	1.15	2.20	0.90
BM	4.67	1.53	2.88	0.63
M523	5.33	2.08	3.19	0.08
TH	5.67	1.53	3.56	1.30
NP	8.33	2.08	5.06	1.39
R2A	9	1	5.21	1.22

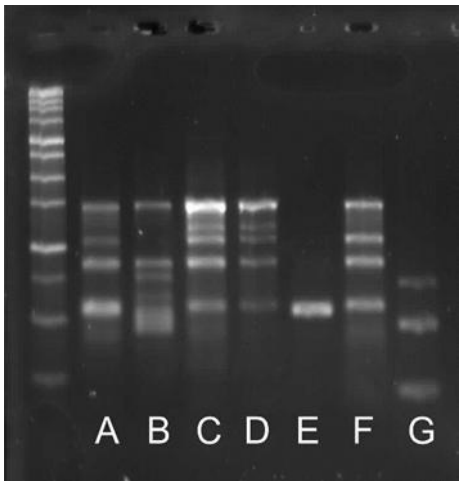


Figure A 3.2 Example of an agarose gel electrophoresis of RAPD (RandomAmplification of Polymorphic DNA) PCR amplicons. The selection of strains for the PGPA (Plant Growth Promoting Actity) test was carried out considering different patterns (e.g. A, B, E and G were selected, but just one among C, D and F was chosen).

Table A 3.6 Selected strains for plant growth promotion (PGP) test including the number of strains per OPU. In blue representative strains of the previous study (Mora-Ruiz *et al.* 2015) incorporated for the PGP analysis.

OPU	OTU	Isolates num	Identification	Strains
1	1	96	*Kushneria indalinina*	4
	29	72	*Kushneria marisflavi*	3
	34	60	*Kushneria indalinina*	6
2	4	44	*Providencia rettgeri*	5
	26	100	*Providencia rettgeri*	4
3	16	4	*Pantoea eucrina*	1
	18	23	*Pantoea eucrina*	6
	31	38	*Pantoea eucrina*	3
5	16	92	*Pseudomonas zahodongensis*	2
	28	24	*Pseudomonas zahodongensis*	4
6	5	75	*Pseudomonas graminis*	4
7	2	56	*Pseudomonas cichorii*	1
14	21	1	*Nesterenkonia halotolerans*	1
15	8	43	*Bacillus safensis*	4
	35	23	*Bacillus safensis*	5
	36	65	*Bacillus safensis*	3
17	22	56	*Staphylococcus saprophyticus subs. saprophyticcus*	4
18	29	19	*Staphylococcus equorum*	2
19	21	26	*Marinilactibacillus piezotolerans*	5
20	9	34	*Paenibacillus borealis*	5
Previous study Mora-Ruiz et al. (2015)			*Bacillus pumilus*	3
			Brevibacterium permense	3
			Chromohalobacter canadensis	6
			Marinococcus tarijensis	5
			Salinicola halophilus	5

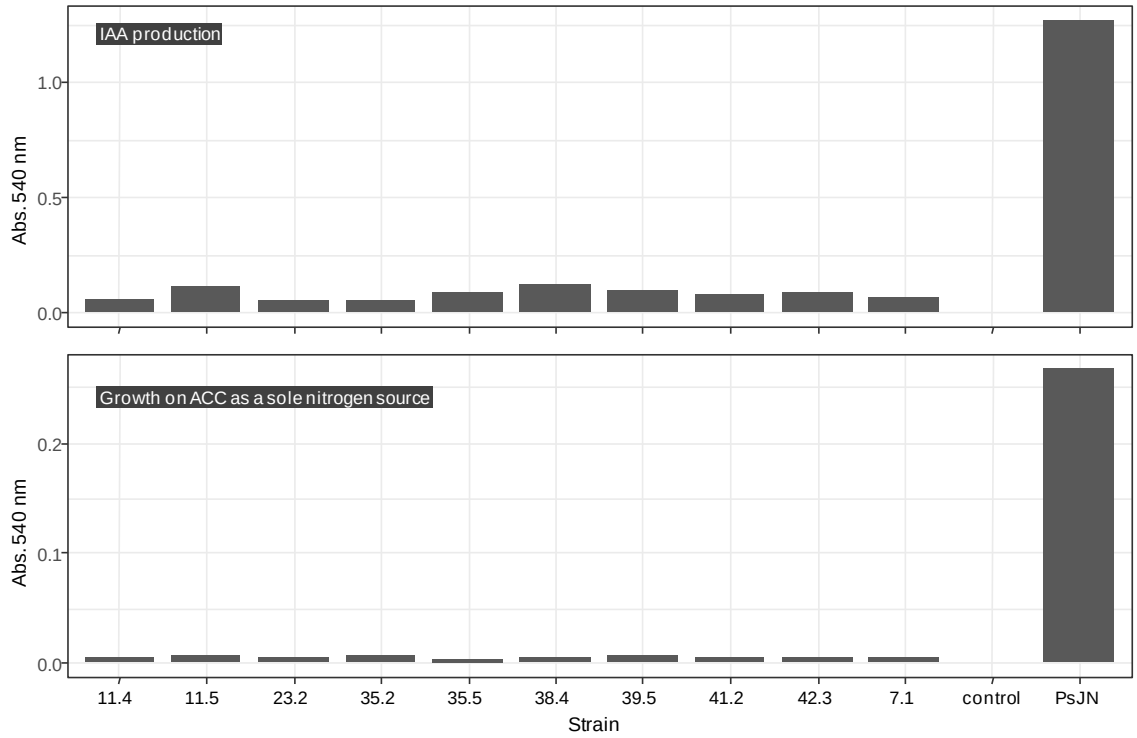


Figure A 3.3 IAA production and growth on ACC as a sole nitrogen source of the bacterial isolates from *A. macrostachyum*. *Pseudomonas graminis* (7.1), *Paenibacillus borealis* (11.4), *Paenibacillus borealis* (11.5), *Staphylococcus equorum* (23.2), *Staphylococcus equ*orum (33.5), *Kushneria indalinina* (35.2), *Pseudomonas zhaodongensis* (38.4), *Bacillus safensis* (39.5), *Salinicola halophilus* (41.2), *Marinococcus tarijensis* (42.3) and *Paraburkholderi phytofirmans* (PsJN).

4. Halophilic endophytic *Archaea* in the halophyte *Arthrocnemum macrostachyum*

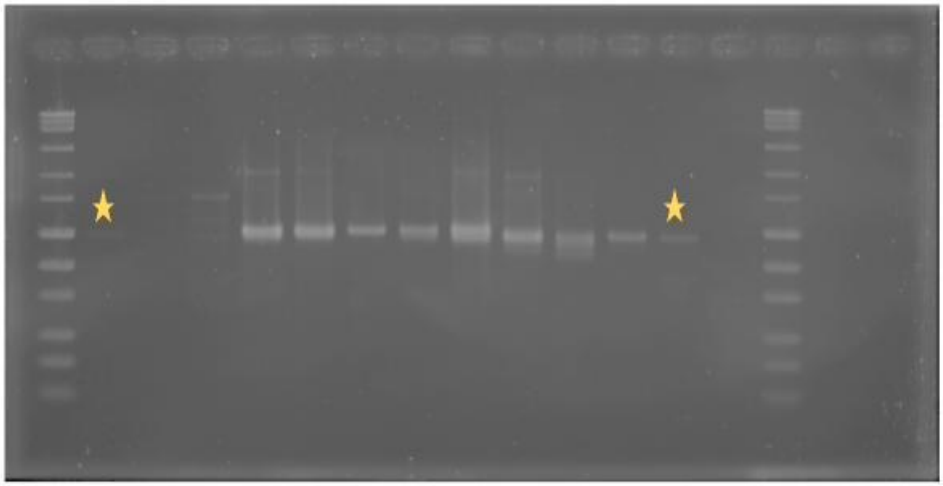


Figure A 4.1 Example of an agarose gel electrophoresis of archaeal PCR amplicons. Stairs (from left to right)= ARO2, ARO4

Table A 4.1 Relative abundances of minor OPUs according to total sequences for each sample (from fourth to eleventh column) and their taxonomic correspondence with the closest relative sequence (from first to third column).

			Samples									
OPU and affiliation	**accession number**	**% identity**	**G1**	**G3**	**G4**	**G5**	**RE2**	**RE3**	**RO2**	**RO4**	**ARE**	**ARO**
8 *Halorubrum lipolyticum*	DQ355814	>90% <98.1%	0.4	0.6	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
10 Uncultured *Halorubrum*	KF814466	>86% <92.3%	0.4	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
12 *Halorubrum* sp. LYG-9 / sp. MG526 / sp. E303-2	JX188273 / GU361143 / JN196506	>99%	0.4	0.0	0.0	0.2	0.9	0.5	0.0	0.4	0.0	0.0
15 *Halorubrum coriense*	JQ068942	>92% <97.1%	0.2	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
18 *Halorubrum* str. GluBr1.1	AJ270245	99.50%	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20 Uncultured *Halonotius*	KF591564 / AM947466	>92% <98.8%	0.4	0.0	0.2	0.3	0.1	0.0	0.0	0.0	0.0	0.0
21 Uncultured *Halorhabdus*	KF591571	>93% <96.2%	0.2	0.0	0.0	0.2	0.0	0.0	0.0	0.0	3.1	0.0
22 Uncultured *Natronomonas*	KJ546113	>97.3% <99.8%	0.0	0.0	0.0	0.0	0.0	0.0	3.0	0.0	0.0	0.0
24 *Natronomonas gomsonensis*	JF950943	99.40%	0.0	0.0	0.0	0.0	1.2	0.0	0.0	0.0	0.0	0.0
25 Uncultured *Halomarina*	KC465602	>91.6% <97%	1.1	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0
28 Uncultured *Haloquadratum*	FN391222	>90% <98.9%	0.6	1.8	1.6	0.2	0.5	0.3	0.3	0.0	1.5	0.0
32 *Halobellus litoreus*	GU951426	94.50%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
33 Uncultured *Halobacteriaceae*	HQ157601 / CU467243	>96 <99.5%	0.0	0.0	0.0	0.7	0.9	0.1	0.5	0.0	0.0	0.0
34 *Haloferax chudinovii / H. mucosum*	JX669135 / DQ860980	>96% <99.1%	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.4	0.0	0.0
35 *Halopelagius longus*	JX518988	>84.8% <88.3%	0.0	2.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
36 *Halolamina salina*	JX192605	98.10%	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.0
37 *Halobaculum gomorrense*	HM159611	98%	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
38 *Haloplanus salinus*	JQ237126	>99% <99.6%	0.6	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
39 Uncultured *Halobacteriaceae*	FN391271 / EF106534	>94% <99.6%	1.1	0.0	0.0	0.2	2.3	0.0	0.0	0.4	0.0	4.2
45 *Halohasta litchfieldiae*	AB935408	>98.9% <99.5%	0.0	0.0	0.0	0.0	0.3	0.8	0.0	0.0	0.0	0.0
46 *Haloarchaeobius iranensis*	KM055652	>93.9% <99.7%	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
47 *Haloarchaeobius* YC82	JQ937361	94.50%	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0
48 Uncultured *Haloarcula*	KF673170 / APHM01010977	>92.7% <98.7%	0.6	0.0	0.2	0.0	1.8	0.0	0.0	0.0	0.0	0.0
49 *Haloarcula* sp. ARA2 / *H.* sp. ARA7 / *H.* sp. CBA115	KM289103 / KM289111	>99.1%	0.7	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	0.0
50 *Haloarcula tradensis*	FJ429313	99.20%	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
51 *Haloarcula marismortui* ATCC43	AY596297	99.10%	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
52 *Halapricum salinum*	KF314042	>91.4% <93.3%	0.0	0.0	0.9	0.0	0.0	0.0	0.0	0.0	1.5	0.0
53 Uncultured *Halomicroarcula*	JN714439 / KC465608	>93.8% <99.1%	0.0	1.4	1.7	0.3	0.0	0.2	0.5	0.0	0.0	0.0

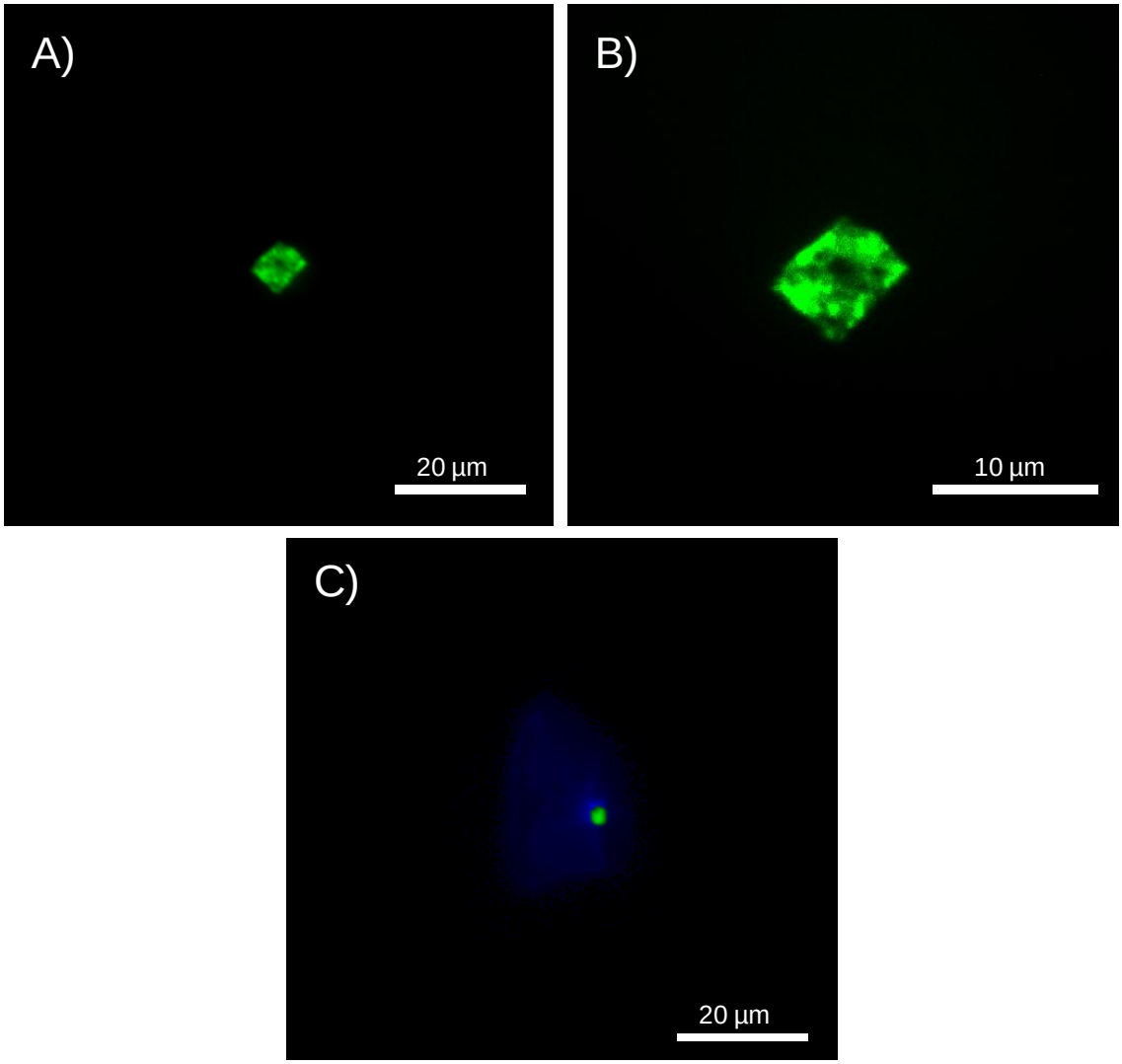


Figure A 4.2 A-B) Square *Archaea* and C) pleomorphic endophytic *Archaea* visualized by CARD-FISH using dye Alexa 488 (green). In blue vegetal fragment of *A. macrostachyum*.

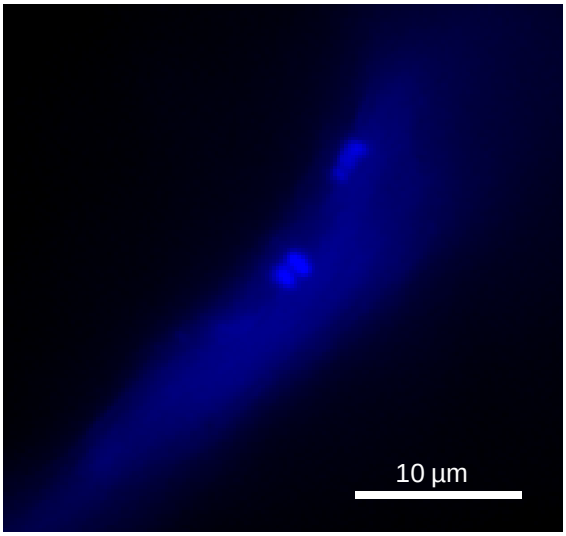


Figure A 4.3 *Bacteria* attached to plant tissue obtained from the maceration of sterilized green shoots. In blue the DAPI dye, in orange the CARD-FISH dye (Alexa 633).

5. Biogeographical patterns of bacterial and archaeal communities of distant hypersaline environments

Table A 5.1 Heatmap of the ionic composition of the brines and sediments studied in percentage of the major components. Values are shown in molar concentration per ion.

ID	Location	sample kind	Cl^-	SO_4^{2-}	Br^-	NO_3^-	NO2-	PO_4^{3-}	F^-	Na^+	Mg^{2+}	Ca^{2+}	K^+	NH_4^+	Li^+
SP-IB1	Spain	S	4.9558	0.5465	0.0079	0.0033	0	0.0016	0	4.1716	1.2047	0.1863	0.1194	0	0
SP-IB2	Spain	S	5.2162	0.4822	0.0097	0.0018	0	0.0014	0	4.2338	1.4623	0.0628	0.1488	0	0
SP-IB3	Spain	S	0.9709	0.7276	0.0019	0.0022	0	0.0017	0	0.7998	0.2496	0.7505	0.0268	0	0
SP-IB4	Spain	S	0.7707	0.4403	0.0018	0.0024	0	0.0018	0.0056	0.5992	0.2778	0.4457	0.0285	0	0
SP-IB5	Spain	B	5.5145	0.5526	0.0137	0.0022	0	0.0011	0.0007	4.1529	1.6625	0.0737	0.1715	0	0
SP-IB6	Spain	S	4.6264	1.5413	0.0139	0.0016	0	0.0016	0	3.7473	2.4683	0.0033	0.2009	0	0
SP-IB7	Spain	S	4.8001	0.3755	0.0065	0.0021	0	0.0018	0	4.1295	1.039	0.0677	0.0935	0	0
SP-IB8	Spain	S	5.2195	0.637	0.0151	0.0028	0	0.0014	0	3.9205	1.8659	0.0051	0.2324	0	0
SP-VC1	Spain	S	4.7027	0.8439	0.0127	0.0027	0	0.0013	0	3.0899	2.4957	0.0061	0.2163	0	0
SP-VC2	Spain	S	5.4053	1.2505	0.0168	0.0022	0	0.0016	0	3.3359	3.2828	0.0099	0.3704	0	0
SP-AR1	Spain	S	5.0615	0.354	0.0071	0.0022	0	0.0016	0	4.2019	1.2668	0.0132	0.1013	0	0
SP-AR2	Spain	S	5.057	0.4504	0.0086	0.0018	0	0.0015	0	3.9863	1.5786	0.0096	0.1324	0	0
SP-CM1	Spain	S	1.2123	0.3952	0.0007	0.0027	0.0002	0.0017	0.1962	1.0909	0.4592	0.2916	0.0436	0.006	0
SP-CM2	Spain	S	1.1291	0.3327	0.0007	0.0027	0	0.0025	0.2064	1.0061	0.4364	0.2719	0.047	0.0064	0
SP-CM3	Spain	S	1.004	0.321	0.0007	0.0027	0.0005	0.0023	0.128	0.9072	0.4407	0.2359	0.0375	0.0047	0
SP-CM4	Spain	S	12.661	1.7753	0.0038	0.002	0.0003	0.0018	0	0.5718	13.653	0.0601	0.2631	0.0026	6E-07
SP-CM5	Spain	B	1.8192	0.3645	0.0011	0.0038	0.0003	0.0013	0.1463	1.5139	0.5593	0.3463	0.0368	0	0
SP-CM6	Spain	S	2.5159	0.9065	0.0002	0.0038	0.0002	0.0013	0.0029	2.1165	1.0015	0.4614	0.0146	0	6E-07
SP-CM7	Spain	B	6.8837	1.4439	0.0013	0.0051	0.0002	0.0011	0.0023	1.0117	7.1928	0.0278	0.098	0	7E-07
SP-CN1	Spain	S	4.6537	2.1257	0.0114	0.0021	0	0.0016	0	3.1653	3.0727	0.0033	0.8237	0	0
SP-CN2	Spain	S	5.1709	0.5166	0.0083	0.0025	0	0.0014	0	4.1236	1.6069	0.0029	0.1403	0	0
SP-CN3	Spain	S	5.1746	0.8721	0.0165	0.0019	0	0.0017	0	3.3449	2.7344	0.0096	0.2563	0	0
SP-CN4	Spain	S	5.4776	1.2432	0.0224	0.0019	0	0.0016	0	2.9906	3.7132	0.0037	0.3836	0	0
ARG1	Argentina	S	2.3326	1.4472	0.0014	0.0031	0.0002	0.0039	0.0018	3.1886	0.5062	0.5886	0.2433	0.0112	9E-06
ARG2	Argentina	B	3.8483	0.0096	0.0203	0.0024	0	0.0024	0.0031	3.7574	0.068	0.0834	0.0591	0	1E-06
ARG3	Argentina	S	3.299	0.2409	0.0028	0.0023	0	0.0112	0.0787	3.7627	0.0129	0.0139	0.8389	0.0022	8E-06
ARG4	Argentina	B	0.699	0.0765	0.0019	0.0041	0.0002	0.0039	0.003	0.7916	0.0482	0.21	0.0783	0	1E-05
ARG5	Argentina	S	4.9945	0.3906	0.0339	0.0044	0	0.0055	0.0054	5.2285	0.0173	0.0098	0.3111	0	1E-05
ARG6	Argentina	B	22.994	0.472	0.0078	0.0019	0	0.0016	0.0014	23.552	0.0216	0.1717	0.138	0	2E-05
ARG7	Argentina	B	5.5045	0.4437	0.0013	0.0037	0	0.001	0.003	5.7601	0.0679	0.0099	0.1421	0	9E-06
ARG8	Argentina	S	4.9959	0.2835	0	0.0018	0	0.0007	0	5.0264	0.0492	0.0881	0.0663	0	1E-05
ARG9	Argentina	B	25.833	1.3166	0	0.0032	0	0.0018	0.0045	26.111	0.1157	1.3125	0.0493	0	1E-06
ARG10	Argentina	S	1.3946	2.3926	0	0.0023	0	0.0021	0	1.2723	0.04	2.4346	0.0081	0	1E-06
ARG11	Argentina	B	0.8623	0.1007	0.0002	0.0031	0.0002	0.0009	0.0008	0.7176	0.1279	0.0991	0.0312	0.0028	1E-05
ARG12	Argentina	S	3.4214	1.8439	0.0025	0.0022	0	0.0007	0.0008	4.1555	0.7303	0.04	0.2896	0.0022	6E-06
ARG13	Argentina	B	4.8049	0.5367	0.0013	0.0034	0.0002	0.0013	0.0011	4.4403	0.2987	0.3718	0.2603	0.0156	1E-05
ARG14	Argentina	S	5.4617	0.4864	0.0005	0.0098	0	0.0007	0	4.8433	0.5689	0.0614	0.3649	0	1E-05
ARG15	Argentina	B	4.5201	0.4288	0.0006	0.003	0.0003	0.0016	0	3.5357	0.2746	1.3197	0.0644	0.002	2E-05
ARG16	Argentina	S	10.822	0.0728	0.0022	0.0096	0	0.0066	0	8.5233	0.9853	1.0867	0.1945	0	4E-06
ARG17	Argentina	B	4.1385	0.3841	0.0028	0.0035	0	0.0139	0.1057	5.9418	0.0242	0.0422	0.9462	0	1E-06
ARG18	Argentina	S	4.0289	0.3056	0.0035	0.0063	0.0003	0.0173	0.1132	4.5161	0.0345	0.0058	1.0553	0.0024	0.0001
ARG19	Argentina	B	4.7075	0.0289	0.0255	0.0027	0	0.0036	0.0033	4.434	0.1511	0.1894	0.2058	0.0198	9E-05
ARG20	Argentina	S	5.3341	0.0409	0.0174	0.0031	0	0.0009	0	4.932	0.1131	0.0457	0.1922	0	2E-06
ARG21	Argentina	B	5.3656	0.0386	0.0002	0.003	0	0.0008	0.0063	4.9544	0.1029	0.0606	0.1975	0	9E-06
ARG22	Argentina	S	3.0013	0.0249	0.0005	0.0019	0	0.0026	0.0014	2.7294	0.1042	0.1116	0.1672	0	3E-06
ARG23	Argentina	B	25.774	1.3013	0.0021	0.0031	0	0.0018	0.0043	26.099	0.1156	1.3099	0.0492	0	4E-07
CHL1	Chile	S	3.8982	0.5169	0.0109	0.0018	0	0.0014	0	2.7854	1.689	0.024	0.1699	0	0
CHL2	Chile	S	4.8305	0.6279	0.012	0.0017	0	0.0015	0	3.4013	2.1357	0.006	0.127	0	0
CHL3	Chile	B	5.3107	1.2349	0.0201	0.0021	0	0.0018	0	2.7275	3.9292	0.0062	0.2276	0	0
CHL4	Chile	B	5.4609	1.4042	0.0247	0.0025	0	0.0017	0	2.4932	4.457	0.0034	0.2742	0	0

S: Sediment; B: Brine.

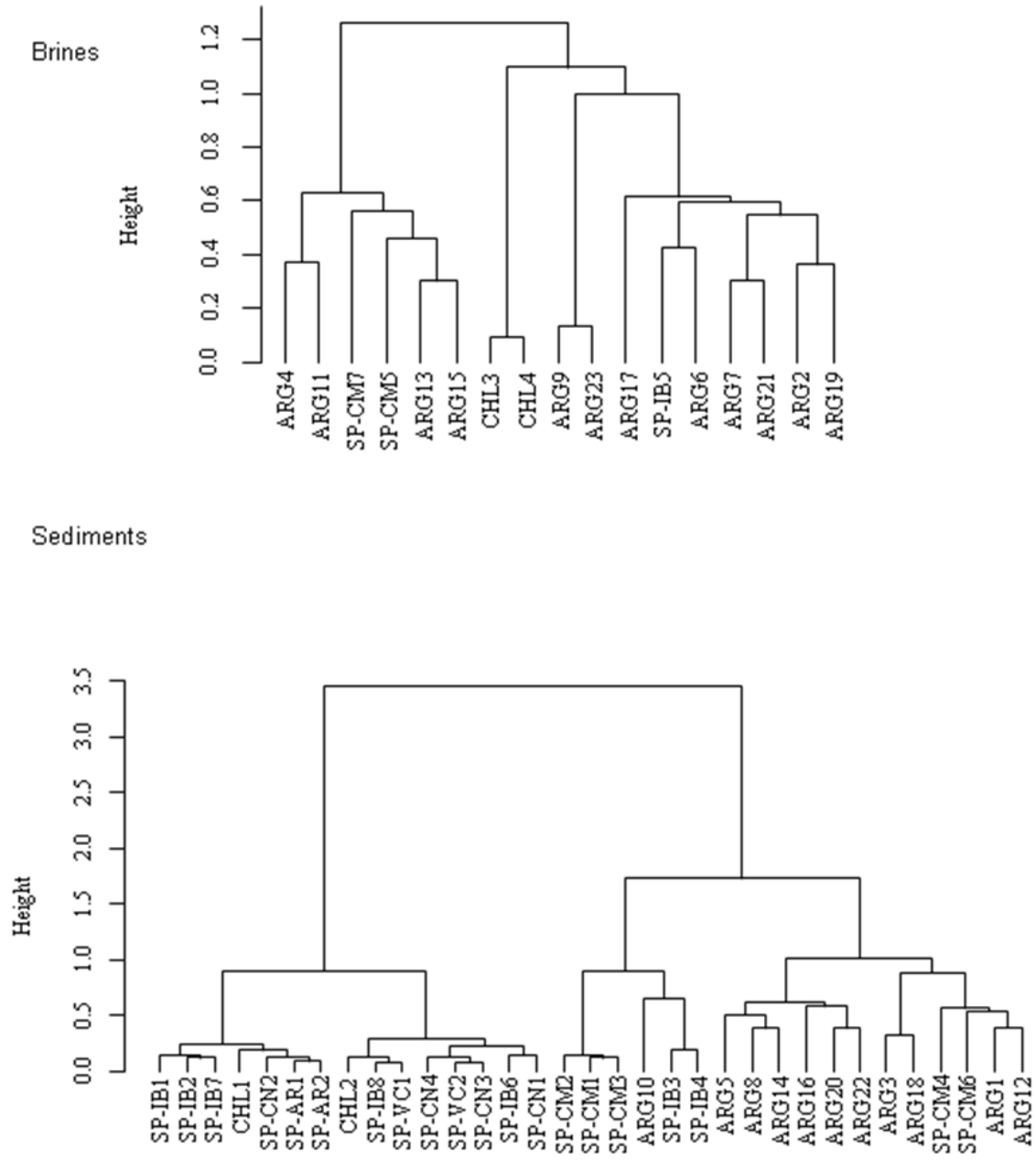


Figure A 5.1 Dendrogram of the hierarchical cluster analysis (Ward linkage) based on the ionic composition for brines and sediments

Table A 5.2 Clustering detail and heatmap for Jost´s indexes q=0 (number of OPUs) and q= 1 for Bacteria and Archaea communities.

		Bacteria				Archaea			
ID	**Type**	**Total seq.**	**OTUs**	***q*=0**	***q*=1**	**Total seq.**	**OTUs**	***q*=0**	***q*=1**
SP-IB1	S	5009	1359	154	25.5	9986	2609	174	29.7
SP-IB2	S	7349	545	101	3.1	5264	343	44	13.7
SP-IB3	S	16409	1545	141	4.7	6808	938	118	14.2
SP-IB4	S	8509	404	30	8	52874	1367	77	5.4
SP-IB6	S	5174	1670	99	19.8	4330	2529	77	9.3
SP-IB7	S	8258	1552	116	33.6	12184	2211	166	44.1
SP-IB8	S	9356	3145	132	17	20647	2513	160	15
SP-VC1	S	1898	517	71	30.7	33449	1259	40	12.2
SP-VC2	S	2999	646	56	15.9	13151	837	67	20.4
SP-AR1	S	5848	2867	152	18.1	4006	1234	160	35.8
SP-AR2	S	5052	1559	157	18.8	10187	1469	150	10
SP-CM1	S	6951	1013	70	9.9	5065	329	33	9.3
SP-CM2	S	6730	1001	55	11	5372	420	33	7.6
SP-CM3	S	4556	848	93	3.7	4457	405	43	14
SP-CM4	S	8911	747	54	3.4	11185	506	22	3.9
SP-CM6	S	15690	2405	165	3.9	20510	2871	120	33.6
SP-CN1	S	1320	249	29	9.6	29606	1563	62	18.3
SP-CN2	S	8521	2426	107	13	6232	1811	171	46
SP-CN3	S	1651	621	77	18.8	21088	4513	**194**	18.6
SP-CN4	S	1055	364	61	19.5	6790	2565	178	**56**
ARG1	S	14774	3431	129	17.5	11361	4342	123	27.6
ARG3	S	17195	3876	249	12.5	17758	1275	16	1.9
ARG5	S	13815	4289	185	19.6	17596	4093	128	19.1
ARG8	S	8720	3508	200	38.6	11846	2769	129	8.6
ARG10	S	14342	3729	181	17.2	7465	813	46	5.8
ARG12	S	15447	4480	174	20.1	8222	1836	65	7.8
ARG14	S	14832	2739	200	21.7	6958	2698	80	18
ARG16	S	15906	5579	297	18.7	11656	904	137	41
ARG18	S	12788	2969	184	34.1	28565	608	26	4.8
ARG20	S	14364	5213	232	**43.6**	14690	4813	121	23.5
ARG22	S	15958	1944	275	16.9	20457	1383	106	29.6
CHL1	S	5151	1650	177	20.3	10304	758	69	14
CHL2	S	10116	2589	161	13.9	15821	1154	74	14.1
SP-IB5	B	9455	2497	151	23.3	6813	573	63	10.2
SP-CM5	B	13412	4717	**312**	18.7	12824	1480	83	17.9
SP-CM7	B	13908	2633	181	2.8	9574	1534	109	25
ARG2	B	11455	2671	161	17.1	10856	1454	88	19
ARG4	B	10313	2298	201	14.8	8145	252	24	2.2
ARG6	B	11190	1273	96	4.6	12884	844	39	3.7
ARG7	B	8863	1883	154	9.8	10323	591	38	3
ARG9	B	6092	997	49	20	12309	666	39	2.9
ARG11	B	9115	1542	107	14.5	12586	2143	91	12.2
ARG13	B	6086	1768	169	12.4	9601	646	40	4
ARG15	B	773	345	77	35.5	26033	868	39	5
ARG17	B	15519	2723	54	5.6	8045	1079	82	16.5
ARG19	B	10924	1521	122	13.4	38881	1090	36	6.6
ARG21	B	12343	2437	217	10.6	24700	2537	86	11.4
ARG23	B	3425	904	113	24.9	3082	330	63	21
CHL3	B	8737	964	121	7.3	13152	288	52	9.7
CHL4	B	6667	964	73	8.5	6713	1726	60	18.8

The highest values for each index are indicated in bold. S: Sediment; B: Brine

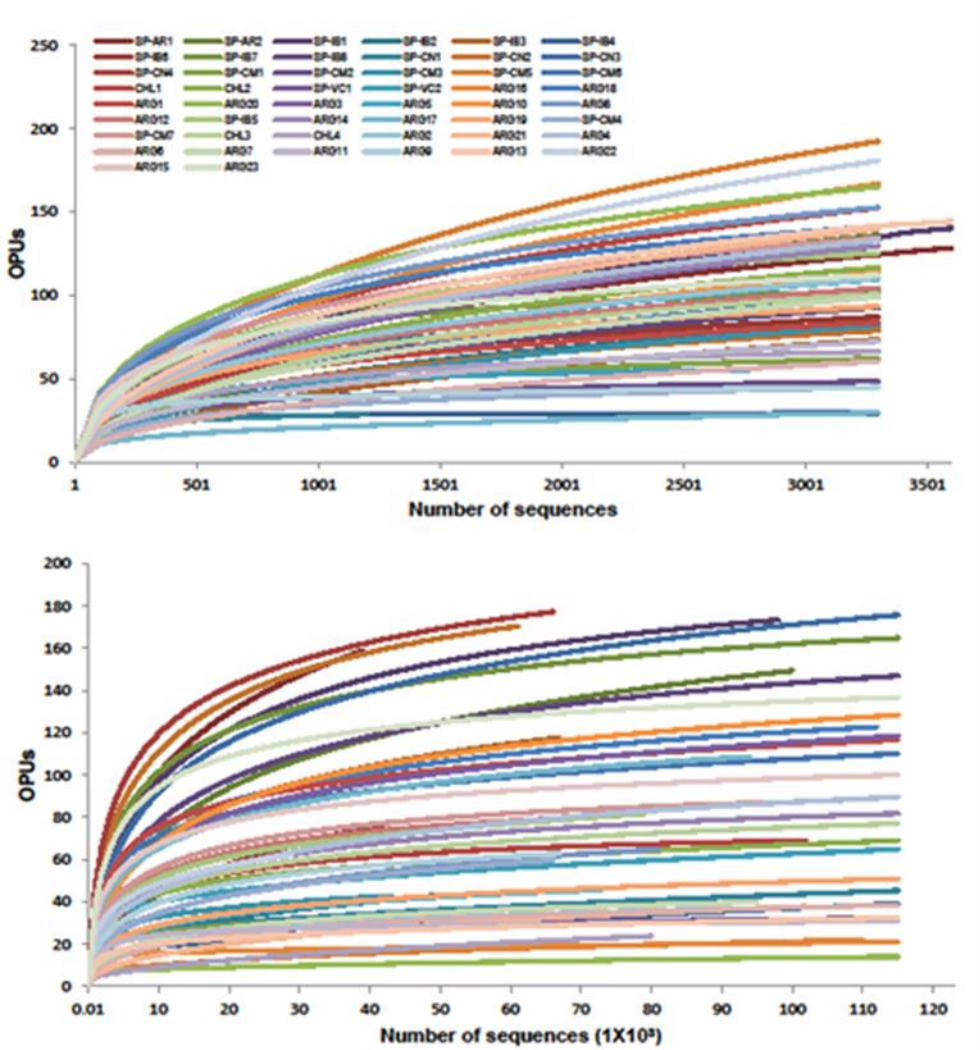


Figure A 5.2 Rarefaction curves of pyrosequencing data for *Bacteria* (up) and *Archaea* (down), OPU-based richness.

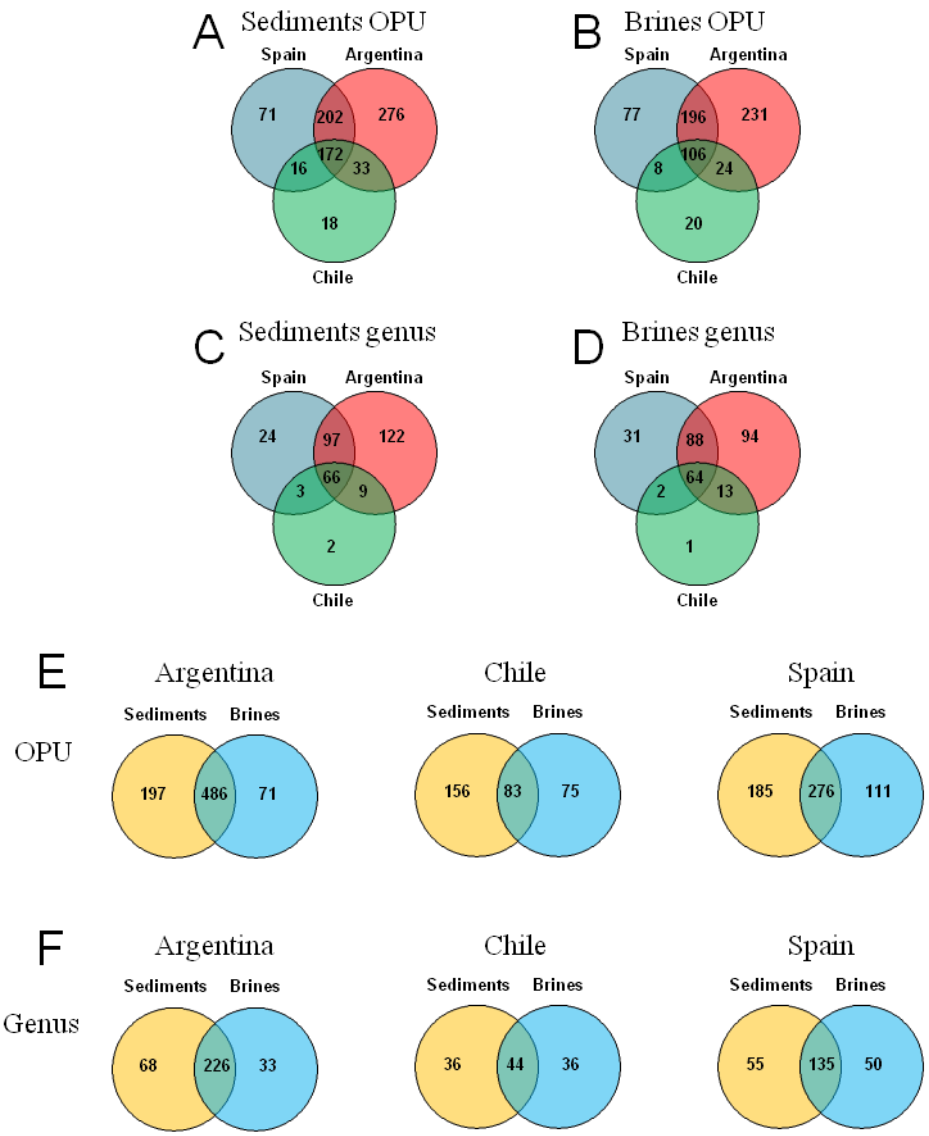


Figure A 5.3 Venn diagram of bacterial taxa detected in sediments (A, C) and brines (B, D) samples at OPU (A, B) and genus (C, D) level. Distribution of OPUs (E) and genera (F) in each country by type of sample (sediments and brines).

Table A 5.3 Heatmap of the relative abundances of most (making >70% of the total sequences per sample) abundant bacterial OPUs and their taxonomic assignment. Light brown cells: sediments samples; white cells: brines samples. Red cells indicate zero while yellow cells are low values (< 0.5) and green cells are the highest values.

OPU	Taxonomic assignment	SP-IB1	SP-IB2	SP-IB3	SP-IB4	SP-IB5	SP-IB6	SP-IB7	SP-IB8	SP-VC1	SP-VC2	SP-AR1	SP-AR2	SP-CM1	SP-CM2	SP-CM3	SP-CM4	SP-CM5	SP-CM6	SP-CM7	SP-CN1	SP-CN2	SP-CN3	SP-CN4	ARG1	ARG2	ARG3	ARG4	ARG5	ARG6	ARG7	ARG8	ARG9	ARG10	ARG11	ARG12	ARG13	ARG14	ARG15	ARG16	ARG17	ARG18	ARG19	ARG20	ARG21	ARG22	ARG23	CHL1	CHL3	CHL2	CHL4
B001	*Proteobacteria Comamonadaceae, Comamonas denitrifians/C. nitrativorans*	0	1	0	17	2	0	1	0	9	3	0	0	16	12	0	1	0	1	0	14	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B002	*Proteobacteria Comamonadaceae Comamonas terrigena*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
B004	*Proteobacteria Comamonadaceae Delftia acidovorans*	0	0	0	0	0	0	0	0	2	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B005	*Proteobacteria Comamonadaceae Diaphorobacter nitroreducens*	0	1	0	10	4	0	0	0	4	3	0	0	11	6	0	0	0	1	0	22	0	0	0	0	0	0	1	0	0	0	0	2	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
B011	*Proteobacteria Comamonadaceae Caenimonas koreensis*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	8	0	0	0	0
B013	*Proteobacteria Comamonadaceae Leptothrix*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	4	0	0	0	0
B015	*Proteobacteria Oxalobacteraceae Massilia oculi/M. aurea/M. timonae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	4	0	0	0	0
B026	*Proteobacteria Moraxellaceae Acinetobacter*	0	5	0	40	0	0	1	0	0	15	0	0	36	36	0	0	0	1	0	29	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B027	*Proteobacteria Moraxellaceae Acinetobacter*	0	0	0	8	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B028	*Proteobacteria Moraxellaceae Acinetobacter venetianus*	0	0	0	4	0	0	0	0	1	2	0	0	2	3	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B029	*Proteobacteria Moraxellaceae Acinetobacter schindleri/A. gyllenbergii/A. lwoffii*	0	0	0	1	0	0	0	0	1	0	0	0	2	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B034	*Proteobacteria Moraxellaceae Acinetobacter junii*	0	2	0	3	0	0	0	0	1	2	0	0	6	9	0	1	0	1	1	8	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
B036	*Proteobacteria Moraxellaceae Moraxella osloensis*	0	0	0	1	0	0	0	0	1	0	0	0	5	6	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0
B040	*Proteobacteria Pseudomonadaceae Pseudomonas*	0	0	51	1	0	0	0	0	0	1	0	0	1	2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0
B044	*Proteobacteria Xanthomonadaceae Stenotrophomonas*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1	0	0	0	0
B045	*Proteobacteria Xanthomonadaceae Lysobacter*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	1	0	0	0	0
B046	*Proteobacteria Xanthomonadaceae Pseudofulvimonas*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0
B048	*Proteobacteria Coxiellaceae Coxiella*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
B049	*Proteobacteria Coxiellaceae Coxiella burnetti*	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B052	*Proteobacteria Halomonadaceae Halomonas*	0	0	0	0	0	0	0	0	0	0	0	0	1	1	3	0	5	6	2	0	0	0	0	2	2	0	0	0	1	0	0	3	3	4	0	1	1	0	1	0	0	0	1	0	1	0	0	0	0
B059	*Proteobacteria Idiomarinaceae Idiomarina ramblicola/I. abyssalis*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	31	0	0
B063	*Proteobacteria Halomonadaceae Hahellaceae Salicola marasensis/Halospina denitrificans*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	7	12	0	2	0	0	1	5	0	1	2	0	0	0	0	0	0

B072	*Proteobacteria Legionellaceae Legionella*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0
B075	*Proteobacteria Incertae sedis Marinicella*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	1	3	1	1	0	0	0	0	0
B076	*Proteobacteria Zeta Marinprofundaceae Mariprofundus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0
B077	*Proteobacteria Halothiobacillaceae, Halothiobacillus halophilus/A. hydrothermalis*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0
B085	*Proteobacteria Ectothiorhodospiraceae Sva0071*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	1	0	0	0	0	0	0	0
B087	*Proteobacteria Ectothiorhodospiraceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	6	0	0	0	0	7	4	6	1	3	3	0	0	0	0	0	0
B089	*Proteobacteria Ectothiorhodospiraceae Halorhodospira neutrophila/H. halophila*	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	8	4	23	11	0	0	0	0	0	7	1	0	0	9	0	0	1	0	0	1	0	0	0	0	0	0	0
B090	*Proteobacteria Ectothiorhodospiraceae*	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14
B092	*Proteobacteria Ectothiorhodospiraceae Spiribacter*	0	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
B093	*Proteobacteria Ectothiorhodospiraceae Arhodomonas*	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	9	0	0	0	1	2	0	0	0	0	0	0
B101	*Proteobacteria Incertae sedis Thiohalorhabdus denitrificans*	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	3	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B106	*Proteobacteria Rhodobacteraceae Roseovarius mucosus*	9	1	1	0	6	0	0	1	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	1	3	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	1	2	0	0	2	0	0	0	0	0
B107	*Proteobacteria Rhodobacteraceae Roseovarius tolerans*	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
B116	*Proteobacteria Rhodobacteraceae Loktanella vesttfoldensis/L. salsilacus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	9	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B117	*Proteobacteria Rhodobacteraceae*	1	0	0	0	8	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	1	0	0	0	0	0	0
B127	*Proteobacteria Rhodobacteraceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	38	0	1
B141	*Proteobacteria Rhodobacteraceae*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0	0	0
B151	*Proteobacteria Rhizobiales*	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3	0	0	0	1	0	0	0	0
B170	*Proteobacteria Sphingomonadaceae Sphingomonas Novosphingobium*	0	0	0	1	1	0	0	0	2	1	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	5	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0
B180	*Proteobacteria Rhodospirillaceae Rhodovibrio*	1	0	0	0	1	3	4	1	0	0	2	0	0	0	0	0	1	0	2	0	7	2	2	1	1	0	0	0	0	0	6	0	4	13	0	0	1	0	1	0	0	0	7	4	0	0	0	1	0	1
B181	*Proteobacteria Rhodospirillaceae Rhodovibrio*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	1	2	0	0	0	0	0	0
B188	*Proteobacteria Rickettsiales*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
B194	*Parcubacteria*	0	0	0	0	0	2	3	3	0	0	1	11	0	0	0	0	0	0	1	0	0	3	0	1	0	0	0	1	0	0	1	0	0	0	1	2	0	0	1	2	0	0	0	0	0	0	0	0	0	3
B196	*Parcubacteria*	1	0	0	0	0	1	2	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B200	*Parcubacteria*	5	0	0	0	0	0	1	3	0	1	6	6	0	0	0	0	0	0	1	0	0	0	4	1	0	0	0	3	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
B207	*Microgenomates*	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	11	0
B211	*Spirochaetae Incertae sedis Candidatus Cloacamonas*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
B216	*Deferribacteres Deferribacteraceae Flexistipes sinusarabici*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19	0
B217	*Actinobacteria* OPB41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	6	0	1	0
B218	*Acidobacteria Acidobacteriaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
B219	*Proteobacteria Desulfohalobiaceae*	6	0	0	0	0	8	3	10	6	17	3	4	0	0	0	0	0	0	0	0	20	0	24	0	0	0	0	0	0	1	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Desulfovermiculus																																																		
B220	*Proteobacteria Desulfohalobiaceae Desulfovermiculus*	6	0	0	0	0	6	2	9	5	2	2	4	0	0	0	0	0	0	0	0	5	0	2	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B222	*Proteobacteria Desulfohalobiaceae Desulfovermiculus*	5	0	0	0	0	6	4	6	0	2	3	1	0	0	0	0	0	0	1	0	1	0	0	1	1	0	0	12	4	27	4	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B224	*Proteobacteria Desulfohalobiaceae Desulfovermiculus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B226	*Cloacimonetes MSBL8*	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B232	*Proteobacteria Desulfobacteraceae Desulfosalsimonas propionicica*	1	0	0	0	0	0	3	0	2	0	1	9	0	0	1	0	0	1	0	0	0	0	0	3	1	0	0	8	0	1	7	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
B233	*Proteobacteria Desulfobacteraceae Desulfosalsimonas*	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B243	*Proteobacteria* GR-WP33-58	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	0	1	0	6	0	0	0	0	0	3
B244	*Proteobacteria* GR-WP33-59	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	2
B247	*Proteobacteria Syntrophobacterales Syntrophobacteraceae Syntrophobacter*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	29	0
B249	*Proteobacteria Desulfobacteraceae Desulfobacterium anilini*	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	4	0
B263	*Bacteroidetes Marinilabiaceae*	3	0	0	0	0	6	2	1	0	1	3	1	1	0	1	0	1	1	2	0	15	0	0	7	2	1	1	7	0	2	8	0	1	0	7	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
B264	*Bacteroidetes* E6aCO2	4	0	1	0	0	2	4	1	0	0	5	14	0	0	0	0	1	0	2	0	1	0	0	10	4	0	0	8	1	5	4	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
B269	*Bacteroidetes Flavobacteriaceae Psychroflexus salinarum P. tropicus P. halocasei*	0	0	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	3	0	0	0	0	0	3	9	0	0	0	0	0	0	0	0	0	0	3	5	36	0	0	1	0	1
B278	*Bacteroidetes Flavobacteriaceae Cloacibacterium normanense/C. rupense*	0	1	0	3	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B289	*Bacteroidetes Bacteroidales Unclassified*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	1	0	0	0	2	0	0	0	0
B291	*Bacteroidetes Schleiferiaceae Xchleiferia thermophila*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0	0	0	0	0	0	0	0	0
B292	*Bacteroidetes Cryomorphaceae Owenweeksia hongkongensis*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
B293	*Bacteroidetes Chitinophagaceae*	0	0	0	0	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	17	1	0	4	0	1	48	0	0	0	6	0	0	0	0	0	0
B294	*Bacteroidetes Chitinophagaceae Ferruginibacter alkalinentus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	5	0	0	0	0
B297	*Bacteroidetes Chitinophagaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	9	0	0	0	0	0	0	0	0
B313	*Bacteroidetes Sphingobacteriales*	0	0	0	0	5	0	0	5	0	0	0	0	0	0	0	0	28	0	28	0	0	5	0	4	1	0	0	0	0	0	1	0	1	10	0	0	0	0	12	0	5	0	1	0	11	0	0	2	0	43
B316	*Bacteroidetes Chitinophagaceae Gracilimonas*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0
B317	*Bacteroidetes Chitinophagaceae Gracilimonas*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	6	4	1	8	0	0	0	0	0	0	0	0	0	0	0	0	0
B318	*Rhodothermaeota Salinibacteraceae Salinibacter*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	15	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B320	*Rhodothermaeota Salinibacteraceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B322	*Chlorobi* OPB56	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
B327	*Lentisphaerae Oligosphaeria*	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	4	6	0	1	3	0	3	0	0	0	0	0	0	0	0	0	0	0
B329	*Lentisphaerae Oligosphaerales*	2	0	0	1	0	3	4	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B331	*Lentisphaerae Oligosphaeraceae Oligosphaera*	1	0	0	0	0	5	4	0	0	0	5	0	0	0	0	0	1	0	6	0	1	0	7	0	0	0	0	10	1	0	5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

B340	*Hydrogenedentes*	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
B343	*Planctomycetes Phycisphaeraceae Phycisphaera*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	4	4	2	0	0	0	0	0	0	0
B346	*Planctomycetes Planctomycetaceae Pirellula/ Rhodopirellula/Blastopirellula*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0
B351	*Gemmatimonadetes* PAUC43f	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	20	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	2	0	0	0	0	0	3	0
B352	*Gemmatimonadetes Gemmatimonadetes*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	3	0	0	0	1	0	0	0
B355	*Gemmatimonadetes Gemmatimonadaceae Gemmatimonas aurantiaca*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	6	0	0	0	0
B358	*Deinococcus-Thermus Trueperaceae Truepera*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	5	0	0	0	0	0
B360	*Aminicenantes*	0	0	0	0	0	0	2	0	2	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0
B378	*Actinobacteria Propionibacteriaceae Propionibacterium acidipropionici/P. acnes/P. avidum*	0	0	0	2	1	0	0	0	2	1	0	0	1	1	0	2	0	0	0	3	0	0	2	0	0	0	0	0	0	0	0	6	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
B385	*Actinobacteria Nitriliruptoraceae Euzebyaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
B387	*Actinobacteria Iamiaceae Ilumatobacter fluminis*	0	0	0	0	1	0	0	0	0	0	0	0	7	4	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B392	*Actinobacteria Gailleales* Unclassified	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	5	0
B398	*Candidate division* TM6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	15	0	0	0	1	0	0	0	0	0	0	1
B399	*Chloroflexi Anaerolineaceae*	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	21	0	0	0
B411	*Caldiserica Caldisericaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
B413	*Firmicutes Halanaerobiaceae Halanaerobium*	19	1	8	0	2	2	1	12	0	0	33	15	2	6	72	76	21	75	18	0	21	0	0	21	23	0	0	14	1	6	14	0	3	4	0	0	0	0	24	0	0	0	6	0	3	0	0	0	3	1
B414	*Proteobacteria Desulfobacterales*	6	0	0	0	0	7	1	1	0	1	6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B416	*Firmicutes Halanaerobiaceae Halanaerobium*	4	0	1	0	1	2	1	12	0	1	6	3	0	0	6	0	2	2	5	1	6	0	0	11	10	0	0	2	7	6	2	0	21	7	12	11	11	0	2	0	0	0	3	0	2	0	0	0	0	1
B417	*Firmicutes Halanaerobiaceae Halanaerobium acetethylicum/H. praevalens/P. alcaliphilum*	1	0	4	0	2	0	0	2	0	0	1	1	0	0	8	1	5	1	3	0	0	1	0	9	18	0	1	13	25	32	1	5	22	6	1	1	0	0	1	0	0	0	5	1	1	0	2	0	2	2
B418	*Firmicutes Halanaerobiaceae Halanaerobium*	3	0	0	0	0	2	2	18	10	16	4	1	0	0	0	0	0	1	2	0	2	1	4	0	1	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B419	*Firmicutes Halanaerobiaceae Halanaerobium*	2	0	0	0	0	2	0	2	0	0	2	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	1	0	2	2	0	3	0	2	2	14	0	0	0	0	0	0	0	0	0	0	0	0	0
B420	*Firmicutes Halanaerobiaceae Halanaerobium*	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	2	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B421	*Firmicutes Halanaerobiaceae Halanaerobium*	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	5	5	4	0	0	0	0	0	0	0	0	0	0	0	0	0
B422	*Firmicutes Halanaerobiaceae Halanaerobium*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	32	9	0	0	0	0	0	2	0	18	42	11	0	0	0	0	0	0	0	0	0	0	0	0	0
B432	*Firmicutes Halanaerobiaceae Halanaerobacter lacunaru H. jeridensis H. salinarius*	0	80	27	0	7	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	1	0	0	0	0	0	54	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	17
B433	*Firmicutes Halobacteroidaceae Halonatrum saccharophilum*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	4	0	0	0	0	0	0	0	0	0	0	1	0	1
B446	*Cyanobacteria Family I*	0	0	0	1	0	26	21	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	8	50	0	0	0	0	0	0
B454	*Firmicutes Clostridia*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B455	*Firmicutes Clostridia Clostridiaceae 2*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	9	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B505	*Firmicutes Bacillaceae Bacillus mannanilyticus/B. horti*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	6	31	0	0	0	0	0	0	0	0	1	8	0	0	0	0	0	0	0	0	0	0	0	0	0
B539	*Proteobacteria Hydrogenophilaceae Thiobacillus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0
B557	*Proteobacteria Haliangiaceae Haliangium*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	24	0	0	0	0
B558	*Proteobacteria Haliangiaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	6	0	0	0	0

	Haliangium																																																		
B563	*Bacteroidetes Chitinophagaceae Gracilimonas*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	35	5	2	1	0	0	0	0	0
B606	*Proteobacteria Rhodobiaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
B656	*Bacteroidetes Cryomorphaceae Brumimicrobium glaciale*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	8	0	0	0	0	0	0	0
B658	*Bacteroidetes Order III*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	2	0	0	0	0	0	0	0
B675	*Cyanobacteria Family I Lyngbya*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0
B741	*Verrucomicrobia Puniceicoccaceae Opitutus terrae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1	0	0	0	0
B829	*Proteobacteria Rhodobacteraceae Roseinatronobacter monicus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	7	11	0	0	0	0	1	0	0	0	2	1	0	0	0	0	0	0	0	1	0	0	0	0	0
B861	*Proteobacteria Gallionellaceae Gallionella*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0

Table A 5.4 Heatmap of bacterial phyla detected in sediments and brines samples. C. d. = Candidate division. Light brown cells: sediment samples; white cells: brine samples. Red cells indicate zero while, yellow cells are low values (< 0.5) and green cells are the highest values.

Phylum	SP-IB1	SP-IB2	SP-IB3	SP-IB4	SP-IB5	SP-IB6	SP-IB7	SP-IB8	SP-VC1	SP-VC2	SP-AR1	SP-AR2	SP-CM1	SP-CM2	SP-CM3	SP-CM4	SP-CM5	SP-CM6	SP-CM7	SP-CN1	SP-CN2	SP-CN3	SP-CN4
Proteobacteria	44	13	56	90	52	35	28	32	64	67	22	25	87	81	7	15	30	17	21	90	47	9	41
Firmicutes	32	82	41	1	16	9	6	48	23	26	49	20	3	10	89	78	31	80	32	2	33	7	10
Bacteroidetes	9	2	2	3	28	10	8	7	3	3	9	16	1	2	2	1	34	1	35	4	16	14	8
Cyanobacteria	0	0	0	2	0	27	22	0	1	0	0	0	0	0	1	0	1	0	0	0	0	14	0
Lentisphaerae	6	1	1	1	0	14	14	1	1	0	11	1	0	0	0	0	1	1	7	0	2	0	8
Actinobacteria	0	0	0	2	2	0	0	0	4	2	0	0	8	5	0	5	0	0	1	4	0	2	5
Parcubacteria	6	0	0	0	0	3	8	10	0	1	8	19	0	0	0	0	1	0	1	0	0	4	5
Gemmatimonadetes	0	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	21	2
Chloroflexi	0	0	0	0	0	0	4	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
Rhodothermaeota	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	21	17
Aminicenantes	0	0	0	0	0	0	2	0	2	0	0	14	0	0	0	0	0	0	0	0	0	0	0
C. d. TM6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	1
Planctomycetes	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Deferribacteres	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microgenomates	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Spirochaetae	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Deinococcus-Thermus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0
Acidobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cloacimonetes	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Verrucomicrobia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Armatimonadetes	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlorobi	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hydrogenedentes	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. OP3	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2
Caldiserica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thermotogae	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Bkrii41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. SR1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Atribacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fibrobacteres	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Saccharibacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. OP8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nitrospirae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. KB1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tenericutes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BD1-5 Group	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria RF3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. WS6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elusimicrobia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. OP11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusobacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. TA06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. TM7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Synergistetes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Phylum	ARG1	ARG2	ARG3	ARG4	ARG5	ARG6	ARG7	ARG8	ARG9	ARG10	ARG11	ARG12	ARG13	ARG14	ARG15	ARG16	ARG17	ARG18	ARG19	ARG20	ARG21	ARG22	ARG23	CHL1	CHL3	CHL2	CHL4
Proteobacteria	22	19	42	39	27	8	34	35	60	36	41	6	13	20	58	33	17	37	40	31	25	34	69	27	84	47	25
Firmicutes	46	67	41	44	32	88	54	22	6	54	19	59	78	55	0	27	0	1	4	19	6	6	1	3	6	7	22
Bacteroidetes	29	11	6	4	17	2	8	17	25	3	33	13	3	14	8	17	80	27	47	23	15	51	10	0	7	0	45
Cyanobacteria	1	1	6	8	0	0	0	2	0	0	0	0	1	3	0	0	0	14	1	10	51	0	0	0	0	0	0
Lentisphaerae	0	0	0	0	12	1	1	9	0	6	6	2	1	3	0	4	0	0	1	1	0	0	0	0	0	0	0
Actinobacteria	0	0	3	3	0	0	0	0	6	0	0	4	1	2	9	1	0	1	0	1	0	1	3	14	1	6	1
Parcubacteria	2	0	0	0	6	0	1	5	1	0	0	2	2	0	1	1	2	0	0	2	0	0	0	4	0	12	4
Gemmatimonadetes	0	0	1	1	0	0	0	0	0	0	0	0	0	0	8	1	0	6	0	5	0	0	6	1	0	3	0
Chloroflexi	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	2	0	0	1	28	1	1	0
Rhodothermaeota	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aminicenantes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0
C. d. TM6	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	15	0	0	0	1	0	0	0	0	0	0	1
Planctomycetes	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	8	5	3	0	1	1	1	0	1	0
Deferribacteres	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	19	0
Microgenomates	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	12	1
Spirochaetae	0	0	0	0	1	0	0	2	0	0	0	3	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
Deinococcus-Thermus	0	0	2	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	5	0	0	0	0	0
Acidobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	6	0	0	0	0
Cloacimonetes	0	0	0	0	0	0	0	1	2	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Verrucomicrobia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	3	1	1	1	0	1	0	0	0	0
Armatimonadetes	0	0	0	0	2	0	0	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Chlorobi	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
Hydrogenedentes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0
C. d. OP3	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Caldiserica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Thermotogae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bkrii41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
C. d. SR1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Atribacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Fibrobacteres	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Saccharibacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. OP8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Nitrospirae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. KB1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tenericutes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
BD1-5 Group	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacteria RF3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. WS6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elusimicrobia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. OP11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusobacterium	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. TA06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. d. TM7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Synergistetes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

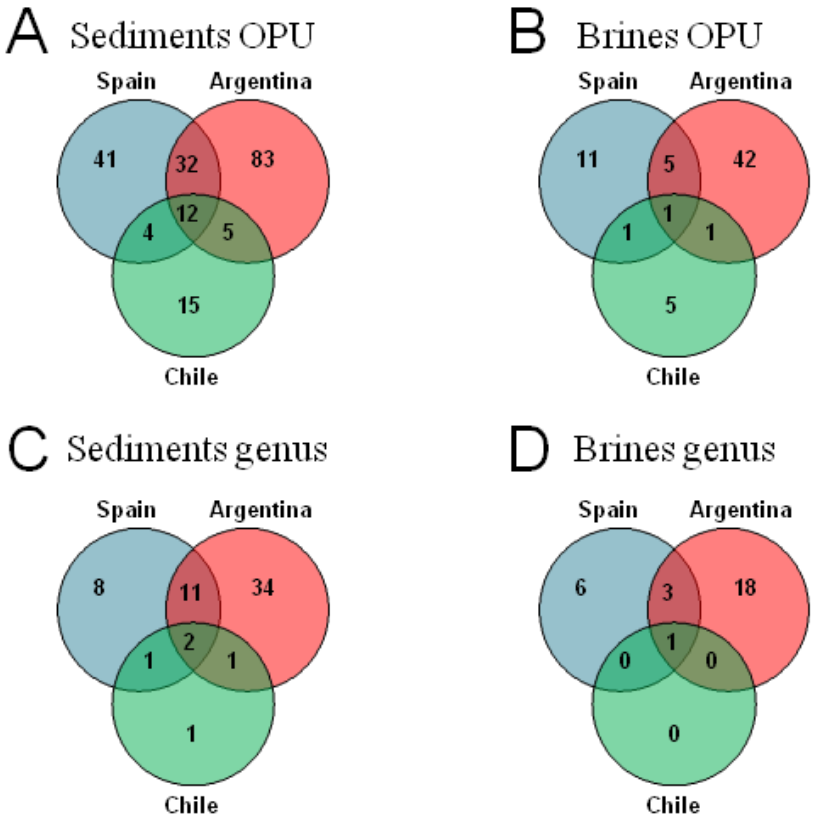


Figure A 5.4 Venn diagram of the distribution of exclusive bacterial OPUs (A, B) and genera (C, D) detected in sediments (A, C) and brines (B, D) per country.

Table A 5.5 Heatmap of the relative abundances of most (making >70% of the total sequences per sample) abundant archaeal OPUs referenced to total sequences for each sample and their taxonomic assignment. Light brown cells: sediments samples; white cells: brines samples. Red cells indicate zero while, yellow cells are low values (< 0.5) and green cells are the highest values.

OPU	*Taxonomic assignment*	SP-IB1	SP-IB2	SP-IB3	SP-IB4	SP-IB5	SP-IB6	SP-IB7	SP-IB8	SP-VC1	SP-VC2	SP-AR1	SP-AR2	SP-CM1	SP-CM2	SP-CM3	SP-CM4	SP-CM5	SP-CM6	SP-CM7	SP-CN1	SP-CN2	SP-CN3	SP-CN4	ARG1	ARG2	ARG3	ARG4	ARG5	ARG6	ARG7	ARG8	ARG9	ARG10	ARG11	ARG12	ARG13	ARG14	ARG15	ARG16	ARG17	ARG18	ARG19	ARG20	ARG21	ARG22	ARG23	CHL1	CHL3	CHL2	CHL4
A001	*Euryarchaeota Halobacteria Halobacteriaceae Halobacterium*	0	0	4	17	0	31	0	0	17	1	1	1	0	0	3	0	0	2	0	0	1	0	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	1	0	1	3	2	0	2	0
A002	*Euryarchaeota Halobacteria Halobacteriaceae Halobacterium*	1	0	0	3	0	2	0	0	0	4	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A003	*Euryarchaeota Halobacteria Halobacteriaceae Halobacterium*	9	0	5	1	0	2	6	0	5	7	8	3	0	2	2	0	0	4	1	0	6	0	2	8	6	0	0	12	0	0	1	0	1	0	0	0	0	5	0	0	0	6	0	1	2	0	0	0	0
A004	*Euryarchaeota Halobacteria Halobacteriaceae Halobacterium*	0	0	0	0	0	0	1	0	0	1	0	0	4	5	0	0	0	0	0	2	4	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A005	*Euryarchaeota Halobacteria Halobacteriaceae Halobacterium*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A006	*Euryarchaeota Halobacteria Halobacteriaceae Halobacterium*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	3	0	0	0	0	0	0	0	1	0	0	0	0	2	0	0	0	0	0
A009	*Euryarchaeota Halobacteria Halobacteriaceae Halobacterium*	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	9	15	0	0	0	0
A010	*Euryarchaeota Halobacteria Halobacteriaceae Halobonum*	0	0	0	0	1	0	0	0	0	0	0	0	22	25	0	0	0	1	0	0	0	15	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
A013	*Euryarchaeota Halobacteria Halobacteriaceae Halovenus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	12	1	0	1	1	1	0	0	0	0	0	1	0	1	1	1	1	4	3	2	1	0	5	1	0	0	0	5	0	2	0
A014	*Euryarchaeota Halobacteria Halobacteriaceae Halovenus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0
A020	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus*	0	0	0	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
A023	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
A024	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus*	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	2	0	0	0	1	0
A025	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus*	0	0	0	0	0	1	0	0	0	0	0	0	1	15	0	0	0	0	0	0	1	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0
A027	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	0	0	1	0	0	5	4	5	0	1	0	6	1	0	0	0	0	4	1	0	0	0	0	0
A028	*Euryarchaeota Halobacteria Halobacteriaceae Halapricum*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A031	*Euryarchaeota Halobacteria Halobacteriaceae Halomicroarcula*	0	2	0	0	0	0	7	0	0	0	0	1	0	0	11	22	1	1	1	4	0	0	4	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	2	0
A032	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus utahensis*	0	0	0	0	0	0	2	0	0	1	0	0	2	7	3	0	0	1	0	7	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
A033	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus*	6	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	3	0	0	0	0	58	1	0	1	7	0	1	0	0	0	0	1	0	2	0	0	0	0	0
A037	*Euryarchaeota Halobacteria Halobacteriaceae Halorhabdus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	8	0	0	0	0	0	0	3	21	0	3	1	7	0	0	5	0	0	0	0	0	0	0	0
A046	*Euryarchaeota Halobacteria Halobacteriaceae Halapricum*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A049	*Nanohaloarchaeota*	4	0	0	0	0	0	5	0	0	6	5	0	0	0	0	0	0	0	0	1	4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0	0	0	0	0
A053	*Euryarchaeota Halobacteria Halobacteriaceae Halorubrum*	0	0	0	0	29	0	0	0	0	0	0	0	0	0	2	0	15	5	13	0	0	1	0	17	23	2	77	10	61	60	3	71	15	23	38	61	27	55	2	1	14	34	20	13	2	0	0	17	0	19
A055	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1	0	0	0	0	1	0	0	4	4	0	2	0	6	0	0	2	5	0	1	0	0	0	0	0
A068	*Euryarchaeota Halobacteria Halobacteriaceae Halonotius/Halohasta*	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	1	6	0	0	0	1	0	1	5	3	2	0	0	0	0	0	0	0	0	0	0	0	3
A069	*Euryarchaeota Halobacteria Halobacteriaceae Halonotius pteroides*	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	2	2	0	0	0	0	0	0	1	0	0	10	0	6
A070	*Euryarchaeota Halobacteria Halobacteriaceae Halonotius*	0	0	0	0	12	0	0	0	0	0	9	0	0	3	0	0	6	1	15	0	0	0	2	1	1	0	0	1	1	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	9	1	18
A073	*Euryarchaeota Halobacteria Halobacteriaceae Halobellus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	4	0	0	0	7	20	2	10	0	1	0	0	0	0	0	4	0	0	16	13	0	4	0	0	0	0
A076	*Euryarchaeota Halobacteria Halobacteriaceae Halobellus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	10	1	0	1	3	0	0	0	0	0	0
A077	*Euryarchaeota Halobacteria Halobacteriaceae Halobellus*	0	0	0	0	1	0	0	0	0	0	0	0	0	24	0	12	1	0	4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	3	1	0	0	4	1	5
A078	*Euryarchaeota Halobacteria Halobacteriaceae Haloquadratum walsbyi*	0	0	0	2	0	1	0	1	0	0	1	0	0	0	0	0	0	0	0	3	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A082	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A085	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	8	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	3	0	0	0	0	0
A086	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	5	0	0	1	20	17	0	12	2	0	1	2	6	7	4	3	0	0	2	24	0	8	0	1	0	15
A090	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
A094	*Euryarchaeota Halobacteria Halobacteriaceae Halomicrobium*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	1	0	0	0	0
A095	*Euryarchaeota Halobacteria Halobacteriaceae Halomicrobium mukhataei*	0	0	0	0	11	0	0	0	2	0	1	0	0	0	1	0	7	1	3	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	10	25	2	0	0	2	2	1	2	0	7
A096	*Euryarchaeota Halobacteria Halobacteriaceae Halomicrobium zhouii*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	1
A097	*Euryarchaeota Halobacteria Halobacteriaceae Halosimplex carlsbadense*	0	0	0	0	0	0	0	0	0	0	0	0	21	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	7	0	0	0	0	0
A100	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	6	0	0	1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0
A101	*Euryarchaeota Halobacteria Halobacteriaceae Halorubellus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	53	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	0	0	1	0	0	0
A103	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
A104	*Euryarchaeota Halobacteria Halobacteriaceae Haloterrigena/Natronococcus*	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	7	2	0	0	0	0	0	45	6	2	0	5	1	0	0	0	5	0	0	0	0	1	0	0	0
A106	*Euryarchaeota Halobacteria Halobacteriaceae Natronomonas*	0	0	2	1	2	1	2	0	0	1	5	1	0	0	0	0	3	1	2	0	2	0	1	1	0	85	13	7	0	0	4	0	22	24	11	17	11	16	4	8	0	35	1	0	0	0	0	0	1	0
A107	*Euryarchaeota Halobacteria Halobacteriaceae Natronomonas*	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	2	0	1	4	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	6	1	0	0	0
A111	*Euryarchaeota Halobacteria Halobacteriaceae Haloarcula*	0	0	1	0	19	0	0	0	0	1	0	0	2	0	1	1	9	4	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	5	19	0	1	0	28	0	15
A116	*Euryarchaeota Halobacteria Halobacteriaceae Haloquadratum*	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
A118	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
A121	*Euryarchaeota Halobacteria Halobacteriaceae Haloplanus natans*	1	0	0	2	2	2	1	0	5	3	1	0	15	1	1	0	1	1	0	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	2	1	2	1

A123	*Euryarchaeota Halobacteria Halobacteriaceae Halomina*	0	0	19	0	0	0	1	0	0	0	0	0	3	0	7	0	2	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	4	3	1	0	0	0	3	5	0	0	0	2
A138	*Euryarchaeota Halobacteria Halobacteriaceae Halorubrum*	6	1	31	9	0	13	8	4	4	9	6	8	0	0	0	0	0	0	0	9	11	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	7	0
A141	*Euryarchaeota Halobacteria Halobacteriaceae*	15	8	1	0	0	0	4	0	0	4	1	0	0	0	8	0	0	5	0	0	6	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0
A148	*Deep Sea Euryarchaeotal Group (DSEG)*	0	0	2	54	0	27	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A151	*Deep Sea Euryarchaeotal Group (DSEG)*	0	0	0	0	0	0	5	4	0	6	5	0	0	0	0	0	0	0	0	0	8	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35	0
A166	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	1	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	1	0	0	0	0	0
A167	*Nanohaloarchaeota*	0	0	0	0	0	0	1	0	0	3	0	0	0	0	0	0	12	2	3	0	0	0	1	2	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	9	3	2
A169	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A172	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A176	*Nanohaloarchaeota*	1	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	1	0	1	1	4	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	1	1	11	2	0	0	0	0
A186	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	4	0	0	0	0	0
A187	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	2	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A188	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	4	0	0	0	0	2	0	0	0	0	0
A192	*Nanohaloarchaeota*	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0
A197	*Nanohaloarchaeota*	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	31	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A201	*Nanohaloarchaeota*	0	0	0	0	0	0	0	1	0	0	0	0	0	0	23	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A202	*Nanohaloarchaeota*	0	0	0	0	0	0	3	0	0	0	0	0	0	0	7	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A205	*Nanohaloarchaeota*	2	8	1	0	0	0	1	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	2	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0
A209	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	1	0	0	4	0	0	0	0	0
A210	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
A211	*Nanohaloarchaeota*	1	0	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
A226	*Nanohaloarchaeota*	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	1	0	0	0	1	0	0	0	0	0	0	0	2	0	7	6	5	0	0	1	0	0	0	0	0	0	0	0	0	0
A230	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	15	0	2	0	1	0	1	2	6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	10	0	1	0	0
A231	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	7	0	1	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	8	0	8	0	7	0	1
A238	*Nanohaloarchaeota*	0	21	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A239	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	12	0	0	0	0	0
A249	*Nanohaloarchaeota*	16	4	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
A251	*Nanohaloarchaeota*	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	0	4	0	8	0	0	0	1	14	9	0	0	1	0	0	0	0	0	1	0	0	0	0	5	19	0	0	8	1	3	4	0	0	0	0
A253	*Euryarchaeota Thermoplasmata Marine Benthic Group D and DHVEG-1*	0	1	0	0	0	0	1	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A256	*Euryarchaeota Thermoplasmata Marine Benthic Group D and DHVEG-1*	1	10	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A261	*MSBL1*	1	1	0	2	0	2	2	0	0	8	0	0	0	0	0	0	0	8	0	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
A272	*Deep Sea Hydrothermal Vent Group 6 (DHVEG 6)*	2	4	1	1	0	2	0	0	5	2	2	1	0	0	2	2	0	6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A277	*Thaumarchaeota Marine Benthic Group B*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0	0	0
A279	*Thaumarchaeota Marine Benthic Group B*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0
A283	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0
A284	*Euryarchaeota Methanomicrobia ST-12K10A*	0	2	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
A286	*Euryarchaeota Methanosarcinales Methanosarcinaceae Methanohalobium evestigatum/M. halophilus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	47	0	0	0	0	0	0	0	0	0
A289	*Euryarchaeota Methanomicrobia group C*	1	0	0	0	0	1	0	2	0	13	0	27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	4	0
A291	*Euryarchaeota Methanomicrobia group C*	0	0	0	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A293	*Euryarchaeota Methanomicrobia group C*	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
A294	*Euryarchaeota Methanomicrobia group C*	0	0	0	0	0	0	0	0	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
A296	*Euryarchaeota*	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	Methanomicrobia group C																																																		
A298	*Euryarchaeota Halobacteria Halobacteriaceae Halovenus*	0	0	0	2	0	3	2	0	0	0	0	0	0	0	11	6	2	2	3	0	0	0	2	1	0	0	0	1	0	0	0	0	0	0	1	0	2	0	0	2	0	1	0	0	1	2	0	0	2	1
A300	*Euryarchaeota Halobacteria Halobacteriaceae Halorubrum*	1	0	0	0	0	0	1	0	0	2	1	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	2	0	0	6	9	0	0	0	2	1	1	0	0	0	0	1	0
A308	*Euryarchaeota Methanomicrobia group C*	1	0	0	0	0	0	1	1	0	6	1	0	0	0	0	0	0	0	0	8	1	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0
A311	*Euryarchaeota Halobacteria Halobacteriaceae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	2	0	0	0	0	0
A312	*Euryarchaeota Halobacteria Halobacteriaceae Haloquadratum*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1	1	2	2	0	0	1	1	0	0	1	0	0	0
A313	*Euryarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	3	0	0	0	0
A316	*Nanohaloarchaeota*	0	0	8	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0
A324	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	1	1	4	0	0	0	0	0	0	0	0	6	3	0	2	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
A325	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	2	0	0	0	24	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A326	*Nanohaloarchaeota*	1	0	1	0	0	0	2	36	0	0	3	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A329	*Nanohaloarchaeota*	3	9	0	0	0	0	0	3	0	0	7	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A338	*Nanohaloarchaeota*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0

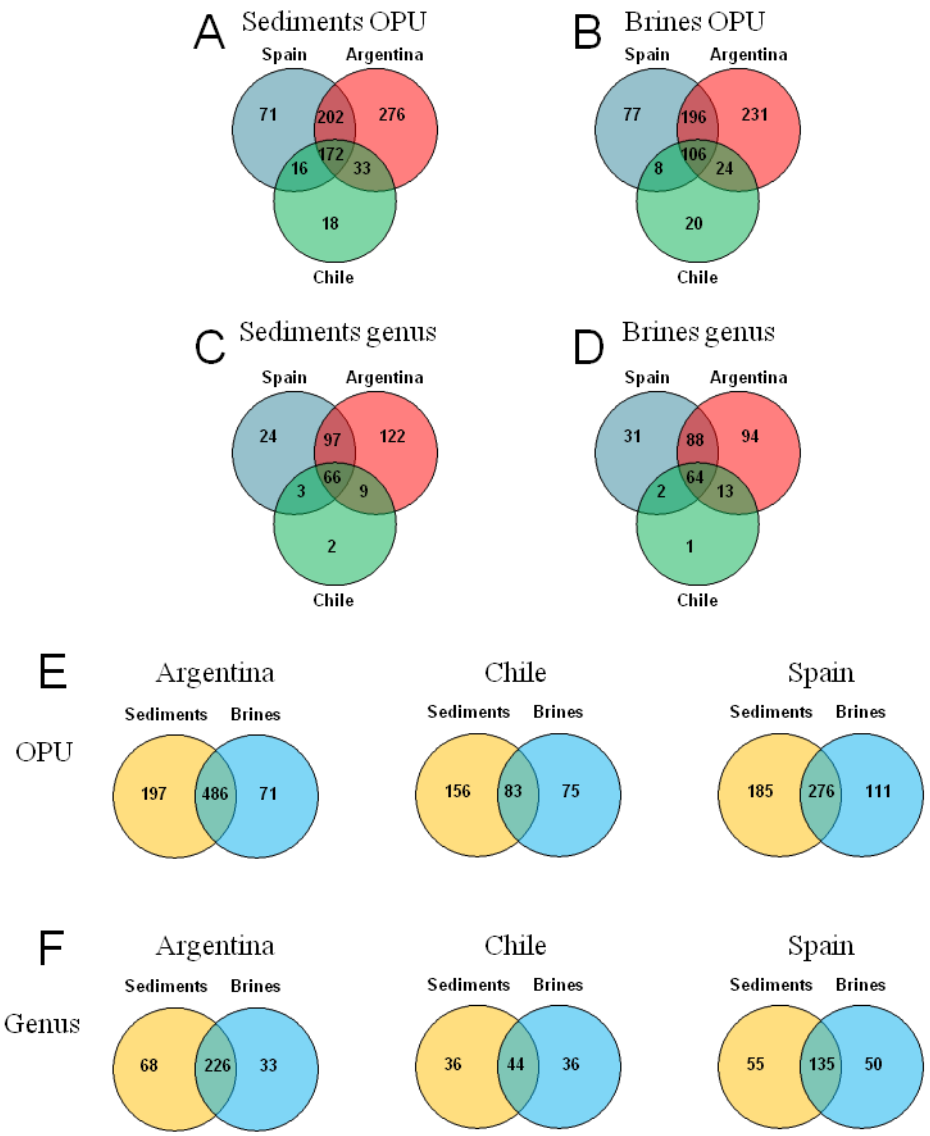


Figure A 5.5 Venn diagram of archaeal taxa detected in sediments (A, C) and brines (B, D) samples at OPU (A, B) and genus (C, D) level. Distribution of OPUs (E) and genera (F) in each country by type of sample (sediments and brines).

Table A 5.6 Heatmap of the most abundant archaeal phyla detected in sediments and brines samples. Light brown cells: sediments samples; white cells: brines samples. Red cells indicate zero while, yellow cells are low values (< 0.5) and green cells are the highest values.

Phylum	SP-IB1	SP-IB2	SP-IB3	SP-IB4	SP-IB5	SP-IB6	SP-IB7	SP-IB8	SP-VC1	SP-VC2	SP-AR1	SP-AR2	SP-CM1	SP-CM2	SP-CM3	SP-CM4	SP-CM5	SP-CM6	SP-CM7	SP-CN1	SP-CN2	SP-CN3	SP-CN4	ARG1	ARG2	ARG3	ARG4	ARG5	ARG6	ARG7	ARG8	ARG9	ARG10	ARG11	ARG12	ARG13	ARG14	ARG15	ARG16	ARG17	ARG18	ARG19	ARG20	ARG21	ARG22	ARG23	CHL1	CHL3	CHL2	CHL4
Euryarchaeota	56	35	79	43	97	68	51	40	76	73	52	48	100	100	62	95	58	49	61	85	60	46	75	71	53	99	100	55	99	99	90	100	97	96	91	88	92	100	65	54	96	98	82	87	50	64	32	82	55	95
Nanohaloarchaeota	41	60	18	0	3	1	41	51	1	11	41	43	0	0	37	2	42	37	39	12	26	53	22	29	47	1	0	44	1	1	10	0	3	4	9	12	8	0	35	46	4	2	18	13	50	35	13	18	8	5
Deep Sea Euryarchaeotal Group (DSEG)	0	0	2	55	0	28	5	7	0	6	5	0	0	0	0	0	0	0	0	0	8	1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	35	0
Thaumarchaeota	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	50	0	0	0
MSBL1	1	1	0	2	0	2	2	0	0	8	0	0	0	0	0	0	0	8	0	2	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Deep Sea Hydrothermal Vent Group 6 (DHVEG 6)	2	4	0	1	0	2	0	1	5	2	2	1	0	0	2	2	0	6	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miscellaneous Crenarchaeotic Group (MCG)	0	0	0	0	1	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	0	0
Marine Hydrothermal Vent Group (MHVG)	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Miscellaneous Euryarchaeotic Group (MEG)	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Diapherotrites	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SM1K20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Deep Sea Hydrothermal Vent Group 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KTK 28A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

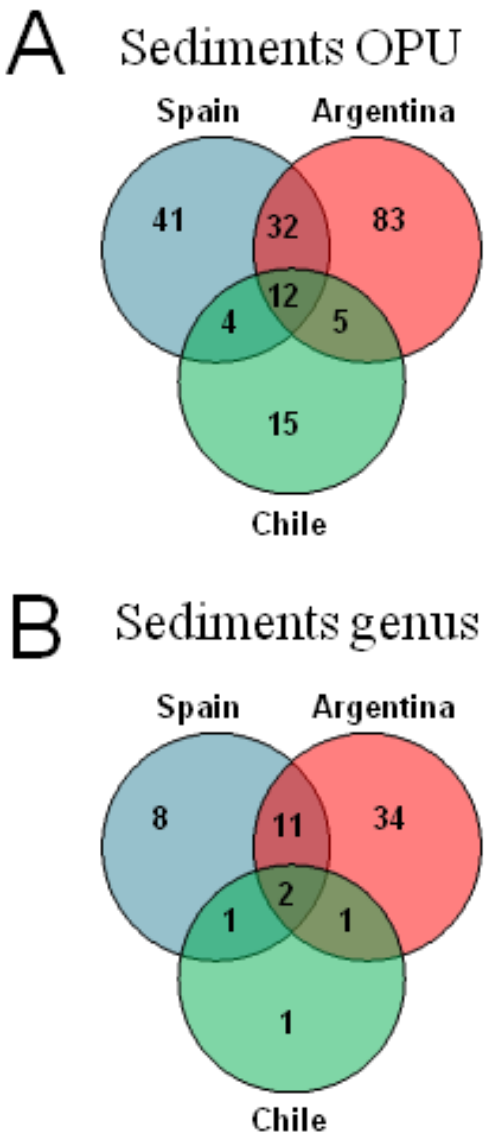


Figure A 5.6 Venn diagram of the distribution of exclusive archaeal OPUs (A) and genera (B) detected in sediments per country.

Table A 5.7 Similarity Percentage analysis for Bacteria identifying which Operation Phylogenetic Units (OPUs) contribute most strongly for differences by country. Contribution of each OPU toward dissimilarity (average dissimilarity and contribution %) and mean abundances per country are showed. Arg. denotes Argentina.

Sediments							Brines						
OPU	Av. dissim.	Contrib. %	Accum. %	Arg.	Chile	Spain	OPU	Av. dissim.	Contrib. %	Accum. %	Arg.	Chile	Spain
B026	3.7	4.2	13.7	0.0	0.0	8.1	B413	4.6	5.2	16.8	2.9	0.4	13.9
B432	2.6	2.9	16.6	0.1	0.0	5.6	B432	4.6	5.2	22.0	5.0	11.0	2.6
B416	2.6	2.9	19.4	5.9	0.1	2.3	B127	4.5	5.0	27.0	0.0	19.4	0.2
B422	2.4	2.7	22.2	5.7	0.0	0.1	B059	3.6	4.0	31.0	0.0	15.6	0.0
B219	2.4	2.6	24.8	0.5	0.0	5.1	B417	3.5	3.9	34.9	7.4	1.1	3.3
B417	2.2	2.5	27.3	4.9	2.2	0.9	B293	2.8	3.1	38.0	5.9	0.0	0.5
B247	2.0	2.2	29.5	0.0	18.1	0.0	B269	2.6	2.9	41.0	1.6	0.6	7.0
B269	1.9	2.1	31.6	4.5	0.0	0.0	B446	2.0	2.2	43.2	4.3	0.0	0.1
B089	1.9	2.1	33.7	4.4	0.0	0.1	B422	1.9	2.2	45.3	4.3	0.0	0.0
B001	1.7	2.0	35.7	0.1	0.0	3.8	B416	1.8	2.0	47.4	3.5	0.6	2.7
B446	1.7	1.9	37.6	1.0	0.0	3.1	B090	1.6	1.8	49.2	0.0	7.0	0.0
B263	1.5	1.7	39.3	2.9	0.0	1.7	B092	1.6	1.8	50.9	0.3	0.4	4.9
B360	1.4	1.6	40.9	0.0	9.9	0.9	B563	1.4	1.6	52.5	3.1	0.0	0.0
B418	1.4	1.6	42.5	0.3	0.2	3.1	B505	1.3	1.5	54.0	2.9	0.0	0.0
B313	1.4	1.5	44.0	3.0	0.1	0.6	B222	1.3	1.5	55.5	2.7	0.0	0.5
B264	1.4	1.5	45.6	2.3	0.0	1.6	B243	1.0	1.2	56.6	0.8	1.5	1.9
B040	1.4	1.5	47.1	0.1	0.0	3.0	B040	1.0	1.1	57.7	0.1	4.1	0.1
B005	1.4	1.5	48.6	0.1	0.0	2.9	B180	1.0	1.1	58.8	1.6	1.0	1.3
B399	1.2	1.4	50.0	0.0	10.6	0.1	B106	0.9	1.0	59.8	0.8	0.0	2.7
B222	1.1	1.3	51.2	1.8	0.1	1.4	B052	0.9	1.0	60.8	0.9	0.0	2.5
B216	1.1	1.2	52.4	0.0	9.7	0.0	B557	0.9	1.0	61.8	2.0	0.0	0.0
B232	1.1	1.2	53.6	1.9	1.1	0.9	B117	0.9	1.0	62.8	0.3	0.0	2.6
B331	1.0	1.1	54.7	1.5	0.0	1.2	B063	0.8	0.9	63.8	1.7	0.0	0.5
B220	1.0	1.1	55.8	0.3	0.0	2.1	B029	0.8	0.9	64.7	1.7	0.0	0.0
B419	0.9	1.1	56.8	2.0	0.3	0.5	B297	0.8	0.9	65.5	1.7	0.0	0.0
B180	0.9	1.0	57.9	1.7	0.1	1.1	B091	0.7	0.8	66.4	0.0	0.0	2.3
B351	0.8	0.9	58.8	0.4	1.6	1.1	B315	0.7	0.8	67.2	1.6	0.0	0.0
B398	0.8	0.9	59.7	1.6	0.0	0.4	B141	0.7	0.8	68.0	1.4	0.0	0.3
B034	0.8	0.9	60.5	0.0	0.0	1.7	B331	0.7	0.8	68.8	0.1	0.1	2.2
B318	0.8	0.8	61.4	0.0	0.0	1.6	B317	0.7	0.7	69.5	1.4	0.0	0.2
B200	0.7	0.8	62.2	0.4	0.7	1.4	B089	0.6	0.7	70.2	1.4	0.0	0.0
B392	0.7	0.8	62.9	0.0	6.3	0.0	B264	0.6	0.7	70.9	0.9	0.1	1.1
B087	0.7	0.8	63.7	1.6	0.0	0.0	B015	0.6	0.7	71.6	1.3	0.0	0.0
B505	0.7	0.8	64.5	1.6	0.0	0.0	B829	0.6	0.7	72.3	1.3	0.0	0.2
B207	0.6	0.7	65.2	0.0	5.6	0.1	B087	0.6	0.6	72.9	1.2	0.1	0.4
B656	0.6	0.7	65.9	1.5	0.0	0.0	B005	0.6	0.6	73.5	0.3	0.1	1.6
B454	0.6	0.7	66.6	1.5	0.1	0.0	B355	0.5	0.6	74.1	1.1	0.0	0.0
B194	0.6	0.7	67.3	0.5	0.0	1.2	B244	0.5	0.5	74.7	0.2	0.9	1.0
B027	0.6	0.6	68.0	0.0	0.0	1.3	B420	0.4	0.5	75.2	0.9	0.0	0.1
B093	0.6	0.6	68.6	1.2	0.0	0.2	B194	0.4	0.5	75.7	0.4	1.3	0.3
B675	0.5	0.6	69.2	1.3	0.0	0.0	B294	0.4	0.5	76.2	0.9	0.2	0.0
B414	0.5	0.6	69.8	0.1	0.1	1.2	B433	0.4	0.5	76.6	0.1	1.0	0.9
B327	0.5	0.6	70.4	1.2	0.0	0.2	B116	0.4	0.5	77.1	0.9	0.0	0.0

Table A 5.8 Similar Percentage (SIMPER) analysis for Bacteria identifying which genera contribute most strongly for differences by country. Contribution of each genera toward dissimilarity (average dissimilarity and contribution %) and mean abundances per country are showed.

Sediments							Brines						
Genus	**Av. dissim.**	**Contrib. %**	**Accum. %**	**Argentina**	**Chile**	**Spain**	**Genus**	**Av. dissim.**	**Contrib. %**	**Accum. %**	**Argentina**	**Chile**	**Spain**
Halanaerobium	17.7	22.4	22.4	27.7	4.5	24.6	*Halanaerobium*	16.7	20.1	20.1	20.1	2.2	21.0
Acinetobacter	7.2	9.1	31.5	0.1	0.0	12.4	*Halanaerobacter*	7.9	9.5	29.6	5.0	11.0	2.6
Desulfovermiculus	6.2	7.9	39.3	3.5	0.3	9.1	*Idiomarina*	6.5	7.8	37.4	0.0	16.9	0.1
Halanaerobacter	3.2	4.1	43.4	0.1	0.0	5.6	*Psychroflexus*	4.1	4.9	42.3	1.6	0.6	7.0
Syntrophobacter	3.1	3.9	47.4	0.0	18.1	0.0	*Spiribacter*	3.9	4.7	46.9	0.4	0.4	7.9
Psychroflexus	2.7	3.4	50.8	4.5	0.0	0.0	*Gracilimonas*	3.7	4.4	51.4	4.6	0.4	0.3
Halorhodospira	2.4	3.0	53.8	4.4	0.0	0.1	*Desulfovermiculus*	2.3	2.8	54.2	3.1	0.0	1.3
Comamonas	2.3	2.9	56.7	0.1	0.0	3.9	*Rhodovibrio*	2.2	2.7	56.8	2.1	1.1	1.3
Desulfosalsimonas	1.8	2.3	59.0	2.1	1.4	1.1	*Bacillus*	1.9	2.3	59.1	3.0	0.0	0.1
Pseudomonas	1.7	2.2	61.2	0.1	0.0	3.0	*Haliangium*	1.8	2.1	61.3	2.6	0.0	0.0
Diaphorobacter	1.7	2.1	63.3	0.1	0.0	2.9	*Hahellaceae*	1.7	2.0	63.2	1.7	0.0	0.5
Flexistipes	1.6	2.0	65.3	0.0	9.7	0.0	*Pseudomonas*	1.6	1.9	65.2	0.1	4.1	0.1
Rhodovibrio	1.5	1.9	67.2	1.9	0.1	1.2	*Acinetobacter*	1.6	1.9	67.1	2.0	0.1	0.8

Table A 5.9 Similar Percentage (SIMPER) analysis for Archaea identifying which genera contribute most strongly for differences by country. Contribution of each genera toward dissimilarity (average dissimilarity and contribution %) and mean abundances per country are showed.

Sediments							Brines						
Genus	Av. dissim.	Contrib. %	Accum. %	Argentina	Chile	Spain	Genus	Av. dissim.	Contrib. %	Accum. %	Argentina	Chile	Spain
Halorubrum	8.6	11.5	11.5	14.9	4.5	6.9	*Halorubrum*	17.74	28.59	28.59	41	18.4	18.9
Halorhabdus	7.7	10.4	21.9	13.2	1.5	4.5	*Halonotius*	11.05	17.8	46.39	1.97	23.7	16.1
Natronomonas	7.5	10.1	32.0	13.2	0.7	1.2	*Haloarcula*	9.16	14.76	61.14	1.76	21.6	10.2
Halobacterium	6.1	8.3	40.3	6.0	2.7	9.3	*Natronomonas*	5.868	9.455	70.6	10.1	0.0299	5.89
Methanomicrobia Group C	5.7	7.7	47.9	0.4	9.0	6.7	*Halomicrobium*	4.234	6.822	77.42	1.37	8.49	7.27
Marine Benthic Group B	4.2	5.6	53.6	0.0	25.2	0.3	*Halobellus*	3.827	6.166	83.59	6.69	4.14	3.65
Haloterrigena	3.4	4.6	58.2	5.4	0.5	0.6	*Halorhabdus*	2.271	3.659	87.25	4.37	0.383	0.893
Halobellus	3.2	4.3	62.5	3.4	0.4	2.4	*Halobacterium*	1.916	3.087	90.33	2.53	0.125	0.491
Halobonum	2.6	3.5	66.0	0.5	0.1	3.7	*Halovenus*	1.626	2.621	92.95	1.99	0.547	2.96
Methanohalobium	2.4	3.3	69.2	4.3	0.0	0.0	*Halomina*	0.8169	1.316	94.27	0.734	0.972	0.743
Halovenus	2.4	3.231	72.46	1.71	4.41	2.81	*Haloterrigena*	0.6563	1.057	95.33	1.17	0	0.0294

Table A 5.10 Similar Percentage (SIMPER) analysis for Archaea identifying which Operation Phylogenetic Units (OPUs) contribute most strongly for differences by country. Contribution of each OPU toward dissimilarity (average dissimilarity and contribution %) and mean abundances per country are showed.

Sediments							Brines						
OPU	Av. dissim.	Contrib. %	Accum. %	Arg.	Chile	Spain	OPU	Av. dissim.	Contrib. %	Accum. %	Arg.	Chile	Spain
A053	5.8	6.3	6.3	13.6	0	0.4	A053	13.3	18.5	18.5	39.8	18.4	18.9
A106	5.5	6	12.3	13.1	0.3	0.8	A111	7	9.7	28.3	1.8	21.6	10.2
A033	2.9	3.2	15.5	6.4	0	0.7	A070	5.7	8	36.2	0.2	13.7	11.2
A138	2.9	3.2	18.7	0	4.2	6.1	A106	4.4	6.1	42.3	9.5	0	2.3
A104	2.5	2.8	21.4	5.4	0.5	0.6	A086	4.2	5.8	48.2	8.3	7.5	0
A151	2.5	2.8	24.2	0	17.7	1.5	A095	2.7	3.7	51.9	1.3	4.6	7
A148	2	2.2	26.4	0	0	4.4	A167	2.5	3.5	55.4	1.2	5.5	5
A001	2	2.1	28.5	0.8	2.1	4	A069	2.4	3.4	58.8	0.4	8.3	3
A286	1.8	2	30.5	4.3	0	0	A073	2.4	3.3	62.1	5.3	0	0
A326	1.8	2	32.5	0	0	3.9	A230	2.2	3.1	65.2	1.4	0.8	5.9
A003	1.8	1.9	34.4	3	0	3.1	A251	2	2.7	68	2.8	0.1	3.9
A010	1.6	1.8	36.1	0.2	0.1	3.4	A231	1.7	2.4	70.3	1.7	4	2.6
A141	1.5	1.6	37.8	0.2	3.9	2.6	A077	1.3	1.8	72.1	0.3	4.1	1.9
A101	1.4	1.5	39.2	0.3	0.3	2.7	A107	1.2	1.7	73.9	0.6	0	3.6
A277	1.3	1.5	40.7	0	12.3	0	A068	1	1.4	75.3	1.4	1.8	1.9
A031	1.3	1.4	42.1	0.4	1.2	2.7	A096	0.9	1.3	76.6	0	3.9	0.2
A037	1.3	1.4	43.5	2.9	0.1	0.1	A076	0.9	1.3	77.8	1.1	0	1.8
A095	1.2	1.3	44.8	2.6	0.7	0.3	A226	0.9	1.2	79	0.6	0	2.4
A289	1.2	1.3	46.2	0	2.3	2.2	A049	0.7	0.9	80	1.5	0	0.1
A325	1.2	1.3	47.5	2.5	0	0.4	A037	0.7	0.9	80.9	1.5	0	0.1
A251	1.2	1.3	48.8	2.7	0	0.2	A013	0.6	0.8	81.7	1	0.2	1.2
A279	1.1	1.2	50	0	10.2	0	A009	0.6	0.8	82.6	1.3	0	0
A073	1.1	1.2	51.2	2.6	0	0	A123	0.6	0.8	83.3	0.7	1	0.7
A308	1.1	1.1	52.4	0	4.3	1.5	A172	0.6	0.8	84.1	0.1	0.1	1.7
A097	1	1.1	53.5	0.7	0.1	1.8	A104	0.5	0.7	84.9	1.2	0	0
A077	1	1.1	54.6	0.5	0.3	1.9	A298	0.5	0.7	85.6	0.5	0.4	1.6
A027	1	1.1	55.7	2.3	0	0	A300	0.5	0.7	86.3	1.1	0.1	0
A123	1	1.1	56.8	0.7	0.1	1.7	A023	0.5	0.7	87	1.1	0	0
A055	0.9	1	57.8	2.1	0	0	A176	0.5	0.7	87.7	0.5	0.2	1.4
A121	0.9	1	58.8	0.1	1.6	1.9	A330	0.5	0.6	88.4	1	0	0.1
A176	0.9	1	59.7	2	0	0.2	A121	0.4	0.6	89	0.1	0.9	1
A013	0.9	1	60.7	0.8	3.6	0.8	A338	0.4	0.5	89.5	0.8	0	0
A205	0.8	0.9	61.6	1.2	0	1.1	A166	0.4	0.5	90	0.2	0	1.1
A197	0.8	0.9	62.5	0.1	0	1.7	A003	0.4	0.5	90.6	0.7	0	0.3
A086	0.8	0.8	63.3	1.8	0	0.1	A027	0.3	0.4	91	0.7	0	0
A261	0.7	0.8	64.1	0	0.6	1.6	A090	0.3	0.4	91.4	0.6	0	0.1
A298	0.7	0.8	64.9	0.5	0.8	1.5	A055	0.2	0.3	91.7	0.6	0	0.1
A283	0.7	0.8	65.7	0	6.6	0	A031	0.2	0.3	92.1	0.2	0	0.7
A272	0.7	0.7	66.4	0.1	0	1.5	A131	0.2	0.3	92.4	0.5	0	0
A131	0.6	0.7	67.2	0.8	0	1	A188	0.2	0.3	92.7	0.4	0.1	0.2
A201	0.6	0.7	67.9	0	0	1.4	A312	0.2	0.3	93.1	0.4	0.1	0.4
A049	0.6	0.7	68.5	0	0	1.3	A118	0.2	0.3	93.3	0.3	0	0.2
A249	0.6	0.6	69.2	0.2	0	1.2	A001	0.2	0.3	93.6	0.4	0	0.1
A209	0.6	0.6	69.8	1.2	0	0.3	A169	0.2	0.3	93.9	0	0.1	0.6

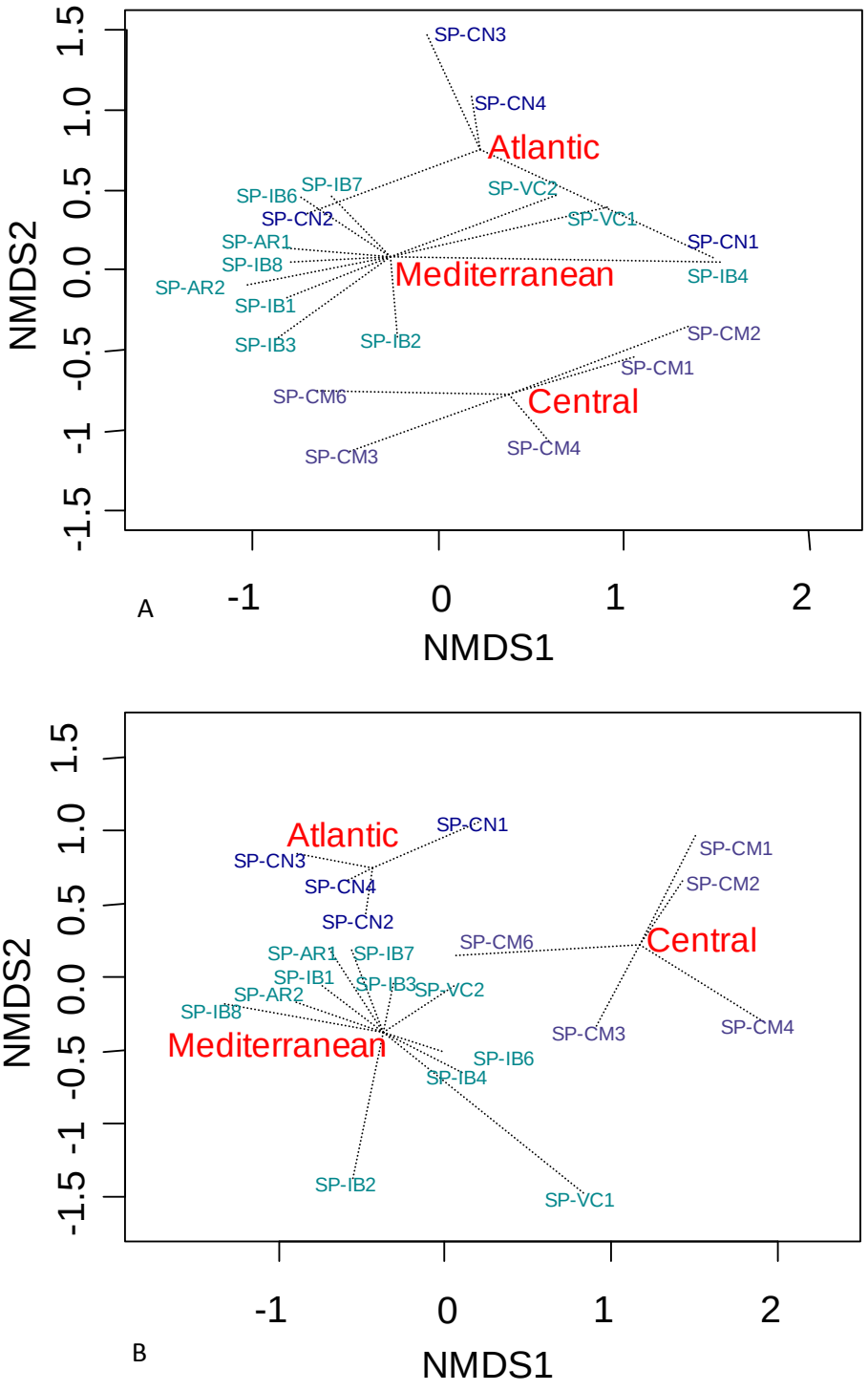


Figure A 5.7 Two dimensional Non-Metric Multidimensional Scaling (NMDS) of bacterial (A) and archaeal (B) communities of Spanish sediments.

Table A 5.11 Whittaker beta diversity for Bacteria (x10). Light brown cells: sediments samples; white cells: brines samples. The increment of blue tonality reflect the increment of values.

	ARG1	ARG2	ARG3	ARG4	ARG5	ARG6	ARG7	ARG8	ARG9	ARG10	ARG11	ARG12	ARG13	ARG14	ARG15	ARG16	ARG17	ARG18	ARG19	ARG20	ARG21	ARG22	ARG23	CHL1	CHL2	CHL3	CHL4	SP-AR1	SP-AR2	SP-CM1	SP-CM2	SP-CM3	SP-CM4	SP-CM5	SP-CM6	SP-CM7	SP-CN1	SP-CN2	SP-CN3	SP-CN4	SP-IB1	SP-IB2	SP-IB3	SP-IB4	SP-IB5	SP-IB6	SP-IB7	SP-IB8	SP-VC1	SP-VC2
ARG1	0																																																
ARG2	4	0																																																
ARG3	6	6	0																																															
ARG4	7	6	4	0																																														
ARG5	4	4	6	6	0	0																																											
ARG6	5	5	7	7	5	0																																											
ARG7	5	4	6	6	4	4	0																																										
ARG8	5	4	6	6	4	6	5	0																																									
ARG9	7	7	8	8	8	8	7	8	0																																								
ARG10	5	5	6	6	6	5	5	5	7	0																																							
ARG11	5	5	7	7	5	5	5	5	7	5	0																																						
ARG12	6	5	5	5	5	6	5	6	8	6	6	0																																					
ARG13	6	5	6	5	6	6	6	6	7	5	6	5	0																																				
ARG14	6	6	4	4	6	6	6	6	7	5	6	5	5	0																																			
ARG15	9	9	8	8	9	9	9	9	9	9	9	8	8	8	0																																		
ARG16	6	6	5	5	5	7	6	5	8	6	6	6	6	6	8	0																																	
ARG17	7	7	8	8	7	7	8	7	7	7	7	8	7	8	9	8	0																															
ARG18	6	6	6	6	7	8	7	6	8	6	7	7	6	7	8	6	7	0																														
ARG19	6	6	7	6	6	7	7	6	7	6	6	6	6	7	8	6	6	5	0																													
ARG20	5	5	6	7	6	6	6	5	8	6	6	6	6	7	9	4	8	4	6	0																												
ARG21	6	5	6	6	6	7	6	5	8	6	6	6	6	6	9	5	7	5	5	4	0																											
ARG22	6	5	5	6	5	7	6	5	8	6	6	6	6	6	8	5	8	6	6	5	5	0																										
ARG23	9	9	7	7	8	9	8	8	9	8	9	8	8	8	5	7	9	8	8	8	8	7	0																									
CHL1	7	6	7	7	6	7	6	6	9	7	7	6	7	7	8	6	8	6	7	6	6	6	8	0																								
CHL2	7	6	7	8	6	7	7	6	9	7	7	6	8	7	9	7	9	7	8	6	7	6	8	4	0																							
CHL3	7	7	7	7	7	7	7	7	8	7	7	7	7	7	8	7	7	7	7	7	6	7	9	7	7	0																						
CHL4	7	7	8	8	7	7	7	7	8	7	6	8	7	8	9	7	7	7	7	7	7	7	9	8	7	6	0																					
SP-AR1	6	5	7	8	5	6	6	5	9	6	6	7	7	7	10	6	7	7	7	6	6	6	9	6	6	7	7	0																				
SP-AR2	6	5	7	8	5	6	5	5	8	6	6	7	7	7	9	6	8	7	7	6	6	6	9	5	5	7	6	4	0																			
SP-CM1	8	7	8	8	8	7	7	8	7	7	7	8	7	7	8	8	7	7	7	8	8	8	8	8	8	8	7	8	8	0																	
SP-CM2	8	8	8	8	8	8	8	9	7	7	8	8	7	8	8	8	8	8	7	9	8	8	9	9	8	8	8	9	8	5	0																
SP-CM3	6	6	8	8	7	7	7	7	8	7	7	7	7	7	9	7	8	7	7	7	7	7	9	7	7	7	8	7	7	7	8	0															
SP-CM4	7	7	8	8	8	7	7	8	7	7	6	8	7	7	9	8	8	7	8	8	8	8	9	8	8	7	7	8	7	6	7	6	0														
SP-CM5	6	5	5	5	5	7	6	5	8	5	6	6	6	5	8	5	8	6	7	5	5	4	8	6	7	6	7	6	6	7	8	6	7	0												
SP-CM6	5	5	6	7	5	7	6	5	7	5	6	6	6	6	9	6	8	7	6	6	6	6	8	6	7	7	7	6	6	6	7	6	7	5	0											
SP-CM7	5	5	6	7	5	6	5	5	7	5	5	6	5	6	9	6	7	6	6	5	6	6	9	7	7	6	6	6	6	7	7	6	6	4	4	0										
SP-CN1	9	9	9	9	9	9	9	8	8	8	9	9	8	9	8	9	8	9	9	10	9	9	9	9	9	8	8	9	9	6	6	9	7	9	8	8	0									
SP-CN2	6	6	7	8	6	6	6	5	8	6	6	7	7	7	10	6	7	7	7	6	7	7	9	7	6	7	6	4	5	7	8	7	7	6	6	6	9	0								
SP-CN3	7	8	8	8	8	8	8	7	9	7	8	8	8	8	9	7	7	8	7	8	8	8	9	8	8	8	8	7	8	8	7	8	8	8	8	7	9	6	0							
SP-CN4	8	8	8	8	8	8	8	7	8	7	7	8	8	8	9	8	7	8	8	8	8	8	9	8	8	8	7	7	7	7	7	8	8	8	7	7	8	6	5	0						
SP-IB1	6	5	7	8	5	6	6	4	8	6	6	7	7	7	9	6	7	6	7	6	6	6	9	6	6	7	7	4	4	8	8	7	8	6	6	6	9	4	8	7	0					
SP-IB2	6	6	8	8	6	6	6	6	8	6	6	7	7	8	9	7	8	7	7	7	7	7	9	7	7	7	7	6	6	7	7	6	7	7	6	6	7	6	8	7	5	0				
SP-IB3	5	5	7	8	5	6	6	5	8	6	6	7	6	7	9	6	8	7	7	6	7	6	9	6	6	6	7	4	5	8	8	6	7	5	5	5	8	4	8	7	4	5	0			
SP-IB4	9	9	9	9	9	9	9	8	8	9	9	8	8	9	9	8	8	9	8	9	9	9	9	9	8	8	8	9	9	7	6	9	7	9	8	8	4	8	9	8	9	6	8	0		
SP-IB5	5	5	7	7	6	6	6	6	7	5	6	7	5	6	9	6	7	6	5	6	5	6	9	7	8	7	6	5	6	7	7	7	6	6	5	4	8	6	8	7	6	6	6	8	0	
SP-IB6	6	6	7	8	5	5	5	5	8	6	6	7	7	7	10	6	6	7	7	6	7	7	9	7	7	8	7	4	5	8	9	7	8	7	7	5	9	3	7	6	4	6	5	9	6	0
SP-IB7	6	6	8	8	6	6	6	5	8	7	6	7	7	7	9	7	8	7	7	6	7	8	9	6	6	7	7	5	5	7	8	7	8	7	6	6	8	5	7	7	5	5	5	7	7	5
SP-IB8	5	5	7	8	5	6	6	4	8	6	5	7	7	7	10	6	7	7	7	6	6	6	9	6	6	7	6	4	5	8	9	7	8	6	5	5	9	4	7	7	4	6	4	9	6	4
SP-VC1	9	8	9	8	8	8	8	8	8	7	8	8	8	8	9	8	8	8	8	8	9	9	9	8	7	8	8	8	8	6	7	8	7	8	7	7	6	7	8	6	8	7	7	7	7	7
SP-VC2	8	7	9	8	8	7	7	8	8	7	7	8	7	8	8	8	8	8	8	9	8	8	9	9	8	8	8	8	8	7	7	8	6	8	7	7	6	7	8	7	8	6	7	6	7	7

Table A 5.12 Whittaker beta diversity for Archaea (x10). Light brown cells: sediments samples; white cells: brines samples. The increment of red tonality reflect the increment of values.

	ARG1	ARG2	ARG3	ARG4	ARG5	ARG6	ARG7	ARG8	ARG9	ARG10	ARG11	ARG12	ARG13	ARG14	ARG15	ARG16	ARG17	ARG18	ARG19	ARG20	ARG21	ARG22	ARG23	CHL1	CHL2	CHL3	CHL4	SP-AR1	SP-AR2	SP-CM1	SP-CM2	SP-CM3	SP-CM4	SP-CM5	SP-CM6	SP-CM7	SP-CN1	SP-CN2	SP-CN3	SP-CN4	SP-IB1	SP-IB2	SP-IB3	SP-IB4	SP-IB5	SP-IB6	SP-IB7	SP-IB8	SP-VC1	SP-VC2
ARG1	0																																																	
ARG2	3	0																																																
ARG3	8	7	0																																															
ARG4	7	7	6	0																																														
ARG5	3	4	8	7	0																																													
ARG6	6	5	6	5	6	0																																											
ARG7	6	5	7	5	6	3	0																																										
ARG8	3	4	8	7	3	6	6	0																																									
ARG9	6	5	6	5	6	4	4	6	0																																								
ARG10	5	4	7	6	5	4	4	5	4	0																																								
ARG11	4	3	7	7	5	5	6	4	5	5	0																																							
ARG12	4	3	7	6	5	4	5	5	5	4	3	0																																						
ARG13	6	5	6	5	6	4	4	6	3	4	5	4	0																																					
ARG14	4	4	7	7	5	5	6	5	5	5	3	3	4	0																																				
ARG15	6	5	6	6	6	5	5	6	4	4	5	3	4	4	0																																			
ARG16	3	3	8	8	4	6	7	4	6	6	3	5	6	4	6	0																																		
ARG17	4	4	8	7	5	6	6	4	5	5	3	4	5	4	5	3	0																																	
ARG18	8	7	8	6	7	7	7	8	7	8	7	7	7	8	8	8	7	0																																
ARG19	7	5	6	7	7	6	6	6	5	6	4	5	5	5	5	6	4	7	0																																
ARG20	3	3	8	7	4	6	6	3	5	5	3	4	6	4	6	2	3	8	6	0																															
ARG21	3	3	7	7	4	5	5	4	4	5	3	4	5	4	5	4	3	7	5	2	0																													
ARG22	4	4	8	8	5	6	6	5	5	6	4	5	6	5	6	4	4	8	6	4	4	0																													
ARG23	5	4	8	8	5	6	6	6	6	6	5	6	7	6	7	5	5	7	7	5	5	4	0																												
CHL1	6	6	8	8	6	8	7	6	8	7	6	7	7	7	7	6	6	7	6	6	6	6	5	0																											
CHL2	5	5	8	8	5	7	7	5	6	6	5	6	7	6	6	5	6	8	7	5	5	6	6	6	0																									
CHL3	5	4	7	6	5	4	5	5	4	5	5	5	5	5	6	5	4	7	6	5	3	5	4	7	6	0																							
CHL4	5	4	8	6	5	5	5	5	5	5	5	5	6	5	4	7	6	4	3	4	4	6	6	2	0																									
SP-AR1	4	4	9	8	4	7	7	4	7	7	4	6	7	5	7	3	5	8	7	4	4	5	6	7	5	6	5	0																					
SP-AR2	5	6	9	9	4	8	7	4	7	7	6	7	8	6	8	5	6	8	7	5	6	6	7	6	6	7	6	4	0																				
SP-CM1	7	6	8	8	7	7	7	7	8	7	7	8	8	8	7	7	7	6	8	7	7	7	6	5	7	8	7	8	8	0																			
SP-CM2	7	7	9	8	7	7	7	7	8	7	7	7	8	8	7	7	7	8	8	7	7	7	7	6	7	7	7	8	8	6	0																		
SP-CM3	6	6	7	7	6	6	7	6	6	5	6	6	7	7	7	6	6	7	7	6	6	6	6	7	6	6	6	7	7	5	6	0																	
SP-CM4	8	7	8	9	7	7	8	8	7	7	8	8	8	8	8	8	8	8	8	8	8	8	7	8	7	7	7	8	8	7	8	7	0															
SP-CM5	4	3	8	7	5	5	5	4	5	5	4	5	5	5	6	3	4	7	6	3	3	4	4	6	5	4	3	5	6	6	7	5	7	0														
SP-CM6	3	4	8	8	3	6	7	3	6	6	5	5	7	5	6	4	5	8	7	4	4	5	6	6	5	6	5	4	5	6	7	5	7	4	0													
SP-CM7	3	3	8	7	4	5	6	4	6	5	4	4	6	4	6	3	4	7	6	3	3	3	4	6	6	4	3	4	6	7	7	6	8	3	4	0												
SP-CN1	6	6	8	8	6	7	7	5	6	7	6	6	7	6	6	6	5	8	7	6	6	7	7	7	5	7	6	6	6	7	7	7	7	6	6	6	0											
SP-CN2	3	5	8	8	4	7	7	4	7	6	4	6	7	5	7	3	5	8	7	3	5	4	6	6	5	6	5	3	5	7	7	7	8	4	4	4	6	0										
SP-CN3	5	5	9	9	5	8	8	5	7	7	5	6	7	5	7	3	5	8	7	4	5	5	6	6	5	7	6	3	5	7	8	7	8	5	5	4	6	2	0									
SP-CN4	4	5	9	9	5	7	8	4	7	7	4	6	7	5	7	3	5	8	7	4	5	5	6	6	5	6	6	3	5	7	7	7	8	5	4	4	6	2	2	0								
SP-IB1	4	5	9	9	4	7	8	3	8	7	5	6	7	6	8	5	6	8	8	5	6	6	6	6	6	7	7	3	3	8	7	7	8	6	4	5	6	4	4	4	0							
SP-IB2	8	8	9	8	7	8	9	6	9	7	8	8	8	8	9	8	8	9	9	8	8	8	8	8	8	9	8	7	6	8	7	6	8	7	7	8	7	8	8	8	6	0						
SP-IB3	4	5	9	8	5	7	7	4	7	6	5	6	7	6	7	4	5	8	7	4	5	5	5	6	5	6	6	4	3	7	7	6	8	4	4	4	6	4	4	4	3	6	0					
SP-IB4	5	5	8	8	5	7	7	5	7	5	5	6	7	6	7	6	6	8	8	5	6	6	7	6	5	7	7	5	5	7	6	6	7	6	5	6	5	6	6	6	5	6	5	0				
SP-IB5	5	4	8	6	5	5	5	4	5	5	5	5	5	6	4	4	7	5	4	3	4	4	6	6	3	3	5	6	6	7	6	7	3	5	4	7	5	6	6	6	8	5	6	0				
SP-IB6	5	5	8	8	5	7	7	5	7	5	5	6	7	6	7	6	6	8	8	5	6	6	7	6	5	7	7	5	5	7	6	6	7	6	5	6	5	6	6	6	5	6	5	0	6	0		
SP-IB7	4	5	9	8	4	7	7	3	7	7	5	6	7	5	7	4	5	8	7	4	5	5	6	6	5	6	6	3	3	8	7	7	8	5	4	4	6	3	3	3	2	7	3	5	6	5	0	
SP-IB8	5	6	9	8	4	7	7	4	7	7	6	7	7	7	8	5	6	8	8	5	6	6	7	7	6	7	6	4	3	8	8	7	8	6	5	5	6	5	5	5	3	7	4	5	7	5	3	0
SP-VC1	8	8	9	9	8	9	8	8	8	8	8	8	9	9	9	8	8	9	9	8	8	8	7	6	7	8	8	8	7	8	8	7	7	7	8	8	7	8	8	8	8	8	7	7	7	7	7	7
SP-VC2	5	6	8	8	5	7	6	5	6	6	6	7	7	6	6	6	6	8	7	6	6	7	7	6	4	7	7	5	6	7	7	7	7	6	5	6	4	5	6	5	5	7	5	5	7	5	5	6

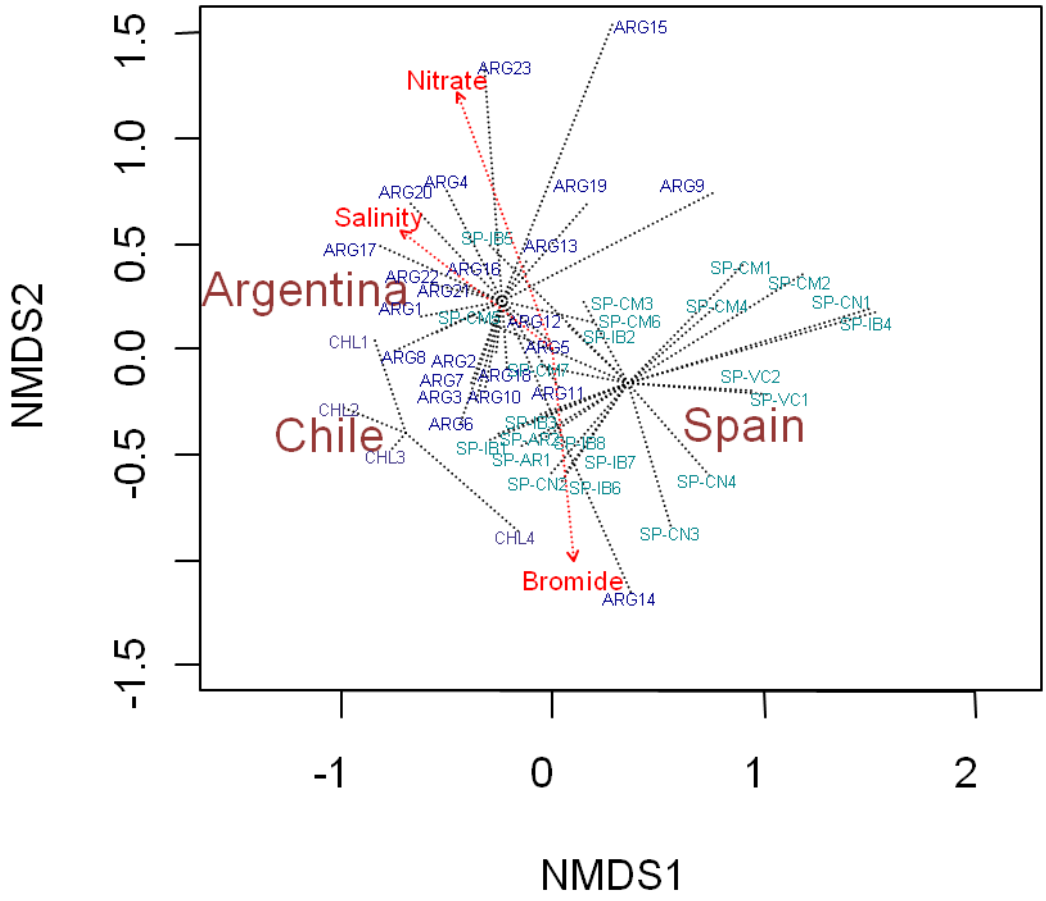


Figure A 5.8 Two dimensional Non-Metric Multidimensional Scaling (NMDS) of bacterial communities from Chile, Argentina and Spain. Red lines show the gradient for the significant parameters in the distribution of the community.

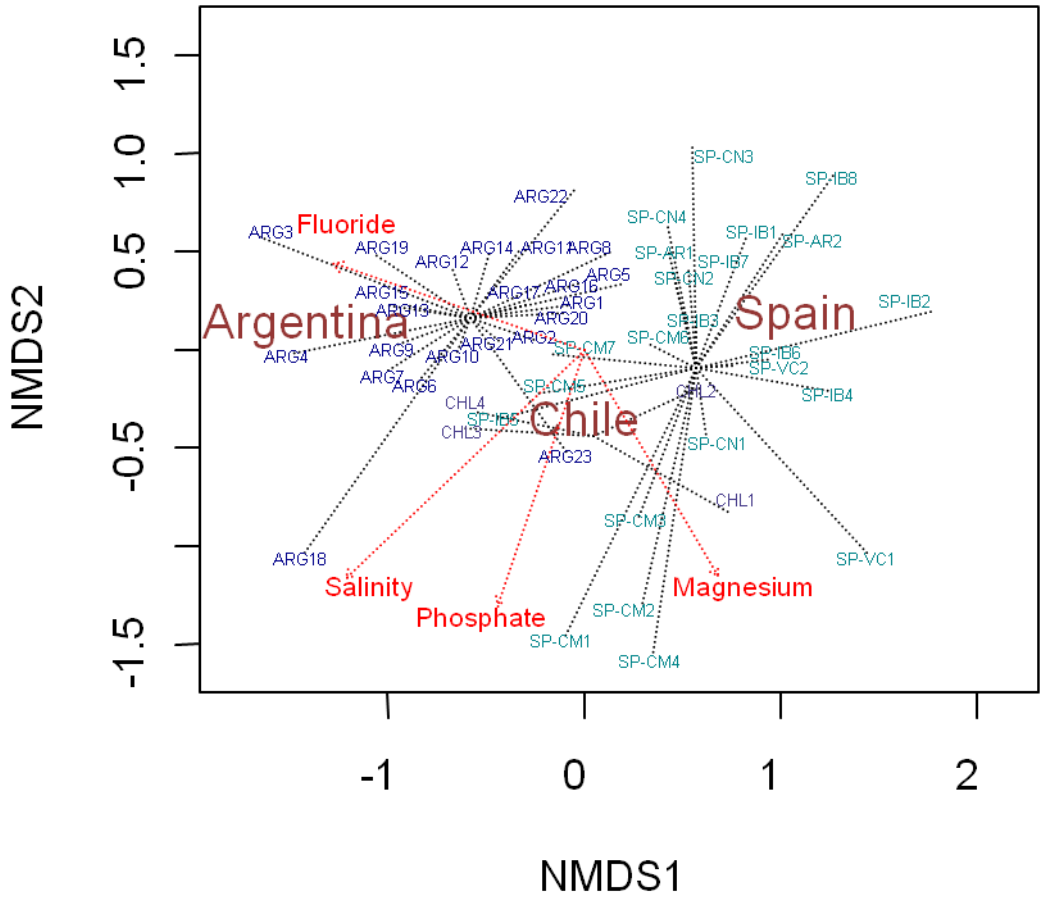


Figure A 5.9 Two dimensional Non-Metric Multidimensional Scaling (NMDS) of archaeal communities from Chile, Argentina and Spain. Red lines show the gradient for the significant parameters in the distribution of the community.

6. Exploring the diversity in other environments: an OPU approach.

Table A 6.1 Primer pair sequences used for 454-pyrosequencing

First amplification	
	Sequence (5' → 3')
GM3 (B)*	AGAGTTTGATCMTGGC
630 (B)	CADAAAGGAGGTGATCC
21F (A)**	TTCCGGTTGATCCTGCCGGA
1492R (A)	TACGGYTACCTTGTTACG

Second amplification				
	Sequence (5' → 3')			
	Adaptor	Key	MID	Primer
GM3-PS	CCTATCCCCTGTGTGCCTTGGCAGTC	TCAG	-	AGAGTTTGATCMTGGC
21F-PS	CCTATCCCCTGTGTGCCTTGGCAGTC	TCAG	-	TTCCGGTTGATCCTGCCGGA
907-PS	CCTATCCCCTGTGTGCCTTGGCAGTC	TCAG	-	CCGTCAATTCMTTTGAGTT

*_Bacteria_ and **_Archaea_ primers

Table A 6.2 Sequences distribution and comparison of the OPUs diversity (H´), dominance (D) and richess (Chao-1) indexes.

Sample	Seqs	OTUs	OPUs	Index		
				H´	D	Chao-1
RB	2495	822	188	4.5	0.02	221.5
RA	1140	32	13	2.0	0.2	13
BB	1072	60	13	1.8	0.2	12
BA	1080	56	13	2.0	0.2	13
Total	5780	970	226	-	-	-
Mean	1445	242.5	56.5	2.6	0.16	64.9
Dev	700.7	386.5	87.7	1.3	0.09	104.4

Table A 6.3 Bacteria and Archaea OPUs and their relative abundances for rhizosphere and brine samples from Es Trenc, Mallorca. *Rhiz= rhizosphere.

OPU	Bacteria	Rhiz*	Brine
1	*Alphaproteobacteria Xanthobacteraceae Xanthobacteraceae*>94 <98	1.48	0.00
2	A*lphaproteobacteria Streptomycetaceae Streptomyces pharmamarensis* FR693804 >94	0.12	0.00
3	*Alphaproteobacteria* Uncultured *Alphaproteobacteria* >81 <99	1.80	0.00
4	*Alphaproteobacteria Hyphomicrobiaceae Hyphomicrobium nitrativorans* JX13369 >92 <95	0.20	0.00
5	*Alphaproteobacteria Hyphomicrobiaceae Devosia* spp. >90 <95	0.56	0.00
6	*Alphaproteobacteria Methyloceanibacter caenitepidi* AB794104 >96 <97	0.08	0.00
7	*Alphaproteobacteria Rhizobiales*>90 <95	0.20	0.00
8	*Alphaproteobacteria* uncultured *Rhizobiales* CU923145 82	0.04	0.00
9	*Alphaproteobacteria Phyllobacteriaceae Aquamicrobium aestuarii* GU199003 >96	0.04	0.00
10	*Alphaproteobacteria Phyllobacteriaceae Hoeflea halophila* GU564401 >99	0.04	0.00
11	*Alphaproteobacteria Aurantimonadaceae Aurantimonadaceae* >97 <99	0.08	0.00
12	*Alphaproteobacteria Aurantimonadaceae Martelella endophytica* HM800924 >93	0.92	0.00
13	*Alphaproteobacteria Alphaproteobacteria* >92 <97	0.20	0.00
14	*Alphaproteobacteria Rhodobacteraceae Labrenzia suaedae* GU322907 >95 <97	0.28	0.00
15	*Alphaproteobacteria Rhodobacteraceae Tropicimonas* sp. >93 <96	0.24	0.00
16	*Alphaproteobacteria Rhodobacteraceae Palleronia marisminoris* AY926462 >96 <97	0.20	0.00
17	*Alphaproteobacteria Rhodobacteraceae Maribius* sp. >95 <98	0.36	0.00
18	*Alphaproteobacteria Rhodobacteraceae Rubellimicrobium* sp. JF139663 >99	0.04	0.00
19	*Alphaproteobacteria Rhodobacteraceae* uncultured *Rhodobacteraceae* KF500634 >94 <97	0.12	0.00
20	*Alphaproteobacteria Rhodobacteraceae Roseivivax pacificus* KC018453 >94 <97	0.16	0.00
21	*Alphaproteobacteria Rhodobacteraceae Pseudoruegeria haliotis* KC196070 >94	0.20	0.00
22	*Alphaproteobacteria Rhodobacteraceae* Uncultured *Rhodobactearaceae*>86 <96	2.40	0.00
23	*Alphaproteobacteria. Rhodobacteraceae* Uncultured *Sediminimonas* AF513933 >0.98	0.00	5.60
24	*Alphaproteobacteria Caulobacteraceae Caulobacter* spp. >95 <97	0.12	0.00
25	*Alphaproteobacteria U*ncultured *Alphaproteobacteria* GQ262926 >94	0.04	0.00
26	*Alphaproteobacteria Swingsia samuiensis* AB786666 92	0.04	0.00
27	*Alphaproteobacteria Rhodospirillaceae Thalassobaculum* spp. >87 <98	0.72	0.00
28	*Alphaproteobacteria Rhodospirillaceae Tistlia consotensis* CBKU010000188 >87 <97	0.80	0.00
29	*Alphaproteobacteria Rhodospirillaceae* Uncultured *Rhodospirillaceae*>87 <97	0.36	0.00
30	*Alphaproteobacteria Rhodospirillaceae* Uncultured *Rhodospirillaceae*>87 <93	0.32	0.00
31	*Alphaproteobacteria Rhodospirillaceae Defloviicoccus* spp. AJ519652 >93 <96	0.04	0.00
32	*Alphaproteobacteria Rhodospirillaceae* Uncultured *Rhodospirillaceae* >93	0.04	0.00
33	*Alphaproteobacteria Rhodospirillaceae Rhodospirillaceae* AACY023776576 >86 <98	0.64	0.00
34	*Alphaproteobacteria. Rhodospirillaceae.* uncultured *Limimonas* EF105687 >0.98	0.00	12.59
35	*Alphaproteobacteria. Rhodospirillaceae. Limimonas halophila* JN605361 95	0.00	1.40
36	*Alphaproteobacteria. Rhodospirillaceae.* uncultured *Rhodovibrio* EF105852 >92	0.00	9.79
37	*Alphaproteobacteria* uncultured *Alphaproteobacteria* >86 <96	0.36	0.00
38	*Alphaproteobacteria Rickettsiales*>77 <97	0.28	0.00
39	*Alphaproteobacteria Erythrobacteraceae Altererythrobacter* spp. >97 <98	1.16	0.00
40	*Alphaproteobacteria Erythrobacteraceae Erythrobacter odishensis* 98	0.12	0.00
41	*Alphaproteobacteria Erythrobacteraceae* uncultured *Altererythrobacter* FJ670864 98	0.08	0.00
42	*Alphaproteobacteria Erythrobacteraceae*	0.20	0.00

	Uncultured *Altererythrobacter* FJ562158 >94 <99		
43	*Alphaproteobacteria Sphingomonadaceae Novosphingobium* spp. >92 <96	0.56	0.00
44	*Alphaproteobacteria* uncultured *Alphaproteobacteria* >84 <95	0.60	0.00
45	*Alphaproteobacteria* uncultured *Alphaproteobacteria* >84 <95	0.80	0.00
46	*Alphaproteobacteria Pahyllobacteriaceae* Uncultured *Mesorhizobium* 98	0.08	0.00
47	*Alphaproteobacteria*. Uncultured DB1-14 JX882755 0.96	0.00	1.40
48	*Betaproteobacteria Comamonadaceae Ramlibacter* spp. >94 <97	0.24	0.00
49	*Betaproteobacteria Comamonadaceae Hydrogenophaga* spp. AB548035 >98	0.04	0.00
50	*Betaproteobacteria Comamonadaceae* uncultured *Comamonadaceae >96 <98*	0.16	0.00
51	*Betaproteobacteria Rhodocyclaceae Zooglea ramigera* X74913 98	0.08	0.00
52	Betaproteobacteria *Nitrosomonadaceae* uncultured *Nitrosomonadaceae* >90 <98	0.08	0.00
53	*Betaproteobacteria Nitrosomonadaceae Nitrosomonas* sp. Nm143 AY123794 98	0.04	0.00
54	*Betaproteobacteria Nitrosomonadaceae Thiobacter* spp. KF287738 >93 <96	0.20	0.00
55	*Betaproteobacteria Methylophilaceae Methylobacillus flagellatus* KC854922 97	0.08	0.00
56	*Gammaproteobacteria Xanthomonadaceae Pseudoxanthomonas dokdonensis* DQ178977 >90	0.04	0.00
57	*Gammaproteobacteria* uncultured *Xanthomonadales* >91 <99	0.68	0.00
58	*Gammaproteobacteria Xanthomonadaceae Rehaibacterium* spp. >91 <100	0.12	0.00
59	*Gammaproteobacteria Ferrimonadaceae Ferrimonas balearica* X93021 >88	0.04	0.00
60	*Gammaproteobacteria Steroidobacter* spp. >90 <98	0.12	0.00
61	*Gammaproteobacteria* RCP1-48 >93 <98	1.60	0.00
62	*Gammaproteobacteria Thiohalomonas* spp. 99	0.04	0.00
63	*Gammaproteobacteria Chromatiaceae Nitrosococcus sp. JN85488 >99*	0.04	0.00
64	*Gammaproteobacteria* SC3-20 FJ793190 93	0.24	0.00
65	*Gammaproteobacteria Plasticicumulans* EU735630 98	0.04	0.00
66	*Gammaproteobacteria Ectothiorhodospiraceae* uncultured *Ectothiorhodospiraceae* >91 <98	0.52	0.00
67	*Gammaproteobacteria Ectothiorhodospiraceae Ectothiorhodospiraceae*>88 <95	0.08	0.00
68	*Gammaproteobacteria. Ectothiorhodospiraceae*. uncultured *Aquisalimonas*a FJ152948 0.97	0.00	1.40
69	*Gammaproteobacteria Chromatiales* spp. >93 <98	0.52	0.00
70	*Gammaproteobacteria* H0C36 HQ800864 98	1.28	0.00
71	*Gammaproteobacteria Halomonadaceae Halomonas* spp. >91 <99	0.32	0.00
72	*Gammaproteobacteria Alteromonadaceae Marinobacter persicus* HQ433441 95	0.04	0.00
73	*Gammaproteobacteria Alteromonadaceae* uncultured *Alteromonadaceae*>89 <95	0.80	0.00
74	*Gammaproteobacteria Sacharospirillaceae* uncultured *Sacharospirilla* GU444084 93	0.24	0.00
75	Gammaproteobacteria uncultured *Gammaproteobacteria*>84 <97	0.60	0.00
76	*Gammaproteobacteria Pseudomaricurvus* sp. >88 <95	1.12	0.00
77	*Gammaproteobacteria* uncultured *Gammaproteobacteria* 98	0.08	0.00
78	*Gammaproteobacteria Methylohalomonas* sp. HQ397439 96	0.24	0.00
79	*Gammaproteobacteria Methylobacterium* spp. 96	0.04	0.00
80	*Gammaproteobacteria Methylohalomonas* sp. HQ397437 97	0.04	0.00
81	*Gammaproteobacteria* ARKICE-90 JQ425960 >89 <98	0.08	0.00
82	*Gammaproteobacteria Salinisphaera* sp. HQ397443 90	0.08	0.00
83	*Gammaproteobacteria Chromatiaceae* uncultured *Nitrosococcus* sp. >97	0.04	0.00
84	*Gammaproteobacteria Pseudomonadaceae Pseudomonas* sp. EU335241 91	0.04	0.00
85	*Gammaproteobacteria* KCM-B-112 HQ397391 >87 <97	0.08	0.00
86	*Deltaproteobacteria* uncultured *Deltaproteobacteria*>92 <96&	0.24	0.00
87	*Deltaproteobacteria* uncultured *Myxococcales*>82 <97	0.72	0.00
88	*Deltaproteobacteria* uncultured *Myxococcales* HQ16681 93	0.08	0.00
89	*Deltaproteobacteria* uncultured *Sandaracinaceae* >87 <96	0.40	0.00
90	*Deltaproteobacteria Sorangiineae* uncultured *Sorangiineae*>93 <99	2.93	0.00
91	*Deltaproteobacteria Nannocystaceae Nannocystis* spp. >88 <97	0.88	0.00
92	*Deltaproteobacteria Haliangianceae Haliangium* sp. >88 <95	0.72	0.00
93	*Deltaproteobacteria Anaeromyxobacter* spp. 98	0.12	0.00
94	*Deltaproteobacteria Cystobacteraceae Cystobacter* spp. >94 <98	0.44	0.00

95	*Deltaproteobacteria Cystobacteraceae* uncultured *Cystobacteraceae* 94	1.84	0.00
96	*Deltaproteobacteria Cystobacteraceae Archangium* sp. EF019058 92	0.04	0.00
97	*Deltaproteobacteria Cystobacteraceae* uncultured *Cystobacteraceae* >94 <98	0.12	0.00
98	*Deltaproteobacteria* GR-WPP3-58 >80 <96	0.36	0.00
99	*Deltaproteobacteria Bdelovibrionaceae Bdelovibrionaceae* 94	0.12	0.00
100	*Deltaproteobacteria Desulfovibrionales*>84 <86	0.16	0.00
101	*Deltaproteobacteria. Desulfohalobiaceae. Desulfovermiculus* HQ425217 >93<97	0.00	4.20
102	*Deltaproteobacteria.* uncultured GR-WP33-58 JX881535 >0.90<0.99	0.00	43.38
103	*Lentisphaerae*. uncultured *Oligosphaerales* JX885062 >0.98	0.00	1.40
104	*Gemmatimonadetes* BD2-11 >82 <96	3.73	0.00
105	*Gemmatimonadetes Gemmatimonadetes* >84 <96	1.12	0.00
106	*Nitrospirae Nitrospiraceae Leptospirillum* sp. HQ672875 85	0.16	0.00
107	*Deinococcus-Thermus Deinococcaceae Deinococcus* spp. >83 <93	0.60	0.00
108	*Planctomycetes Planctomycetaceae* uncultured *Planctomyces* >80 <96	3.69	0.00
109	*Planctomycetes Planctomycetaceae* uncultured *Rhodopirellula*>89 <95	0.28	0.65
110	*Planctomycetes Planctomycetaceae* uncultured *Pirellula* JN494200 >94 <95	0.12	0.00
111	*Planctomycetes Planctomycetaceae Blastopirellula cremea* JF78733 >88 <90	0.20	0.00
112	*Planctomycetes Planctomycetaceae* uncultured *Planctomyces* JF319269 <99	0.12	0.00
113	*Planctomycetes Planctomycetaceae* uncultured *Planctomyces*>78 <95	0.36	0.00
114	*Planctomycetes Phycisphaeraceae* uncultured *Phycisphaera* >75 <93	1.12	0.00
115	*Planctomycete*s Uncultured *Planctomycetes*>79 <92	0.60	0.00
116	Candidate division BRC1 92	0.04	0.00
117	*Verrucomicrobia Opitutaceae Opitutus* spp. EF516121 >81 <96	1.84	0.00
118	Candidate division TM7 >78 <88	0.60	0.00
119	Candidate division 0D1 AY532577 >78 <87	0.36	0.00
120	*Chloroflexi Ardenticatena maritima* AB576167 >74 <83	5.01	0.00
121	*Chloroflexi* uncultured *Chloroflexi*>79 <97	0.96	0.00
122	*Spirochaetes Spirochaetaceae Sphirochaeta* sp. JN523325 >91	0.44	0.00
123	*Acidobacteria Acidobacteriaceae* uncultured *Acidobacteriaceae* >79 <96	2.08	0.00
124	*Firmicutes Bacillaceae Bacillus thermotolerans* JX261934 >86 <94	0.56	0.00
125	*Firmicutes Bacillaceae Bacillus halosaccharovorans* HQ433447 >99	3.25	0.00
126	*Firmicutes Bacillaceae Paenibacillus* spp. >92 <97	0.36	0.00
127	*Firmicutes Bacillaceae Tumebacillus flagellatus* JQ421297 >88 <96	0.20	0.00
128	*Firmicutes Bacillaceae* uncultured *Bacillaceae*>87 <96	0.52	0.00
129	*Firmicutes Planococcaceae Bhargavaea ullalensis* JX144975 >99	0.08	0.00
130	*Firmicutes Planococcaceae Sporosarcina saromensis* AB243859 >94 <100	0.40	0.00
131	*Firmicutes Planococcaceae Paenisporosarcina macmudoensis AJ514408 >97*	0.04	0.00
132	*Firmicutes Planococcaceae Planomicrobium* spp. >95 <97	0.16	0.00
133	*Firmicutes Planococcaceae Bhargavaea* spp. >85 <92	0.36	0.00
134	*Firmicutes Sporolactobacillaceae Sinobaca qinghaiensis* DQ168584 >89	0.04	0.00
135	*Firmicutes Erysipelotrichaceae* Clostridium XVIII >96	0.08	0.00
136	*Firmicutes* uncultured *Firmicutes* >88 <96	0.20	0.00
137	*Cyanobacteria Nostocaceae Anabaena cylindrica* AF091150 >95	0.08	0.00
138	*Cyanobacteria* Family II *Rivularia* sp. HF678513 >95	0.12	0.00
139	*Cyanobacteria* Family I. *Microcoleus* sp. EF654070 >96	0.32	0.00
140	*Cyanobacteria* uncultured *Cyanobacteria*>81 <98	0.20	0.00
141	*Fibrobacteres Fibrobacterace*ae uncultured *Fibrobacteraceae* >74 <93	0.16	0.00
142	*Bacteroidetes. Cryomorphaceae.* uncultured *Owenweeksia* HM127168 0.965	0.00	1.40
143	*Bacteroidetes.*uncultured *Chitinophagaceae* JX882395 >0.92<0.98	0.00	12.59
144	*Bacteroidetes. Rhodohermaceae. Salinibacter* sp. >0.94<0.999	0.20	4.20
145	*Bacteroidetes Rhodothermaceae* uncultured *Rhodothermaceae* >89 <98	0.68	0.00
146	*Bacteroidetes Sphingobacteriales Fodinibius salinus* HM153810 >86 <96	1.64	0.00
147	*Bacteroidetes Sphingobacteriales Alliifodinibius sediminis* JQ923476 >99	0.04	0.00
148	*Bacteroidetes Sphingobacteriales Fodinibius* spp. >86 <97	0.92	0.00
149	*Bacteroidete*s uncultured *Bacteroidetes*>87 <92	0.80	0.00
150	*Chlorobi Ignavibacteriaceae Ignavibacterium* spp. >93	0.28	0.00
151	*Bacteroidetes* Marine Bacterium JK1007 JX050172 >97	0.04	0.00

152	*Bacteroidetes Cyclobacteriaceae Mongoliicoccus alkaliphilus* HE996970 95	0.04	0.00
153	*Bacteroidetes Cytophagaceae* uncultured *Cytophagaceae*>87 <96	3.61	0.00
154	*Bacteroidetes* uncultured *Cytophagales*>85 <98	0.52	0.00
155	*Bacteroidetes* uncultured *Cytophagales* >88 <97	0.40	0.00
156	*Bacteroidetes Cytophagales* uncultured *Cytophagales*>78 <97	1.28	0.00
157	*Bacteroidetes Cytophagaceae Pontibacter odishensis* HE681883 >93	0.04	0.00
158	*Bacteroidetes Flammeovirgaceae Marinoscillum luteum* HM16878 >94 <98	0.20	0.00
159	*Bacteroidetes Flammeovirgaceae Fulvivirga kasyanovii* >88 <98	0.12	0.00
160	*Bacteroidetes Flammeovirgaceae* Candidatus *Amoebophilus asiaticus* 5a2 SP001102 >94	0.08	0.00
161	*Bacteroidetes Flavobacteriaceae Salegentibacter* sp. RV2 GQ365193 >88 <91	0.92	0.00
162	*Bacteroidetes Flavobacteriaceae Gramella flava* JX397931 >98	0.08	0.00
163	*Bacteroidetes Flavobacteriaceae Salinimicrobium* sp. >93	0.04	0.00
164	*Bacteroidetes Flavobacteriaceae Gelidibacter* spp. >91 <97	0.48	0.00
165	*Bacteroidetes Flavobacteriaceae Sinomicrobium oceani* JQ352762 >90	0.24	0.00
166	*Bacteroidetes Flavobacteriaceae Muricauda* spp. >97	0.04	0.00
167	*Bacteroidetes Flavobacteriaceae Flavobacteriaceae*>80 <88	0.92	0.00
168	*Bacteroidetes Cryomorphaceae Owenweeksia* sp. KC331461 >90	0.04	0.00
169	*Bacteroidetes Sapropiraceae Lewinella* spp. >81 <95	0.12	0.00
170	*Actinobacteria Microbacteriaceae Microbacteriaceae* >85 <92	0.72	0.00
171	*Actinobacteria Vibrionaceae Vibrio ponticus* AJ630103 >84 <86	0.16	0.00
172	*Actinobacteria* uncultured *Actinobacteria*>79 <90	2.97	0.00
173	*Actinobacteria* 480-2 >86 <97	2.20	0.00
174	*Actinobacteria Micromonosporaceae Pilimelia* sp. HM445002 95	0.08	0.00
175	*Actinobacteria Nitriliruptoraceae Nitriliruptor* spp. >88 <98	1.76	0.00
176	*Actinobacteria Euzebyaceae Euzebyaceae* >93 <98	2.04	0.00
177	*Actinobacteria* uncultured *Actinobacteria*>90 <98	1.36	0.00
178	*Actinobacteria Aquihabitans daechungensis* JN033775 94	0.56	0.00
179	*Actinobacteria* Sva0996 GQ472831 >90 < 96	0.20	0.00
180	*Actinobacteria Acidimicrobiales*>93 < 99	0.96	0.00
181	*Actinobacteria Acidimicrobiaceae Ilumatobacter* spp. >90 <99	1.32	0.00
182	*Actinobacteria Streptomycetaceae Streptomyces* spp. >97 <999	0.28	0.00
183	*Actinobacteria Nocardiaceae Nocardioides* spp. >98 <99	0.24	0.00
184	*Marmoricola* sp. KC820854 98	0.04	0.00
185	*Actinobacteria Nocardiaceae Kribbella albertanoniae* 99	0.04	0.00
186	*Actinobacteria Nocardiaceae Nocardia* spp. 93	0.04	0.00
187	*Actinobacteria Nocardiopsaceae Haloactinospora alba* DQ923130 99	0.32	0.00
188	*Actinobacteria Glycomycetaceae* uncultured *Glycomycetaceae* GQ263395 >81 <91	0.52	0.00
189	*Actinobacteria Pseudonocardiaceae Amycolatopsis salitolerans* FJ606836 99	0.16	0.00
190	*Actinobacteria Pseudonocardiaceae Amycolaptosis* spp 99	0.04	0.00
191	*Actinobacteria* uncultured *Actinobacteria* 98	0.40	0.00
192	*Actinobacteria Actinopolysporaceae Actinopolyspora* spp. >98 < 999	0.08	0.00
193	*Actinobacteria Geodermatophilaceae Blastococcus* spp. >98 < 999	0.56	0.00
194	*Actinobacteria* uncultured *Actinobacteria*>98 < 999	0.72	0.00
195	*Actinobacteria Micrococcaceae Kocuria* spp. 99	0.04	0.00
196	*Actinobacteria Micrococcaceae Arthrobacter* spp. 97	0.04	0.00
197	*Actinobacteria Micrococcineae Luteimicrobium* spp. 99	0.44	0.00
198	*Actinobacteria Microbacteriaceae Glaciihabitans tibetensis* KC256953 99	0.68	0.00
199	*Actinobacteria Intransporanginaceae Aquipuribacter* sp. EU930868 99	0.12	0.00
200	*Actinobacteria Corynebacteriaceae Corynebacterium matruchotii* X82065 93	0.04	0.00
	Total	100.0	100.0
	Archaea		
201	*Euryarchaeota. Halobacteriaceae. Halococcus dombrowskii* AJ420376 >98	1.32	0.00
202	*Euryarchaeota. Halobacteriaceae. Halococcus hamelinensis* DQ017835 >95<97	6.58	0.00
203	*Euryarchaeota. Halobacteriaceae. Halococcus* spp. >93 <95	1.32	0.00
204	*Euryarchaeota. Halobacteriaceae. Haladaptatus paucihalophilus* DQ344973 >98	27.63	0.00

205	*Euryarchaeota. Halobacteriaceae.* uncultured *Haladaptatus* HQ400419 >94<96	25.00	0.00
206	*Euryarchaeota. Halobacteriaceae. Halobonum* JN714431 >99	14.47	0.00
207	*Euryarchaeota. Halobacteriaceae. Halobonum* HQ400558 >97	1.32	0.00
208	*Euryarchaeota. Halobacteriaceae. Halomarina oriensis* AB519798 923	0.00	3.70
209	Euryarchaeota. Halobacteriacea. Halorubrum aquaticum AM268115 94	0.00	14.81
210	*Euryarchaeota. Halobacteriacea. Halorubrum orientale* AM235789 >99	0.00	37.04
211	*Euryarchaeota. Halobacteriacea*. uncultured *Halorubrum* HQ157591 975	0.00	1.85
212	*Euryarchaeota. Halobacteriacea. Halorubrum xinjiongense* AY510707 >98	0.00	5.56
213	*Euryarchaeota. Halobacteriacea. Halorubrum* spp >99	0.00	1.85
214	*Euryarchaeota. Halobacteriacea. Haloferax mediterranei* D11107 >98	1.32	0.00
215	*Euryarchaeota. Halobacteriacea. Halopelagius fulvigenes* JQ996497 94	3.95	0.00
216	*Euryarchaeota. Halobacteriacea. Halopelagius inordinatus* EU887284 92	6.58	0.00
217	*Euryarchaeota. Halobacteriacea. Halobellus salinus* HQ451075 >94<99	0.00	5.56
218	*Euryarchaeota. Halobacteriacea. Halobellus clavatus* GQ282620 >98	0.00	1.85
219	Euryarchaeota.*Halobacteriacea*. uncultured *Halobellus* FN391236 >98	1.32	0.00
220	*Euryarchaeota. Halobacteriacea.* uncultured *Haloquadratum* CU467219 >99	0.00	16.67
221	*Euryarchaeota. Halobacteriaceae.* uncultured *Haloarcula* HE604439 >98	0.00	1.85
222	*Euryarchaeota. Halobacteriaceae*. uncultured *Haloarcula* HQ400420 978	0.00	1.85
223	*Euryarchaeota. Halobacteriaceae. Halonotius* AM947464 >99	0.00	3.70
224	*Euryarchaeota. Halobacteriaceae* CU467243 >98< 94	0.00	3.70
225	*Euryarchaeota. Methanosarcinaceae.* uncultured *Methanosarcina* EU420698 >98	3.95	0.00
226	*Euryarchaeota. Methanoregulaceae. Methanolinea mesophila* AB447467 >98	5.26	0.00
	Total	100.0	100.0

Table A 6.4 Sample distribution in groups based on the clustering analysis, their number of OTUs and OPUs, and the main diversity indices (from Vidal et al., 2015)

Study Group	Sample	Nr of Sequences	Nr OPUs	Dominance-D index	Shannon-Weiner index	Chao-1	Nr OTUs
CD1	24C	8706	66	0.250	2.209	79.6	178
	2C	7631	84	0.194	2.482	89.1	170
	10C	4801	59	0.142	2.542	62.1	113
	12C	10414	75	0.132	2.581	78.0	190
	3C	7552	98	0.288	2.326	123.5	235
	Mean	**7820**	**76**	**0.201**	**2.428**	**86.5**	**177**
	SD	2045	15	0.068	0.156	22.9	44
CD2	5C	3048	106	0.044	3.697	107.7	240
	9C	4037	75	0.131	2.865	84.0	192
	6C	6008	86	0.060	3.293	91.6	280
	8C	6952	79	0.050	3.359	80.4	264
	4C	4699	101	0.090	3.204	121.0	249
	Mean	**4949**	**89**	**0.075**	**3.284**	**97.0**	**245**
	SD	1553	14	0.036	0.299	17.1	33
CD3	**1C**	**12030**	**59**	**0.099**	**2.601**	**74.0**	**239**
HC	14S	7047	100	0.103	2.953	113.1	183
	15S	2921	79	0.167	2.444	82.9	115
	16S	4617	110	0.060	3.485	121.7	257
	17S	3772	93	0.094	3.173	94.9	184
	20S	6182	115	0.124	3.062	117.0	251
	22S	6223	76	0.203	2.447	80.7	172
	23S	5171	129	0.080	3.422	136.8	312
	Mean	**5133**	**102**	**0.120**	**2.990**	**112.2**	**199**
	SD	1470	19	0.047	0.391	25.0	69
CD4	7C	8917	105	0.110	2.974	112.8	264
	11C	11107	85	0.101	2.871	110.5	296
	Mean	**10012**	**95**	**0.105**	**2.923**	**111.7**	**280**
	SD	1549	14	0.006	0.073	1.6	23

Table A 6.5 List of OPUs and their correspondence with the closest relative sequence, and their affiliation with higher taxa from (Vidal et al., 2015)

OPU	Closest relative sequence	Family	Class	Phylum
OPU-001	*Escherichia coli (X80725/Type sp.) Shigella dysenteriae (X96966/Type sp.)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-002	*Enterobacter cloacae subsp. cloacae (AJ251469/Type sp.) Klebsiella pneumoniae subsp. pneumoniae (X87276/Type sp.)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-003	*Citrobacter amalonaticus (FR870441) Citrobacter farmeri (AF025371)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-004	*Klebsiella singaporensis (AF250285)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-005	*Enterobacter aerogenes KCTC 2190 (CP002824)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-006	*Citrobacter freundii (AB626119)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-007	*uncultured bacterium (EU773762)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-008	*Klebsiella oxytoca (AF129440)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-009	*Kluyvera georgiana (AF047186)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-010	*Pseudomonas flectens (AB021400)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-011	*uncultured organism (HQ757307)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-012	*Morganella morganii subsp. morganii (AJ301681/Type sp.)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-013	*Proteus vulgaris (DQ885257/Type sp.)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-014	*Haemophilus parainfluenzae (AY362908) Haemophilus sputorum (JF506642)*	*Pasteurellaceae*	*Gammaprot.*	*Proteobacteria*
OPU-015	*Haemophilus paraphrohaemolyticus (M75076)*	*Pasteurellaceae*	*Gammaprot.*	*Proteobacteria*
OPU-016	*Aggregatibacter segnis (M75043)*	*Pasteurellaceae*	*Gammaprot.*	*Proteobacteria*
OPU-017	*Providencia sp. enrichment culture clone SRC_NBB16 (GU374076)*	*Enterobacteriaceae*	*Gammaprot.*	*Proteobacteria*
OPU-018	*Aeromonas fluvialis (FJ230078)*	*Aeromonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-019	*Succinivibrio dextrinosolvens (Y17600/Type sp.)*	*Succinivibrionaceae*	*Gammaprot.*	*Proteobacteria*
OPU-020	*Pseudomonas japonica (AB126621) Pseudomonas monteilii (AF064458) Pseudomonas oryzihabitans (D84004)*	*Pseudomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-021	*Pseudomonas alcaliphila (AB030583) Pseudomonas stutzeri (AF094748) Pseudomonas xanthomarina (AB176954)*	*Pseudomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-022	*Pseudomonas aeruginosa (X06684/Type sp.) Pseudomonas alcaligenes (D84006)*	*Pseudomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-023	*Pseudomonas halophila (AB021383)*	*Pseudomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-024	*Marinicella litoralis (AB500095/Type sp.)*	*Moraxellaceae*	*Gammaprot.*	*Proteobacteria*
OPU-025	*Enhydrobacter aerosaccus (AJ550856/Type sp.)*	*Moraxellaceae*	*Gammaprot.*	*Proteobacteria*
OPU-026	*Acinetobacter baylyi (AF509820) Acinetobacter gerneri (AF509829) Acinetobacter lwoffii (X81665) Acinetobacter schindleri (AJ278311) Acinetobacter towneri (AF509823) Acinetobacter ursingii (AJ275038)*	*Moraxellaceae*	*Gammaprot.*	*Proteobacteria*
OPU-027	*Beggiatoa alba (AF110274/Type sp.)*	*Thiotrichaceae*	*Gammaprot.*	*Proteobacteria*
OPU-028	*Cupriavidus necator (AF191737/Type sp.) Ralstonia pickettii (AY741342/Type sp.)*	*Burkholderiaceae*	*Betaprot.*	*Proteobacteria*
OPU-029	*Burkholderia acidipaludis (AB513180) Burkholderia ferrariae (DQ514537) Burkholderia graminis (U96939) Burkholderia heleia (AB495123) Burkholderia kururiensis (AB024310) Burkholderia phenoliruptrix (AY435213) Burkholderia tuberum (AJ302311)*	*Burkholderiaceae*	*Betaprot.*	*Proteobacteria*
OPU-030	*Bordetella hinzii (AF177667)*	*Alcaligenaceae*	*Betaprot.*	*Proteobacteria*
OPU-031	*Acidovorax anthurii (AJ007013) Acidovorax facilis (AF078765/Type sp.)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-032	*Xenophilus aerolatus (EF660342) Xylophilus ampelinus (AJ420330/Type sp.)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-033	*Curvibacter delicatus (AF078756) Curvibacter fontanus (AB120963) Curvibacter gracilis (AB109889/Type sp.)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-034	*Variovorax sp. MLBW2 (AB610599)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-035	*Limnohabitans australis (FM178226) Limnohabitans curvus (AJ938026/Type sp.) Limnohabitans parvus (FM165536) Limnohabitans planktonicus (FM165535)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-036	*Ramlibacter henchirensis (AF439400) Ramlibacter tataouinensis (AF144383/Type sp.)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*

OPU-037	*Ottowia thiooxydans (AJ537466/Type sp.)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-038	*Hydrogenophaga palleronii (AF078769)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-039	*Comamonas composti (EF015884)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-040	*Comamonas denitrificans (AF233877)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-041	*Comamonas kerstersii (AJ430347)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-042	*Diaphorobacter nitroreducens (AB064317/Type sp.)* *Diaphorobacter oryzae (EU342381)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-043	*uncultured bacterium (AB491412)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-044	*Delftia acidovorans (AB021417/Type sp.)* *Delftia lacustris (EU888308)* *Delftia tsuruhatensis (AB075017)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-045	*Pelomonas puraquae (AM501439)* *Pelomonas saccharophila (AB021407/Type sp.)*	*Comamonadaceae*	*Betaprot.*	*Proteobacteria*
OPU-046-1	*Sutterella stercoricanis (AJ566849)* *Sutterella wadsworthensis (GU585669/Type sp.)*	*Sutterellaceae*	*Betaprot.*	*Proteobacteria*
OPU-046-2	*Sutterella wadsworthensis (HM037997)*	*Sutterellaceae*	*Betaprot.*	*Proteobacteria*
OPU-047	*Parasutterella excrementihominis (AB370250/Type sp.)*	*Sutterellaceae*	*Betaprot.*	*Proteobacteria*
OPU-048	*uncultured organism (HQ760564)*	*Sutterellaceae*	*Betaprot.*	*Proteobacteria*
OPU-049	*uncultured bacterium (DQ801351)*	*Sutterellaceae*	*Betaprot.*	*Proteobacteria*
OPU-050	*Neisseria gonorrhoeae (X07714/Type sp.)* *Neisseria iguanae (GU233442)* *Neisseria sicca ATCC 29256 (ACKO02000016)*	*Neisseriaceae*	*Betaprot.*	*Proteobacteria*
OPU-051	*Eikenella corrodens (AB525415/Type sp.)*	*Neisseriaceae*	*Betaprot.*	*Proteobacteria*
OPU-052	*Sulfuritalea hydrogenivorans (AB552842/Type sp.)*	*Rodocyclaceae*	*Betaprot.*	*Proteobacteria*
OPU-053	*Hydrogenophilus hirschii (FR749905)* *Hydrogenophilus thermoluteolus (AB009828/Type sp.)*	*Hydrogenophilaceae*	*Betaprot.*	*Proteobacteria*
OPU-054	*Pseudomonas beteli (AB021406)* *Stenotrophomonas chelatiphaga (EU573216)* *Stenotrophomonas maltophilia (AB294553/Type sp.)* *Stenotrophomonas rhizophila (AJ293463)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-055	*Stenotrophomonas maltophilia (GU945534)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-056	*Thermomonas brevis (AJ519989)* *Thermomonas fusca (AJ519986)* *Thermomonas haemolytica (AJ300185/Type sp.)* *Thermomonas koreensis (DQ154906)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-057	*Dyella koreensis (AY884571)* *Dyella marensis (AM939778)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-058	*Dyella ginsengisoli (AB245367)* *Dyella soli (EU604272)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-059	*Dyella thiooxydans (EF397574)* *Frateuria aurantia (AB091194/Type sp.)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-060	*Dyella terrae (EU604273)* *Fulvimonas soli (AJ311653/Type sp.)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-061	*Rhodanobacter thiooxydans (AB286179)*	*Xhanthomonadaceae*	*Gammaprot.*	*Proteobacteria*
OPU-062	*Arhodomonas aquaeolei (M26631/Type sp.)*	*Ectothiorhodospiraceae*	*Gammaprot.*	*Proteobacteria*
OPU-063	*Ensifer arboris (Z78204)*	*Rhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-064	*Rhizobium borbori (EF125187)* *Rhizobium larrymoorei (Z30542)* *Rhizobium pusense (FJ969841)*	*Rhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-065	*Shinella granuli (AB187585/Type sp.)*	*Rhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-066	*Ochrobactrum anthropi (CP000758/Type sp.)* *Ochrobactrum ciceri (DQ647056)* *Ochrobactrum pecoris (FR668302)*	*Brucellaceae*	*Alphaprot.*	*Proteobacteria*
OPU-067	*Mesorhizobium plurifarium (Y14158)*	*Phyllobacteriaceae*	*Alphaprot.*	*Proteobacteria*
OPU-068	*Mesorhizobium ciceri (U07934)* *Mesorhizobium loti (X67229/Type sp.)*	*Phyllobacteriaceae*	*Alphaprot.*	*Proteobacteria*
OPU-069	*Rhizobium leguminosarum (U29386/Type sp.)* *Rhizobium tibeticum (EU256404)* *Rhizobium tubonense (EU256434)*	*Rhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-070-1	*Agromonas oligotrophica (D78366/Type sp.)* *Bradyrhizobium japonicum (U69638/Type sp.)*	*Bradyrhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-070-2	*Afipia birgiae (AF288304)* *Afipia broomeae (U87759)*	*Bradyrhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-070-3	*uncultured Bradyrhizobiaceae bacterium (AM935305)*	*Bradyrhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-071	*Methylorosula polaris (EU586035/Type sp.)* *Methylovirgula ligni (FM252034/Type sp.)*	*Beijerinckiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-072	*Amorphus coralli (DQ097300/Type sp.)*	*Unclassified Rhizobiales*	*Alphaprot.*	*Proteobacteria*

	Amorphus orientalis (FJ998414)			
OPU-073	*Oceanibaculum indicum (EU656113/Type sp.) Oceanibaculum pacificum (FJ463255)*	*Rhodospirillaceae*	*Alphaprot.*	*Proteobacteria*
OPU-074	*Kiloniella laminariae (AM749667/Type sp.)*	*Sphingomonadaceae*	*Alphaprot.*	*Proteobacteria*
OPU-075	*Sphingomonas aromaticivorans (CP000248) Sphingomonas capsulata (D16147)*	*Caulobacteraceae*	*Alphaprot.*	*Proteobacteria*
OPU-076	*Sphingomonas leidyi (AJ227812)*	*Sphingomonadaceae*	*Alphaprot.*	*Proteobacteria*
OPU-077	*Sphingomonas oligophenolica (AB018439)*	*Sphingomonadaceae*	*Alphaprot.*	*Proteobacteria*
OPU-078	*Brevundimonas nasdae (AB071954) Brevundimonas vesicularis (AJ227780)*	*Caulobacteraceae*	*Alphaprot.*	*Proteobacteria*
OPU-079	*Brevundimonas diminuta (AB021415/Type sp.) Brevundimonas vancanneytii (AJ227779)*	*Caulobacteraceae*	*Alphaprot.*	*Proteobacteria*
OPU-080	*uncultured bacterium (DQ658781)*	*Caulobacteraceae*	*Alphaprot.*	*Proteobacteria*
OPU-081	*uncultured bacterium (DQ490042)*	*Caulobacteraceae*	*Alphaprot.*	*Proteobacteria*
OPU-083	*Bosea minatitlanensis (AF273081) Bosea thiooxidans (AJ250796/Type sp.)*	*Bradyrhizobiaceae*	*Alphaprot.*	*Proteobacteria*
OPU-084	*Acidocella aluminiidurans (AB362219) Acidocella facilis (D30774/Type sp.)*	*Acetobacteraceae*	*Alphaprot.*	*Proteobacteria*
OPU-085	*Reyranella massiliensis (EF394922/Type sp.)*	*Unclassified Rhodospirillales*	*Alphaprot.*	*Proteobacteria*
OPU-086	*Bacteroides dorei (AB242142) Bacteroides vulgatus (AJ867050)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-087	*Bacteroides coprocola (AB200224)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-088	*Bacteroides plebeius (AB200217)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-089	*Bacteroides faecis (GQ496624) Bacteroides finegoldii (AB222699) Bacteroides thetaiotaomicron (AE015928)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-090	*Bacteroides ovatus (AB050108) Bacteroides xylanisolvens (AM230650)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-091	*Bacteroides acidifaciens (AB021164) Bacteroides caccae (X83951)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-092	*Bacteroides fragilis (CR626927/Type sp.)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-093	*Bacteroides faecichinchillae (AB574480) Bacteroides nordii (AY608697) Bacteroides salyersiae (AY608696)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-094	*Bacteroides cellulosilyticus (AJ583243) Bacteroides intestinalis (AB214328)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-095	*Bacteroides stercoris (X83953)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-096	*Bacteroides clarus (AB490801)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-097	*Bacteroides eggerthii (AB050107)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-098	*Bacteroides fluxus (AB490802) Bacteroides helcogenes (AB200227) Bacteroides rodentium (AB531489) Bacteroides uniformis (AB050110)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-099	*Bacteroides coprophilus (AB260026)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-100	*Bacteroides salanitronis (AB253731)*	*Bacteroidaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-101	*Paraprevotella clara (AB331896/Type sp.) Paraprevotella xylaniphila (AB331897)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-102	*Prevotella copri (AB064923)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-103	*Prevotella fusca (FJ545433) Prevotella histicola (EU126661) Prevotella melaninogenica (CP002122/Type sp.) Prevotella scopos (FJ545434) Prevotella veroralis (L16473)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-104	*Hallella seregens (X81877/Type sp.) Prevotella bergensis (AY350613) Prevotella denticola (AY323524)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-105	*Prevotella brevis (AJ011682) Prevotella bryantii (AJ006457)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-106	*Prevotella stercorea (AB244774)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-107	*Prevotella buccae (L16477)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-108	*Prevotella tannerae (AJ005634)*	*Prevotellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-109	*Parabacteroides distasonis (AB238922/Type sp.)*	*Porphyromonadaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-110	*Parabacteroides johnsonii (AB261128) Parabacteroides merdae (AB238928)*	*Porphyromonadaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-111	*Barnesiella intestinihominis (AB370251)*	*Porphyromonadaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-112	*uncultured bacterium (EU774794)*	*Porphyromonadaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-113	*Butyricimonas virosa (AB443949)*	*Porphyromonadaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-114	*Odoribacter splanchnicus (L16496/Type sp.)*	*Porphyromonadaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-115	*uncultured organism (HQ792271)*	*Porphyromonadaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-116	*Alistipes finegoldii (AY643083) Alistipes onderdonkii (AY974071) Alistipes putredinis (L16497/Type sp.)*	*Rikenellaceae*	*Bacteroidia*	*Bacteroidetes*

	Alistipes shahii (AY974072)			
OPU-117	*Alistipes indistinctus (AB490804)*	*Rikenellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-118	*uncultured bacterium (FJ793177)*	*Rikenellaceae*	*Bacteroidia*	*Bacteroidetes*
OPU-119	*Psychroflexus halocasei (FR714910)*	*Flavobacteriaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-120	*Owenweeksia hongkongensis (AB125062/Type sp.)*	*Cryomorphaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-121	*Chryseobacterium arthrosphaerae (FN398101)* *Chryseobacterium vietnamense (HM212415)*	*Flavobacteriaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-122	*Cloacibacterium normanense (AJ575430/Type sp.)* *Cloacibacterium rupense (EU581834)*	*Flavobacteriaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-123	*Chryseobacterium koreense (AF344179)*	*Flavobacteriaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-124	*Elizabethkingia anophelis (EF426425/Type sp.)* *Elizabethkingia meningoseptica (AJ704540/Type sp.)* *Elizabethkingia miricola (AB071953)* *Soonwooa buanensis (FJ713810/Type sp.)*	*Flavobacteriaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-125	*Sediminibacterium salmoneum (EF407879/Type sp.)*	*Chitinophagaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-126	*Hydrotalea flava (FN665659/Type sp.)*	*Chitinophagaceae*	*Flavobacteria*	*Bacteroidetes*
OPU-127	*uncultured bacterium (AY820722)*	*Uncultured Deltaprot.*	*Deltaprot.*	*Proteobacteria*
OPU-128	*uncultured bacterium (EU803844)*	*Uncultured Deltaprot.*	*Deltaprot.*	*Proteobacteria*
OPU-129	*uncultured bacterium (JF800713)*	*Uncultured Deltaprot.*	*Deltaprot.*	*Proteobacteria*
OPU-130	*uncultured bacterium (HM126783)*	*Uncultured Deltaprot.*	*Deltaprot.*	*Proteobacteria*
OPU-131	*uncultured bacterium (JF833867)*	*Uncultured Deltaprot.*	*Deltaprot.*	*Proteobacteria*
OPU-132	*uncultured bacterium (FJ437978)*	*Uncultured Deltaprot.*	*Deltaprot.*	*Proteobacteria*
OPU-133	*Elusimicrobium minutum (AM490846/Type sp.)*	*Elusimicrobiaceae*	*Elusimicrobia*	*Elusimicrobia*
OPU-134	*Schlesneria paludicola (AM162407/Type sp.)*	*Planctomycetaceae*	*Planctomycetes*	*Planctomycetes*
OPU-135	*uncultured bacterium (GU553741)*	*Candidate division OD1*	*OD1*	*OD1*
OPU-136	*Victivallis vadensis (AY049713/Type sp.)*	*Victivallaceae*	*Lentisphaeria*	*Lentisphaerae*
OPU-137	*Clostridium innocuum (M23732)*	*Clostridiaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-138	*Eubacterium dolichum (L34682)* *Eubacterium tortuosum (L34683)*	*Eubacteriaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-139	*Eubacterium biforme (M59230)*	*Eubacteriaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-140	*uncultured bacterium (GQ492216)*	*Uncultured Firmicutes*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-141	*Holdemania filiformis (Y11466/Type sp.)*	*Erysipelotrichaceae*	*Erysipelotrichia*	*Firmicutes*
OPU-142	*Clostridium ramosum (X73440)*	*Clostridiaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-143	*Clostridium saccharogumia (DQ100445)*	*Clostridiaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-144	*Clostridium spiroforme (X75908)*	*Clostridiaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-145	*uncultured bacterium (DQ806500)*	*Clostridiaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-146	*Catenibacterium mitsuokai (AB030224/Type sp.)*	*Erysipelotrichaceae*	*Clostridia (Erysipelotrichia)*	*Firmicutes*
OPU-147	*uncultured bacterium (JF210269)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-148	*Fusobacterium canifelinum (AY162221)* *Fusobacterium nucleatum subsp. nucleatum (AE009951/Type sp.)* *Fusobacterium nucleatum subsp. polymorphum (AF287812)* *Fusobacterium nucleatum subsp. vincentii (AABF01000111)* *Fusobacterium simiae (M58685)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-149	*uncultured bacterium (EU773871)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-150	*Fusobacterium nucleatum subsp. animalis (GQ301042)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-151	*Fusobacterium periodonticum (X55405)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-152	*Fusobacterium ulcerans (X55412)* *Fusobacterium varium (AJ867036)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-153	*Fusobacterium equinum (AJ295750)* *Fusobacterium gonidiaformans (X55410)* *Fusobacterium necrophorum subsp. funduliforme (AB525413)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-154	*Leptotrichia sp. oral clone IK040 (AY349387)*	*Leptotrichiaceae*	*Fusobacteria*	*Firmicutes*
OPU-155	*uncultured bacterium (EU773871)*	*Fusobacteriaceae*	*Fusobacteria*	*Firmicutes*
OPU-156	*Veillonella atypica (AF439641)* *Veillonella caviae (AY355140)* *Veillonella denticariosi (EF185167)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*

	Veillonella dispar (AF439639) Veillonella montpellierensis (AF473836) Veillonella parvula (AY995767/Type sp.)			
OPU-157	*uncultured bacterium (FJ365765)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-158	*Megasphaera elsdenii (U95027/Type sp.)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-159	*Megasphaera micronuciformis (AF473834)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-160	*Allisonella histaminiformans (AF548373/Type sp.) Dialister pneumosintes (X82500/Type sp.)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-161	*Dialister invisus (AY162469)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-162	*Centipeda periodontii (AJ010963/Type sp.) Selenomonas infelix (AF287802) Selenomonas noxia (AF287799)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-163	*Mitsuokella jalaludinii (AF479674) Mitsuokella multacida (X81878/Type sp.)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-164	*Megamonas funiformis (AB300988) Megamonas rupellensis (EU346729)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-165	*Phascolarctobacterium succinatutens (AB490811)*	*Acidaminococcaceae*	*Negativicutes*	*Firmicutes*
OPU-166	*Phascolarctobacterium faecium (X72865/Type sp.)*	*Acidaminococcaceae*	*Negativicutes*	*Firmicutes*
OPU-167	*Acidaminococcus fermentans (X78017/Type sp.) Acidaminococcus intestini (AF473835)*	*Acidaminococcaceae*	*Negativicutes*	*Firmicutes*
OPU-168	*uncultured bacterium (HM277793)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-169	*Selenomonas lipolytica (AF001901)*	*Veillonellaceae*	*Negativicutes*	*Firmicutes*
OPU-170	*uncultured bacterium (AY916294)*	*Uncultured Firmicutes*	*Negativicutes*	*Firmicutes*
OPU-172	*uncultured bacterium (AJ583204)*	*Uncultured Firmicutes*	*Uncultured Firmicutes*	*Firmicutes*
OPU-173	*Daucus carota (carrot) (X73670)*	*Chloroplast*	*Cyanobacteria*	*Cyanobacteria*
OPU-174	*uncultured organism (JN486390)*	*Uncultured Cyanobacteria*	*Cyanobacteria*	*Cyanobacteria*
OPU-175	*uncultured bacterium (EU768575)*	*Uncultured Gammaprot.*	*Uncultured Gammaprot.*	*Proteobacteria*
OPU-176	*uncultured bacterium (HM445484)*	*Uncultured Actinomycete*		*Actinobacteria*
OPU-177	*Halanaerobaculum tunisiense (EU327343)*	*Halobacteroidaceae*	*Clostridia*	*Firmicutes*
OPU-178	*Gordonia cholesterolivorans (EU244645)*	*Nocardiaceae*	*Actinobacteria*	*Actinobacteria*
OPU-179	*Corynebacterium macginleyi (AJ439345) Corynebacterium tuberculostearicum (AJ438050)*	*Corynebacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-180	*uncultured bacterium (HM445560)*	*Corynebacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-181	*Actinomyces odontolyticus (AJ234040)*	*Actinomycetaceae*	*Actinobacteria*	*Actinobacteria*
OPU-182	*Brevibacterium picturae (AJ620364) Brevibacterium sandarakinum (FN293377)*	*Brevibacteraceae*	*Actinobacteria*	*Actinobacteria*
OPU-183	*Micrococcus luteus (AJ536198/Type sp.) Micrococcus lylae (X80750)*	*Micrococcaceae*	*Actinobacteria*	*Actinobacteria*
OPU-184	*Janibacter limosus (Y08539/Type sp.)*	*Intrasporangiaceae*	*Actinobacteria*	*Actinobacteria*
OPU-185	*Janibacter limosus (Y08539/Type sp.)*	*Microbacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-186	*uncultured compost bacterium (FN667026)*	*Microbacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-187	*Leucobacter alluvii (AM072820) Leucobacter komagatae (D45063/Type sp.)*	*Microbacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-188	*Leucobacter alluvii (AM072820)*	*Microbacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-190	*Propionibacterium acnes (AB042288)*	*Propionibacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-191	*Propionibacterium granulosum (AJ003057)*	*Propionibacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-192	*Tessaracoccus bendigoensis (AF038504/Type sp.) Tessaracoccus oleiagri (GU111566)*	*Propionibacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-193	*Eggerthella sinensis (AY321958) Gordonibacter pamelaeae (AM886059/Type sp.) Paraeggerthella hongkongensis (AY288517/Type sp.)*	*Coriobacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-194	*Eggerthella lenta (AF292375/Type sp.)*	*Coriobacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-195	*Adlercreutzia equolifaciens (AB306661/Type sp.) Enterorhabdus mucosicola (AM747811/Type sp.)*	*Coriobacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-196	*Collinsella aerofaciens (AB011816/Type sp.)*	*Coriobacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-197	*Atopobium parvulum (CP001721) Atopobium rimae (AF292371)*	*Coriobacteriaceae*	*Actinobacteria*	*Actinobacteria*
OPU-198	*Ruminococcus faecis (FJ611794)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-199	*Ruminococcus lactaris (L76602)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-200	*uncultured bacterium (FJ509801)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-201	*Coprococcus comes (EF031542)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-202	*Clostridium glycyrrhizinilyticum (AB233029)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-203	*Ruminococcus torques (L76604)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*

OPU-204	*uncultured bacterium (DQ824050)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-205	*Clostridium nexile (X73443)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-206	*Ruminococcus gnavus (X94967)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-207	*Dorea longicatena (AJ132842)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-208	*uncultured bacterium (EU775530)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-209	*Dorea formicigenerans (L34619/Type sp.)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-210	*uncultured bacterium (DQ794633)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-211	*Clostridium scindens (AF262238)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-212	*uncultured bacterium (DQ795704)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-213	*uncultured bacterium (DQ800695)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-215	*Blautia wexlerae (EF036467)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-216	*uncultured bacterium (AY982765)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-217	*Blautia glucerasea (AB439724)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-218	*uncultured bacterium (EU778842)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-219	*Ruminococcus obeum (X85101)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-220	*Blautia luti (AJ133124)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-221	*Blautia coccoides (AB571656)* *Blautia hansenii (AB534168)* *Blautia producta (L76595)* *Blautia stercoris (HM626177)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-222	*uncultured bacterium (DQ802533)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-223	*uncultured bacterium (DQ071475)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-224	*Lactonifactor longoviformis (DQ100449/Type sp.)* *Ruminococcus gauvreauii (EF529620)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-225	*Clostridium clostridioforme (M59089)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-226	*Clostridium citroniae (DQ279737)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-227	*Clostridium sp. (AJ002591)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-228	*Clostridium bolteae (AJ508452)* *Clostridium lavalense (EF564277)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-229	*uncultured bacterium (EU510219)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-230	*Clostridium aldenense (DQ279736)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-231	*Clostridium symbiosum (M59112)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-232	*uncultured bacterium (EU531926)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-233	*uncultured bacterium (GQ491418)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-234	*uncultured bacterium (AY982766)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-235	*uncultured bacterium (DQ795209)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-236	*uncultured organism (HQ791041)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-237	*uncultured bacterium (FJ504169)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-238	*uncultured bacterium (DQ823771)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-239	*uncultured bacterium (EU767878)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-240	*uncultured organism (HQ789787)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-241	*Clostridium hathewayi (AJ311620)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-242	*Marvinbryantia formatexigens (AJ505973/Type sp.)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-243	*uncultured bacterium (EU765024)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-244	*Coprococcus catus (AB038359)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-245	*Acetivibrio ethanolgignens (FR749897)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-246	*Eubacterium hadrum (FR749932)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-247	*Anaerostipes caccae (AJ270487/Type sp.)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-248	*Eubacterium hallii (L34621)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-249	*Roseburia faecis (AY305310)* *Roseburia hominis (AJ270482)* *Roseburia intestinalis (AJ312385)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-250	*Butyrivibrio fibrisolvens (AY169412)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-251	*uncultured bacterium (EU462328)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-252	*Roseburia inulinivorans (AJ270473)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-253	*Eubacterium rectale (L34627)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-254	*uncultured bacterium (DQ793364)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-255	*uncultured bacterium (DQ793889)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-256	*Bacteroides galacturonicus (DQ497994)* *Lactobacillus rogosae (GU269544)*	*Lactobacillaceae*	*Clostridia*	*Firmicutes*
OPU-257	*Eubacterium eligens (L34420)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-258	*uncultured bacterium (DQ824130)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-259	*Eubacterium ventriosum (L34421)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-260	*uncultured bacterium (GQ898730)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-261	*uncultured bacterium (DQ797221)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-262	*uncultured bacterium (DQ824050)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-263	*uncultured bacterium (DQ799863)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-264	*uncultured bacterium (EU469607)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-265	*Butyrivibrio fibrisolvens (U41172/Type sp.)* *Butyrivibrio proteoclasticus (U37378)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*

OPU-266	*uncultured bacterium (AY980384)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-267	*Eubacterium saburreum (AB525414)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-268	*uncultured bacterium (EU469425)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-269	*Eubacterium ruminantium (AB008552)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-270	*Eubacterium xylanophilum (L34628)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-271	*uncultured bacterium (EU766736)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-272	*Howardella ureilytica (DQ925472/Type sp.)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-273	*uncultured bacterium (DQ797026)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-274	*Clostridium aminophilum (L04165)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-275	*uncultured bacterium W028 (AF125202)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-276	*Cellulosilyticum lentocellum (X71851)* *Cellulosilyticum ruminicola (EF382648)*	*Lachnospiraceae*	*Clostridia*	*Firmicutes*
OPU-277	*Clostridium lactatifermentans (AY033434)* *Clostridium neopropionicum (X76746)* *Clostridium propionicum (X77841)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-278	*Clostridium bartlettii (AY438672)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-279	*Clostridium lituseburense (M59107)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-280	*Clostridium glycolicum (X76750)* *Clostridium mayombei (FR733682)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-281	*Clostridium difficile (AB075770)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-282	*Peptostreptococcus anaerobius (AY326462/Type sp.)* *Peptostreptococcus stomatis (DQ160208)*	*Peptostreptococcaceae*	*Clostridia*	*Firmicutes*
OPU-283	*Eubacterium brachy (Z36272)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-284	*Anaerovorax odorimutans (AJ251215/Type sp.)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-285	*Eubacterium infirmum (U13039)* *Eubacterium sulci (AJ006963)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-286	*Peptoniphilus gorbachii (DQ911241)* *Peptoniphilus harei (Y07839)* *Peptoniphilus olsenii (DQ911242)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-287	*Anaerococcus octavius (Y07841)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-288	*uncultured Clostridiales bacterium (EF419367)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-289	*Parvimonas micra (AY323523/Type sp.)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-290	*Faecalibacterium prausnitzii (AJ413954/Type sp.)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-291	*Gemmiger formicilis (GU562446/Type sp.)* *Subdoligranulum variabile (AJ518869/Type sp.)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-292	*uncultured bacterium (EU475053)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-293	*uncultured bacterium (DQ810046)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-294	*uncultured bacterium (FJ511830)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-295	*Eubacterium siraeum (L34625)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-296	*Clostridium leptum (AJ305238)* *Clostridium sporosphaeroides (X66002)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-297	*Ruminococcus bromii (L76600)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-298	*Eubacterium coprostanoligenes (HM037995)*	*Eubacteriaceae*	*Clostridia*	*Firmicutes*
OPU-299	*Ruminococcus albus (L76598)* *Ruminococcus callidus (L76596)* *Ruminococcus champanellensis (AJ515913)* *Ruminococcus flavefaciens (L76603/Type sp.)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-300	*Anaerotruncus colihominis (AJ315980/Type sp.)*	*Ruminococcaceae*	*Clostridia*	*Firmicutes*
OPU-301	*Clostridium cellulosi (L09177)* *Ethanoligenens harbinense (AY295777/Type sp.)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-302	*uncultured bacterium (EF404944)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-303	*Flavonifractor plautii (AY724678/Type sp.)*	*Unclassified Clostridiales*	*Clostridia*	*Firmicutes*
OPU-304	*uncultured bacterium (DQ796985)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-305	*uncultured bacterium (EU462423)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-306	*Oscillibacter valericigenes (AB238598/Type sp.)*	*Oscillospiraceae*	*Clostridia*	*Firmicutes*
OPU-307	*uncultured bacterium (DQ797232)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-308	*uncultured bacterium (DQ793223)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-309	*uncultured organism (HQ759228)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-310	*uncultured organism (HQ759228)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-311	*uncultured bacterium (FJ366843)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-312	*uncultured bacterium (FJ363126)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-313	*uncultured bacterium (GQ897604)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-314	*Butyricicoccus pullicaecorum (EU410376/Type sp.)* *Eubacterium desmolans (L34618)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-315	*uncultured Clostridiales bacterium (AM420036)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-316	*uncultured bacterium (EF403120)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-317	*uncultured bacterium (DQ797210)*	*Uncultured Firmicutes*	*Clostridia*	*Firmicutes*
OPU-318	*Clostridium celatum (X77844)* *Clostridium disporicum (Y18176)* *Clostridium quinii (X76745)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*

OPU-319	*Clostridium paraputrificum (X75907) Clostridium vincentii (X97432)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-320	*Clostridium perfringens (CP000246)*	*Clostridiaceae*	*Clostridia*	*Firmicutes*
OPU-321	*Mahella australiensis (AY331143/Type sp.)*	*Thermoanaerobacterales*	*Clostridia*	*Firmicutes*
OPU-322	*Christensenella minuta (AB490809/Type sp.)*	*Christensenellaceae*	*Clostridia*	*Firmicutes*
OPU-323	*Bacillus muralis (AJ316309) Bacillus psychrosaccharolyticus (AB021195)*	*Bacillaceae*	*Bacilli*	*Firmicutes*
OPU-324	*Bacillus cereus (AE016877) Bacillus thuringiensis (D16281)*	*Bacillaceae*	*Bacilli*	*Firmicutes*
OPU-325	*Staphylococcus aureus subsp. aureus (L36472/Type sp.) Staphylococcus capitis subsp. capitis (L37599)*	*Staphylococcaceae*	*Bacilli*	*Firmicutes*
OPU-326	*Streptococcus infantis (AY485603)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-327	*Streptococcus lactarius (GU045364) Streptococcus peroris (AB008314)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-328	*Streptococcus sanguinis (AF003928)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-329	*Streptococcus parasanguinis (AF003933)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-330	*uncultured bacterium (FJ558307)*	*Uncultured Firmicutes*	*Bacilli*	*Firmicutes*
OPU-331	*Streptococcus gordonii (AF003931)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-332	*Streptococcus salivarius subsp. salivarius (AY188352) Streptococcus salivarius subsp. thermophilus (AY188354)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-333	*uncultured bacterium (EU473173)*	*Uncultured Firmicutes*	*Bacilli*	*Firmicutes*
OPU-334	*Streptococcus anginosus (AF104678) Streptococcus massiliensis (AY769997)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-335	*Streptococcus mutans (AY188348)*	*Streptococcaceae*	*Bacilli*	*Firmicutes*
OPU-336	*Enterococcus faecalis (AB012212/Type sp.) Vagococcus fluvialis (Y18098/Type sp.)*	*Enterococcaceae*	*Bacilli*	*Firmicutes*
OPU-337	*Granulicatella adiacens (D50540/Type sp.) Granulicatella balaenopterae (Y16547) Granulicatella elegans (AF016390)*	*Carnobacteriaceae*	*Bacilli*	*Firmicutes*
OPU-338	*Abiotrophia defectiva (D50541/Type sp.)*	*Aerococcaceae*	*Bacilli*	*Firmicutes*
OPU-339	*Gemella haemolysans (L14326/Type sp.) Gemella morbillorum (L14327)*	*Unclassified Bacillales*	*Bacilli*	*Firmicutes*

7. Prokaryotic microbiota in the digestive cavity of the jellyfish *Cotylorhiza tuberculata*

Table A 7.1 Primer pair sequences used.

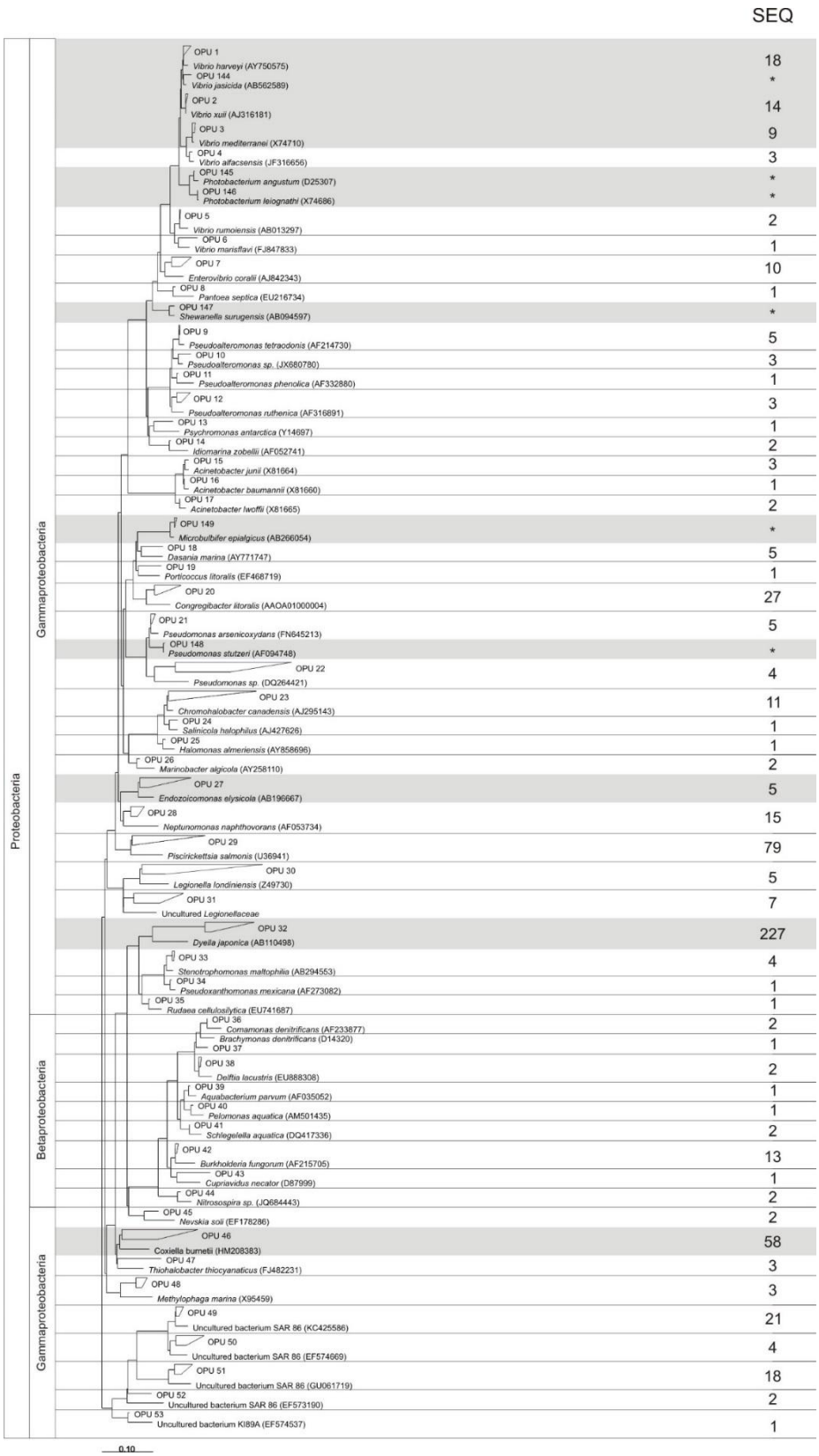
First amplification

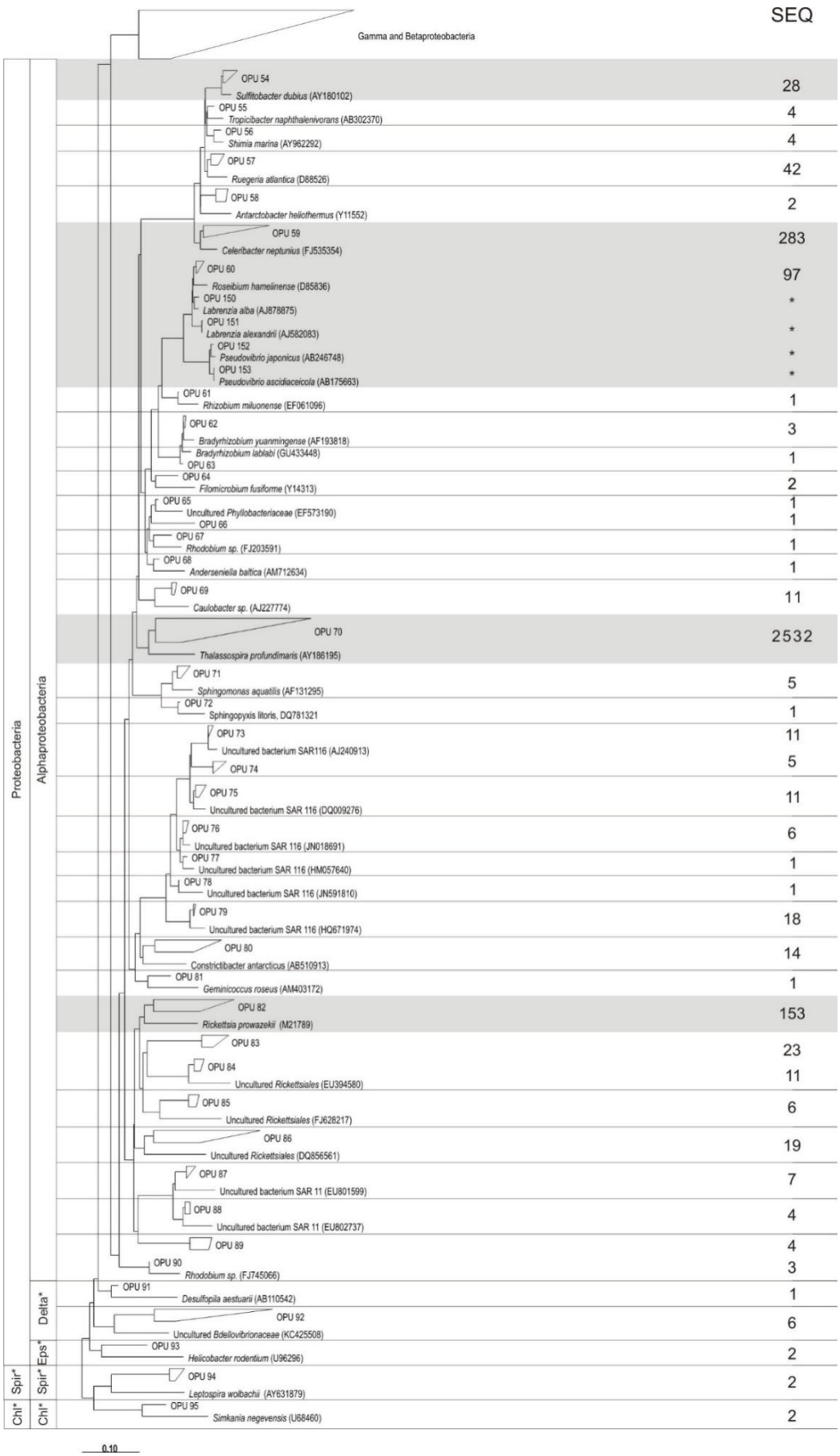
	Sequence (5' → 3')
GM3 *	AGAGTTTGATCMTGGC
630R *	CADAAAGGAGGTGATCC
S *	GGTACCTTGTTACGACTT
21F **	TTCCGGTTGATCCTGCCGGA
1492R **	TACGGYTACCTTGTTACG

Second amplification

	Sequence (5' → 3')			
	Adaptor	Key	MID	Primer
GM3-PS *	CCTATCCCCTGTGTGCCTTGGCAGTC	TCAG	-	AGAGTTTGATCMTGGC
907-PS *	CCATCTCATCCCTGCGTGTCTCCGAC	TCAG	-	CCGTCAATTCMTTTGAGTT
Sample				
M1BT			ACGAGTGCGT	
M1BF			ACGCTCGACA	
M2BT			AGACGCACTC	
M2BF			AGCACTGTAG	
M3BF			TACTGAGCTA	
M4BT			ATATCGCGAG	
M4BF			CGTGTCTCTA	

**Bacteria* and ***Archaea* primers


SEQ
Proteobacteria
Gammaproteobacteria
Betaproteobacteria
Gammaproteobacteria
OPU 1
Vibrio harveyi (AY750575)
OPU 144
Vibrio jasicida (AB562589)
18
*
OPU 2
Vibrio xuii (AJ316181)
14
OPU 3
Vibrio mediterranei (X74710)
9
OPU 4
Vibrio alfacsensis (JF316656)
3
OPU 145
Photobacterium angustum (D25307)
*
OPU 146
Photobacterium leiognathi (X74686)
*
OPU 5
Vibrio rumoiensis (AB013297)
2
OPU 6
Vibrio marisflavi (FJ847833)
1
OPU 7
Enterovibrio coralii (AJ842343)
10
OPU 8
Pantoea septica (EU216734)
1
OPU 147
Shewanella surugensis (AB094597)
*
OPU 9
Pseudoalteromonas tetraodonis (AF214730)
5
OPU 10
Pseudoalteromonas sp. (JX680780)
3
OPU 11
Pseudoalteromonas phenolica (AF332880)
1
OPU 12
Pseudoalteromonas ruthenica (AF316891)
3
OPU 13
Psychromonas antarctica (Y14697)
1
OPU 14
Idiomarina zobellii (AF052741)
2
OPU 15
Acinetobacter junii (X81664)
3
OPU 16
Acinetobacter baumannii (X81660)
1
OPU 17
Acinetobacter lwoffii (X81665)
2
OPU 149
Microbulbifer epialgicus (AB266054)
*
OPU 18
Dasania marina (AY771747)
5
OPU 19
Porticoccus litoralis (EF468719)
1
OPU 20
Congregibacter litoralis (AAOA01000004)
27
OPU 21
Pseudomonas arsenicoxydans (FN645213)
5
OPU 148
Pseudomonas stutzeri (AF094748)
*
OPU 22
Pseudomonas sp. (DQ264421)
4
OPU 23
Chromohalobacter canadensis (AJ295143)
11
OPU 24
Salinicola halophilus (AJ427626)
1
OPU 25
Halomonas almeriensis (AY858696)
1
OPU 26
Marinobacter algicola (AY258110)
2
OPU 27
Endozoicomonas elysicola (AB196667)
5
OPU 28
Neptunomonas naphthovorans (AF053734)
15
OPU 29
Piscirickettsia salmonis (U36941)
79
OPU 30
Legionella londiniensis (Z49730)
5
OPU 31
Uncultured Legionellaceae
7
OPU 32
Dyella japonica (AB110498)
227
OPU 33
Stenotrophomonas maltophilia (AB294553)
4
OPU 34
Pseudoxanthomonas mexicana (AF273082)
1
OPU 35
Rudaea cellulosilytica (EU741687)
1
OPU 36
Comamonas denitrificans (AF233877)
2
Brachymonas denitrificans (D14320)
OPU 37
1
OPU 38
Delftia lacustris (EU888308)
2
OPU 39
Aquabacterium parvum (AF035052)
1
OPU 40
Pelomonas aquatica (AM501435)
1
OPU 41
Schlegelella aquatica (DQ417336)
2
OPU 42
Burkholderia fungorum (AF215705)
13
OPU 43
Cupriavidus necator (D87999)
1
OPU 44
Nitrosospira sp. (JQ684443)
2
OPU 45
Nevskia soli (EF178286)
2
OPU 46
Coxiella burnetii (HM208383)
58
OPU 47
Thiohalobacter thiocyanaticus (FJ482231)
3
OPU 48
Methylophaga marina (X95459)
3
OPU 49
Uncultured bacterium SAR 86 (KC425586)
21
OPU 50
Uncultured bacterium SAR 86 (EF574669)
4
OPU 51
Uncultured bacterium SAR 86 (GU061719)
18
OPU 52
Uncultured bacterium SAR 86 (EF573190)
2
OPU 53
Uncultured bacterium KI89A (EF574537)
1
0.10


SEQ
Gamma and Betaproteobacteria
Proteobacteria
Alphaproteobacteria
Delta*
Eps*
Spir*
Spir*
Chl*
Chl*
OPU 54
Sulfitobacter dubius (AY180102)
28
OPU 55
Tropicibacter naphthalenivorans (AB302370)
4
OPU 56
Shimia marina (AY962292)
4
OPU 57
Ruegeria atlantica (D88526)
42
OPU 58
Antarctobacter heliothermus (Y11552)
2
OPU 59
Celeribacter neptunius (FJ535354)
283
OPU 60
Roseibium hamelinense (D85836)
97
OPU 150
Labrenzia alba (AJ878875)
*
OPU 151
Labrenzia alexandrii (AJ582083)
*
OPU 152
Pseudovibrio japonicus (AB246748)
*
OPU 153
Pseudovibrio ascidiaceicola (AB175663)
*
OPU 61
Rhizobium miluonense (EF061096)
1
OPU 62
Bradyrhizobium yuanmingense (AF193818)
3
Bradyrhizobium lablabi (GU433448)
1
OPU 63
OPU 64
Filomicrobium fusiforme (Y14313)
2
OPU 65
1
Uncultured Phyllobacteriaceae (EF573190)
OPU 66
1
OPU 67
Rhodobium sp. (FJ203591)
1
OPU 68
Anderseniella baltica (AM712634)
1
OPU 69
Caulobacter sp. (AJ227774)
11
OPU 70
Thalassospira profundimaris (AY186195)
2532
OPU 71
Sphingomonas aquatilis (AF131295)
5
OPU 72
Sphingopyxis litoris, DQ781321
1
OPU 73
Uncultured bacterium SAR116 (AJ240913)
11
OPU 74
5
OPU 75
Uncultured bacterium SAR 116 (DQ009276)
11
OPU 76
Uncultured bacterium SAR 116 (JN018691)
6
OPU 77
Uncultured bacterium SAR 116 (HM057640)
1
OPU 78
Uncultured bacterium SAR 116 (JN591810)
1
OPU 79
Uncultured bacterium SAR 116 (HQ671974)
18
OPU 80
Constrictibacter antarcticus (AB510913)
14
OPU 81
Geminicoccus roseus (AM403172)
1
OPU 82
Rickettsia prowazekii (M21789)
153
OPU 83
23
OPU 84
Uncultured Rickettsiales (EU394580)
11
OPU 85
Uncultured Rickettsiales (FJ628217)
6
OPU 86
Uncultured Rickettsiales (DQ856561)
19
OPU 87
Uncultured bacterium SAR 11 (EU801599)
7
OPU 88
Uncultured bacterium SAR 11 (EU802737)
4
OPU 89
4
OPU 90
Rhodobium sp. (FJ745066)
3
OPU 91
Desulfopila aestuarii (AB110542)
1
OPU 92
Uncultured Bdellovibrionaceae (KC425508)
6
OPU 93
Helicobacter rodentium (U96296)
2
OPU 94
Leptospira wolbachii (AY631879)
2
OPU 95
Simkania negevensis (U68460)
2
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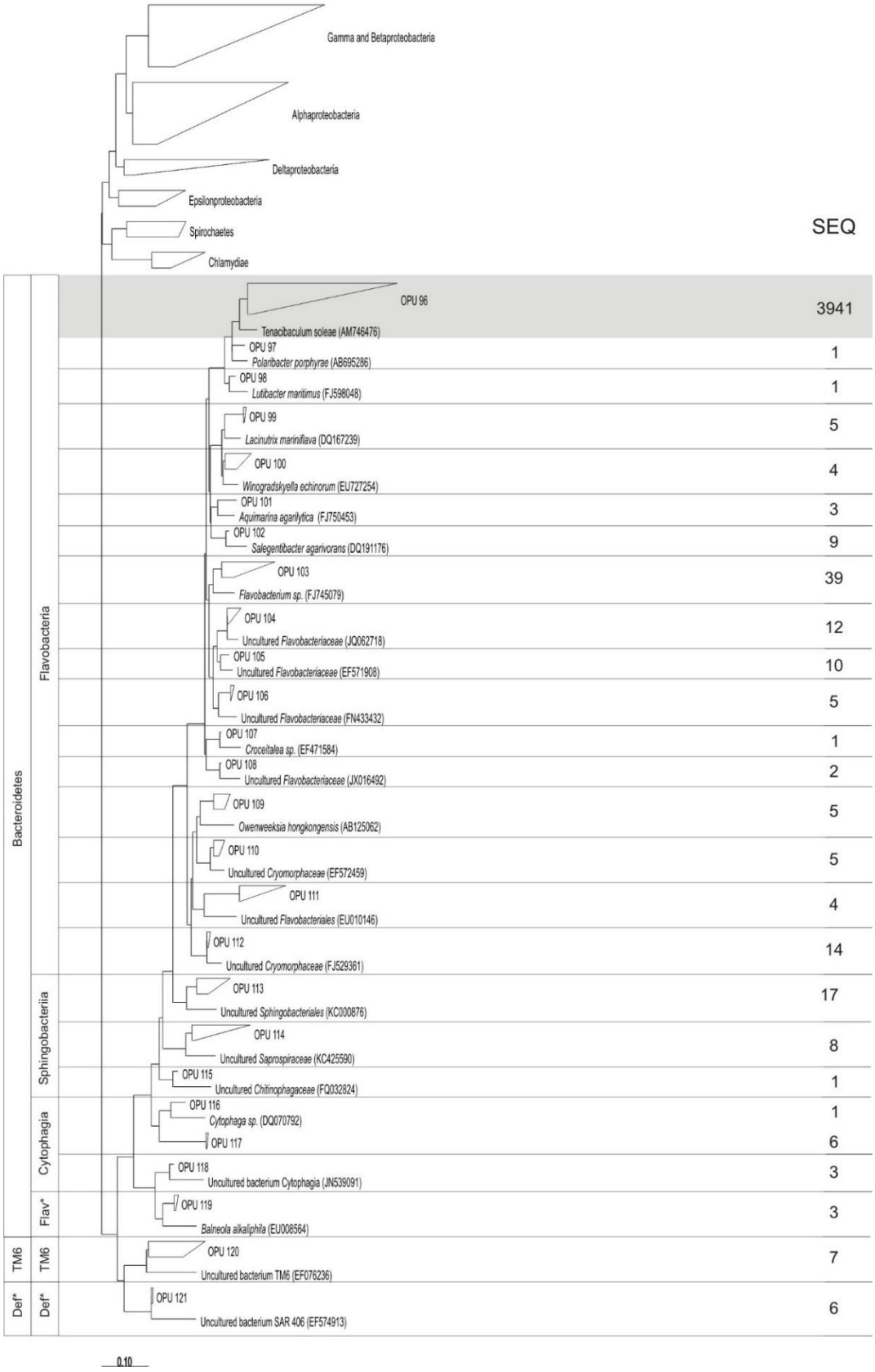
Gamma and Betaproteobacteria
Alphaproteobacteria
Deltaproteobacteria
Epsilonproteobacteria
Spirochaetes
Chlamydiae
SEQ
Bacteroidetes
Flavobacteria
Sphingobacteriia
Cytophagia
Flav*
TM6
Def*
OPU 96
3941
Tenacibaculum soleae (AM746476)
OPU 97
Polaribacter porphyrae (AB695286)
1
OPU 98
Lutibacter maritimus (FJ598048)
1
OPU 99
Lacinutrix marinifiava (DQ167239)
5
OPU 100
Winogradskyella echinorum (EU727254)
4
OPU 101
Aquimarina agarilytica (FJ750453)
3
OPU 102
Salegentibacter agarivorans (DQ191176)
9
OPU 103
Flavobacterium sp. (FJ745079)
39
OPU 104
Uncultured Flavobacteriaceae (JQ062718)
12
OPU 105
Uncultured Flavobacteriaceae (EF571908)
10
OPU 106
Uncultured Flavobacteriaceae (FN433432)
5
OPU 107
Croceitalea sp. (EF471584)
1
OPU 108
Uncultured Flavobacteriaceae (JX016492)
2
OPU 109
Owenweeksia hongkongensis (AB125062)
5
OPU 110
Uncultured Cryomorphaceae (EF572459)
5
OPU 111
Uncultured Flavobacteriales (EU010146)
4
OPU 112
Uncultured Cryomorphaceae (FJ529361)
14
OPU 113
Uncultured Sphingobacteriales (KC000876)
17
OPU 114
Uncultured Saprospiraceae (KC425590)
8
OPU 115
Uncultured Chitinophagaceae (FQ032824)
1
OPU 116
Cytophaga sp. (DQ070792)
1
OPU 117
6
OPU 118
Uncultured bacterium Cytophagia (JN539091)
3
OPU 119
Balneola alkaliphila (EU008564)
3
OPU 120
Uncultured bacterium TM6 (EF076236)
7
OPU 121
Uncultured bacterium SAR 406 (EF574913)
6
0.10

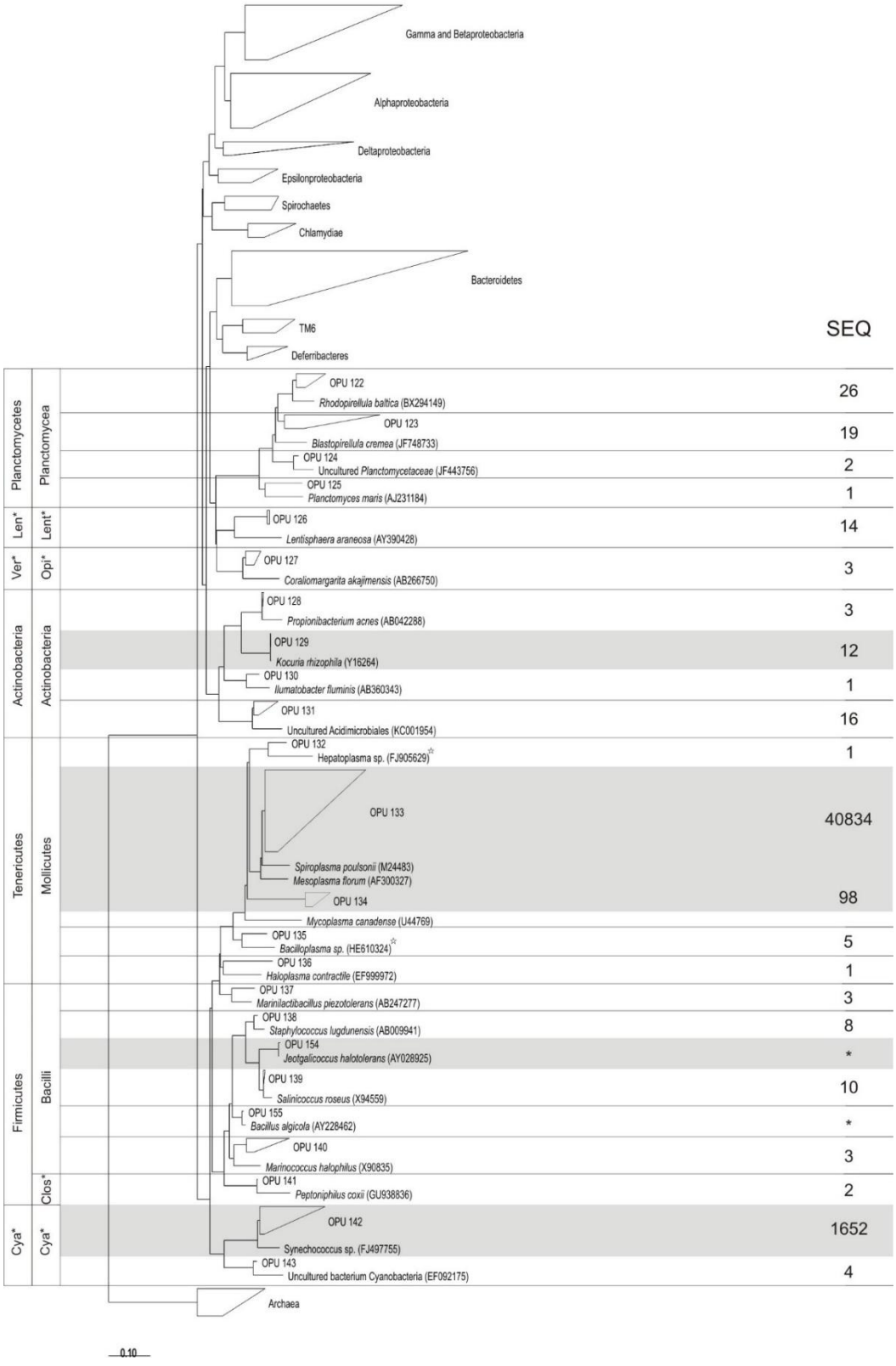

Figure A 7.1 Phylogenetic affiliation of the representative sequences of each OTU observed in this study, and recognition of the distinct OPUs in the phylogenetic tree. The right column indicates the number of sequences enclosed in each OPU. * indicates that the respective OPU was only recovered by culture, but their presence was not detected in the pyrosequencing approach. Shaded in grey are highlighted those most relevant groups also included in Figure 1 of the main text. This figure shows the tree branch comprising *Gamma-* and *Betaproteobacteria.*

Table A 7.2 List of OPUs indicating the affiliation: phylum, class, family and species (access number of the closest relative sequence, and the identity value of the representatives with the closest sequence.

		BT			BF			
		M1	M2	M4	M1	M2	M3	M4
OPU 1	*Proteobacteria, Gammaproteobacteria, Vibrionaceae* *Vibrio harveyi* (AY750575), >99%	0.11	0.00	0.00	0.00	0.00	0.03	0.00
OPU 2	*Proteobacteria, Gammaproteobacteria, Vibrionaceae* *Vibrio xuii* (AJ316181), >99%	0.02	0.00	0.07	0.00	0.00	0.00	0.00
OPU 3	*Proteobacteria, Gammaproteobacteria, Vibrionaceae* *Vibrio mediterranei* (HM771351), >99%	0.00	0.00	0.06	0.00	0.00	0.00	0.00
OPU 4	*Proteobacteria, Gammaproteobacteria, Vibrionaceae* *Vibrio alfacsensis* (JF316656), >99%	0.00	0.00	0.02	0.00	0.00	0.00	0.00
OPU 5	*Proteobacteria, Gammaproteobacteria, Vibrionaceae* *Vibrio rumoiensis* (AB013297), >99%	0.00	0.00	0.00	0.01	0.05	0.00	0.00
OPU 6	*Proteobacteria, Gammaproteobacteria, Vibrionaceae* *Vibrio marisflavi* (FJ847833), >95%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 7	*Proteobacteria, Gammaproteobacteria, Vibrionaceae* *Enterovibrio coralii* (AJ842343), >95%	0.00	0.00	0.07	0.00	0.00	0.00	0.00
OPU 8	*Proteobacteria, Gammaproteobacteria, Enterobacteriaceae* *Pantoea septica* (EU216734), >99%	0.00	0.00	0.00	0.00	0.05	0.00	0.00
OPU 9	*Proteobacteria, Gammaproteobacteria, Pseudoalteromonadaceae* *Pseudoalteromonas tetraodonis* (AF214730), >99%	0.02	0.00	0.01	0.00	0.00	0.00	0.00
OPU 10	*Proteobacteria, Gammaproteobacteria, Pseudoalteromonadaceae* Pseudoalteromonas sp. (FX680780), >96%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 11	*Proteobacteria, Gammaproteobacteria, Pseudoalteromonadaceae* *Pseudoalteromonas phenolica* (AF332880), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 12	*Proteobacteria, Gammaproteobacteria, Pseudoalteromonadaceae* *Pseudoalteromonas ruthenica* (AF316891), >98%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 13	*Proteobacteria, Gammaproteobacteria, Idiomarinaceae* *Idiomarina zobellii* (AF052741), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 14	*Proteobacteria, Gammaproteobacteria, Psychromonadaceae* *Psychromonas antarctica* (Y14697), type sp., >91%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 15	*Proteobacteria, Gammaproteobacteria, Moraxellaceae* *Acinetobacter junii* (X81664), >99%	0.00	0.00	0.00	0.00	0.16	0.00	0.00
OPU 16	*Proteobacteria, Gammaproteobacteria, Moraxellaceae* *Acinetobacter baumanii* (X81660), >99%	0.00	0.00	0.01	0.00	0.00	0.00	0.00
OPU 17	*Proteobacteria, Gammaproteobacteria, Moraxellaceae* *Acinetobacter lwoffii* (X81665), >99%	0.00	0.00	0.00	0.03	0.00	0.00	0.00
OPU 18	*Proteobacteria, Gammaproteobacteria, Alteromonadaceae* *Dasania marina* (AY771747), type sp., >92%	0.00	0.00	0.03	0.00	0.00	0.00	0.00
OPU 19	*Proteobacteria, Gammaproteobacteria, Alteromonadaceae* *Porticoccus litoralis* (EF468719), type sp., >92%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 20	*Proteobacteria, Gammaproteobacteria, Alteromonadaceae* *Congregibacter litoralis* (AA0A01000004), type sp., >92-97%	0.13	0.00	0.04	0.00	0.00	0.00	0.00
OPU 21	*Proteobacteria, Gammaproteobacteria, Pseudomonadaceae* *Pseudomonas arsenicoxydans* (FN645213), >99%	0.02	0.04	0.00	0.00	0.00	0.00	0.00
OPU 22	*Proteobacteria, Gammaproteobacteria, Pseudomonadaceae* *Pseudomonas sp.* (DQ264421), >82-92%	0.01	0.00	0.01	0.01	0.00	0.00	0.02
OPU 23	*Proteobacteria, Gammaproteobacteria, Halomonadaceae* *Chromohalobacter canadensis* (AJ295143), >94-99%	0.01	0.15	0.00	0.04	0.16	0.00	0.00
OPU 24	*Proteobacteria, Gammaproteobacteria, Halomonadaceae* *Salinicola halophilus* (AJ427626), >97%	0.00	0.04	0.00	0.00	0.00	0.00	0.00
OPU 25	*Proteobacteria, Gammaproteobacteria, Halomonadaceae* *Halomonas almeriensis* (AY858696), >98%	0.00	0.04	0.00	0.00	0.00	0.00	0.00
OPU 26	*Proteobacteria, Gammaproteobacteria, Alteromonadaceae* *Marinobacter algicola* (AY258110), 99%	0.00	0.07	0.00	0.00	0.00	0.00	0.00
OPU 27	*Proteobacteria, Gammaproteobacteria, Haellaceae* *Endozoicomonas elysicola* (AB196667), *type sp.*, >96%	0.02	0.07	0.00	0.00	0.00	0.00	0.00
OPU 28	*Proteobacteria, Gammaproteobacteria, Oceanospirillaceae* *Neptunomonas naphthovorans* (AF053734), type sp., >90%	0.04	0.04	0.05	0.00	0.00	0.00	0.00
OPU 29	*Proteobacteria, Gammaproteobacteria, Piscirickettsiaceae* *Piscirickettsia salmonis* (AY498633), >85-96%	0.01	0.00	0.49	0.01	0.05	0.00	0.00
OPU 30	*Proteobacteria, Gammaproteobacteria, Legionellaceae* *Legionella londiniensis* (Z49730), >90%	0.02	0.04	0.00	0.00	0.00	0.00	0.00
OPU 31	*Proteobacteria, Gammaproteobacteria, Legionellaceae* uncultured bacterium (FJ744812), >87%	0.04	0.00	0.00	0.00	0.00	0.00	0.00
OPU 32	*Proteobacteria, Gammaproteobacteria, Xanthomonadaceae* *Dyella japonica* (AB110498), type sp., >80%	0.19	1.57	0.88	0.01	0.10	0.50	0.04
OPU 33	*Proteobacteria, Gammaproteobacteria, Xanthomonadaceae* *Stenotrophomonas maltophilia* (AB294553), type sp., >99%	0.00	0.07	0.00	0.03	0.00	0.00	0.00
OPU 34	*Proteobacteria, Gammaproteobacteria, Xanthomonadaceae* *Pseudoxanthomonas mexicana* (AF273082), >99%	0.00	0.00	0.00	0.00	0.05	0.00	0.00
OPU 35	*Proteobacteria, Gammaproteobacteria, Xanthomonadaceae* *Rudaea cellulosilytica* (EU741687), *type sp.*, >98%	0.00	0.00	0.00	0.00	0.00	0.00	0.02
OPU 36	*Proteobacteria, Betaproteobacteria, Comamonadaceae* *Comamonas denitrificans* (AF233877), >99%	0.00	0.00	0.00	0.03	0.00	0.00	0.00
OPU 37	*Proteobacteria, Betaproteobacteria, Comamonadaceae* *Brachymonas denitrificans* (D14320), >98%	0.00	0.04	0.00	0.00	0.00	0.00	0.00
OPU 38	*Proteobacteria, Betaproteobacteria, Comamonadaceae*	0.01	0.00	0.00	0.01	0.00	0.00	0.00

	Delftia lacustris (EU888308), >98%							
OPU 39	*Proteobacteria, Betaproteobacteria, Comamonadaceae Aquabacterium parvum* (AF035052), >99%	0.00	0.00	0.00	0.00	0.00	0.00	0.02
OPU 40	*Proteobacteria, Betaproteobacteria, Comamonadaceae Pelomonas aquatica* (AM501435), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 41	*Proteobacteria, Betaproteobacteria, Comamonadaceae Schlegelella aquatica* (DQ417336) >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 42	*Proteobacteria, Betaproteobacteria, Burkholderiaceae Burkholderia fungorum* (AF215705), >99%	0.00	0.15	0.06	0.00	0.00	0.00	0.00
OPU 43	*Proteobacteria, Betaproteobacteria, Burkholderiaceae Cupriavidus necator* (D87999), >93%	0.00	0.00	0.00	0.00	0.00	0.00	0.02
OPU 44	*Proteobacteria, Betaproteobacteria, Nitrosomonadaceae Nitrosospira sp.* (JQ684443), >98%	0.00	0.00	0.01	0.00	0.00	0.00	0.00
OPU 45	*Proteobacteria, Gammaproteobacteria, Nevskiaceae Nevskia soli* (EF178286), >93%	0.00	0.00	0.01	0.00	0.00	0.00	0.00
OPU 46	*Proteobacteria, Gammaproteobacteria, Coxiellaceae Coxiella burnetii* (HM208383), >86-92%	0.19	0.15	0.15	0.00	0.00	0.00	0.02
OPU 47	*Proteobacteria, Gammaproteobacteria, Chromatiaceae Thiohalobacter thiocyanaticus* (FJ482231), >90%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 48	*Proteobacteria, Gammaproteobacteria, Piscirickettsiaceae Methylophaga marina* (X95459), *type sp*., >95%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 49	*Proteobacteria, Gammaproteobacteria,* SAR86 *Uncultured bacterium* (KC425586), >98%	0.07	0.30	0.01	0.00	0.00	0.00	0.00
OPU 50	*Proteobacteria, Gammaproteobacteria,* SAR86 *Uncultured bacterium* (EF574669), >93-97%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 51	*Proteobacteria, Gammaproteobacteria,* SAR86 *Uncultured bacterium* (GU061719), >96%	0.08	0.00	0.03	0.00	0.00	0.03	0.00
OPU 52	*Proteobacteria, Gammaproteobacteria,* SAR87 *Uncultured bacterium* (EF573190), >93%	0.00	0.00	0.01	0.00	0.00	0.00	0.00
OPU 53	*Proteobacteria, Gammaproteobacteria,* KI89A *Uncultured bacterium* (EF574537), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 54	*Proteobacteria, Alphaproteobacteria, Rhodobacteraceae Sulfitobacter dubius* (AY180102), >96%	0.05	0.07	0.12	0.00	0.00	0.00	0.00
OPU 55	*Proteobacteria, Alphaproteobacteria, Rhodobacteraceae Tropicibacter naphtalenivorans* (AB302370), *type sp*., >99%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 56	*Proteobacteria, Alphaproteobacteria, Rhodobacteraceae Shimia marina* (AY962292), *type sp*., >98%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 57	*Proteobacteria, Alphaproteobacteria, Rhodobacteraceae Ruegeria atlantica* (D88526), *type sp*., >96%	0.14	0.00	0.06	0.00	0.00	0.32	0.00
OPU 58	*Proteobacteria, Alphaproteobacteria, Rhodobacteraceae Antarctobacter heliothermus* (Y11552), *type sp*., >97%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 59	*Proteobacteria, Alphaproteobacteria, Rhodobacteraceae Celeribacter neptunius* (FJ535354), *type sp*., >96%	1.73	0.00	0.00	0.00	0.00	0.13	0.02
OPU 60	*Proteobacteria, Alphaproteobacteria, Rhodobacteraceae Roseibium hamelinense* (D85836), >97%	0.47	0.00	0.14	0.00	0.00	0.00	0.00
OPU 61	*Proteobacteria, Alphaproteobacteria, Rhizobiaceae Rhizobium miluonense* (EF061096), >99%	0.00	0.00	0.00	0.00	0.00	0.00	0.02
OPU 62	*Proteobacteria, Alphaproteobacteria, Bradyrhizobiaceae Bradyrhizobium yuanmingense* (AF193818), >99%	0.00	0.04	0.00	0.03	0.00	0.00	0.00
OPU 63	*Proteobacteria, Alphaproteobacteria, Bradyrhizobiaceae Bradyrhizobium lablabi* (GU433448), >99%	0.00	0.00	0.00	0.00	0.05	0.00	0.00
OPU 64	*Proteobacteria,Alphaproteobacteria, Hyphomicrobiaceae Filomicrobium fusiforme* (Y14313), type sp., >94%	0.00	0.00	0.01	0.00	0.00	0.00	0.00
OPU 65	*Proteobacteria,Alphaproteobacteria, Phyllobacteriaceae Uncultured bacterium* (EF573190), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 66	*Proteobacteria,Alphaproteobacteria, Phyllobacteriaceae Uncultured bacterium* (EF573190), >91%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 67	*Proteobacteria,Alphaproteobacteria, Rhodobiaceae Rhodobium sp.* (FJ203591), >96%	0.00	0.00	0.00	0.01	0.00	0.00	0.00
OPU 68	*Proteobacteria, Alphaproteobacteria, Rhizobiaceae Anderseniella baltica* (AM712634), type sp., >97%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 69	*Proteobacteria, Alphaproteobacteria, Caulobacteraceae Caulobacter mirabilis* (AJ227774), >96%	0.02	0.00	0.00	0.10	0.00	0.00	0.00
OPU 70	*Proteobacteria,Alphaproteobacteria, Rhodospirillaceae Thalassospira profundimaris* (AY186195), >79-88%	1.37	33.06	2.71	0.16	32.82	11.16	0.32
OPU 71	*Proteobacteria,Alphaproteobacteria, Sphingomonadaceae Sphingomonas aquatilis* (AF131295), >99%	0.02	0.04	0.00	0.00	0.00	0.00	0.00
OPU 72	*Proteobacteria,Alphaproteobacteria, Sphingomonadaceae Sphingopyxis litoris* (DQ781321), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 73	*Proteobacteria,Alphaproteobacteria,* SAR116 *Uncultured bacterium* (AJ240913), >99%	0.05	0.00	0.00	0.00	0.00	0.09	0.00
OPU 74	*Proteobacteria,Alphaproteobacteria,* SAR116 *Uncultured bacterium* (AJ240913), >94%	0.02	0.04	0.00	0.00	0.00	0.00	0.00
OPU 75	*Proteobacteria,Alphaproteobacteria,* SAR116 *Uncultured bacterium* (DQ009276), >98%	0.06	0.00	0.00	0.00	0.05	0.00	0.00
OPU 76	*Proteobacteria,Alphaproteobacteria,* SAR116 *Uncultured bacterium* (JN018691), >99%	0.01	0.07	0.01	0.00	0.00	0.00	0.00
OPU 77	*Proteobacteria,Alphaproteobacteria,* SAR116 *Uncultured bacterium* (HM057640), >99%	0.00	0.04	0.00	0.00	0.00	0.00	0.00
OPU 78	*Proteobacteria,Alphaproteobacteria,* SAR116 *Uncultured bacterium* (JN591810), >99%	0.00	0.04	0.00	0.00	0.00	0.00	0.00

OPU 79	*Proteobacteria,Alphaproteobacteria,* SAR116 *Uncultured bacterium* (HQ671974), >99%	0.10	0.04	0.01	0.00	0.00	0.00	0.00
OPU 80	*Proteobacteria,Alphaproteobacteria, Rhodospirillaceae Constrictibacter antarcticus* (AB510913), >80-85%	0.05	0.00	0.01	0.00	0.00	0.13	0.00
OPU 81	*Proteobacteria,Alphaproteobacteria, * Geminicoccus roseus* (AM403172), >92%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 82	*Proteobacteria,Alphaproteobacteria, Rickettsiaceae Rickettsia prowazekii* (M21789), *type sp.*, >84-89%	0.07	0.19	0.89	0.00	0.00	0.00	0.00
OPU 83	*Proteobacteria, Alphaproteobacteria, Rickettsiales Uncultured Rickettsiales* (EU394580), >96%	0.12	0.00	0.02	0.00	0.00	0.00	0.00
OPU 84	*Proteobacteria, Alphaproteobacteria, Rickettsiales Uncultured Rickettsiales* (EU394580), >96%	0.00	0.00	0.07	0.00	0.00	0.00	0.00
OPU 85	*Proteobacteria, Alphaproteobacteria, Rickettsiales Uncultured Rickettsiales* (FJ628217), >90%	0.00	0.00	0.04	0.00	0.00	0.00	0.00
OPU 86	*Proteobacteria, Alphaproteobacteria, Rickettsiales Uncultured Rickettsiales* (DQ856561), >80%	0.06	0.00	0.06	0.00	0.00	0.00	0.00
OPU 87	*Proteobacteria,Alphaproteobacteria,* SAR11 *Uncultured bacterium* (EU801599), >97%	0.00	0.04	0.04	0.00	0.00	0.00	0.00
OPU 88	*Proteobacteria,Alphaproteobacteria,* SAR11 *Uncultured bacterium* (EU802737), >98%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 89	*Proteobacteria, Alphaproteobacteria, Rhodobiaceae Rhodobium sp.* (FJ745066), >82%	0.00	0.07	0.00	0.01	0.00	0.00	0.00
OPU 90	*Proteobacteria, Alphaproteobacteria, Rhodobiaceae Rhodobium sp.* (FJ745066), >99%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 91	*Proteobacteria, Deltaproteobacteria, Desulfobulbaceae Desulfopila aestuarii* (AB110542), type sp., >98%	0.00	0.04	0.00	0.00	0.00	0.00	0.00
OPU 92	*Proteobacteria, Deltaproteobacteria, Bdellovibrionaceae Uncultured bacterium* (KC425508), >84-97%	0.04	0.00	0.00	0.00	0.00	0.00	0.00
OPU 93	*Proteobacteria, Epsilonproteobacteria, Helicobacteraceae Helicobacter rodentium* (U96296), >89%	0.00	0.00	0.01	0.00	0.00	0.00	0.00
OPU 94	*Spirochaetes, Spirochaetes, Leptospiraceae Leptospira wolbachii* (AY631879), >86%	0.00	0.00	0.01	0.00	0.00	0.03	0.00
OPU 95	*Chlamydiae, Chlamydiae, Simkaniaceae Simkania negevensis* (U68460), type sp., >93%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 96	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Tenacibaculum soleae* (AM746476), >81-96%	10.87	9.37	6.00	8.50	2.64	9.56	2.10
OPU 97	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Polaribacter porphyrae* (AB695286), >95%	0.00	0.04	0.00	0.00	0.00	0.00	0.00
OPU 98	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Lutibacter maritimus* (FJ598048), >98%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 99	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Lacinutrix mariniflava* (DQ167239), >95%	0.02	0.00	0.01	0.00	0.00	0.00	0.00
OPU 100	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Winogradskyella echinorum* (EU727254), >96%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 101	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Salegentibacter agarivorans* (DQ191176), >99%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 102	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Aquimarina agarilytica* (FJ750453), >94%	0.06	0.00	0.00	0.00	0.00	0.00	0.00
OPU 103	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Flavobacterium sp.* (FJ745079), >92-98%	0.21	0.00	0.04	0.00	0.00	0.00	0.00
OPU 104	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Uncultured bacterium* (FQ062718), >98%	0.07	0.00	0.01	0.00	0.00	0.00	0.00
OPU 105	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Uncultured bacterium* (EF571908), >98%	0.06	0.00	0.00	0.00	0.00	0.00	0.00
OPU 106	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Uncultured bacterium* (FN433432), >97%	0.01	0.04	0.01	0.00	0.00	0.00	0.00
OPU 107	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Croceitalea sp.* (EF471584), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 108	*Bacteroidetes, Flavobacteriia, Flavobacteriaceae Uncultured bacterium* (JX016492), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 109	*Bacteroidetes, Flavobacteriia, Cryomorphaceae Owenweeksia hongkongensis* (AB125062), type sp., >90%	0.02	0.04	0.00	0.00	0.00	0.00	0.00
OPU 110	*Bacteroidetes, Flavobacteriia, Cryomorphaceae Uncultured bacterium* (EF572459), type sp., >97%	0.03	0.00	0.00	0.00	0.00	0.00	0.00
OPU 111	*Bacteroidetes, Flavobacteriia, Flavobacteriales Uncultured bacterium* (EU010146), >92-99%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 112	*Bacteroidetes, Flavobacteriia, Cryomorphaceae* Owenweeksia sp. (FJ529361), >99%	0.01	0.00	0.08	0.00	0.00	0.00	0.00
OPU 113	*Bacteroidetes, Sphingobacteriia,Sphingobacteriales Uncultured bacterium* (KC000876), >91-98%	0.11	0.00	0.00	0.00	0.00	0.00	0.00
OPU 114	*Bacteroidetes, Sphingobacteriia,Saprospiraceae Uncultured bacterium* (KC425590), >87-98%	0.05	0.00	0.00	0.00	0.00	0.00	0.00
OPU 115	*Bacteroidetes, Sphingobacteriia, Chitinophagaceae Uncultured Chitinophagaceae* (FQ032824), >98%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 116	*Bacteroidetes, Cytophagia, Cytophagaceae Cytophaga sp.* (DQ070792), >97%	0.00	0.00	0.00	0.01	0.00	0.00	0.00
OPU 117	*Bacteroidetes, Cytophagia, Cytophagaceae Cytophaga sp.* (DQ070792), >87%	0.04	0.00	0.00	0.00	0.00	0.00	0.00
OPU 118	*Bacteroidetes, Cytophagia, * Uncultured bacterium* (JN539091), >99%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 119	*Bacteroidetes, Sphingobacteriia, Chitinophagaceae*	0.02	0.00	0.00	0.00	0.00	0.00	0.00

	Balneola alkaliphila (EU008564), >95%							
OPU 120	*Bacteria, TM6 ** *Uncultured bacterium* (EF076236), >88%	0.02	0.00	0.03	0.00	0.00	0.00	0.00
OPU 121	*Deferribacteres, Deferribacteres,* SAR406 *Uncultured bacterium* (EF574913), >99%	0.03	0.00	0.01	0.00	0.00	0.00	0.00
OPU 122	*Planctomycetes, Planctomycea, Planctomycetaceae* *Rhodopirellula baltica* (BX294149), type sp., >90-94%	0.12	0.00	0.04	0.00	0.00	0.00	0.00
OPU 123	*Planctomycetes, Planctomycea, Planctomycetaceae* *Blastopirellula cremea* (JF748733), >90%	0.11	0.00	0.01	0.00	0.00	0.00	0.00
OPU 124	*Planctomycetes, Planctomycea, Planctomycetaceae* *Uncultured Planctomycetales* (JF443756), >98%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 125	*Planctomycetes, Planctomycea, Planctomycetaceae* *Planctomyces maris* (AJ231184), >88%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 126	*Lentisphaerae, Lentisphaeria, Lentisphaeraceae* *Lentisphaera araneosa* (AY390428),type sp.,>87%	0.09	0.00	0.00	0.00	0.00	0.00	0.00
OPU 127	*Verrucomicrobia, Opitutae, Puniceicoccaceae* *Coraliomargarita akajimensis* (AB266750), type sp., >95%	0.02	0.00	0.00	0.00	0.00	0.00	0.00
OPU 128	*Actinobacteria, Actinobacteria, Propionibacteriaceae* *Propionibacterium acnes* (AB042288), >99%	0.01	0.04	0.00	0.01	0.00	0.00	0.00
OPU 129	*Actinobacteria, Actinobacteria, Micrococcaceae* *Kocuria rhizophila* (Y16264), >99%	0.00	0.00	0.00	0.00	0.62	0.00	0.00
OPU 130	*Actinobacteria, Acidimicrobiia, Acidimicrobiaceae* *Ilumatobacter fluminis* (AB360343), type sp., >96%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 131	*Actinobacteria, Acidimicrobiia, Acidimicrobiales* *Uncultured bacterium* (KC001954), >98%	0.07	0.00	0.03	0.00	0.00	0.00	0.00
OPU 132	*Tenericutes, Mollicutes, Entomoplasmatales* *Candidatus Hepatoplasma* (FJ905629), >92%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 133	*Tenericutes, Mollicutes, Spiroplasmataceae* *Spiroplasma poulsonii* (M24483), >85%	75.42	48.09	83.99	90.8	62.79	78.02	97.39
OPU 134	*Tenericutes, Mollicutes, Entomoplasmataceae* *Mesoplasma florum* (AF300327), type sp., >75%	0.00	3.41	0.00	0.00	0.36	0.00	0.00
OPU 135	*Tenericutes, Mollicutes, Mycoplasmataceae* *Candidatus Bacilloplasma* (HE610324), >91%	0.03	0.00	0.00	0.00	0.00	0.00	0.00
OPU 136	*Tenericutes, Mollicutes, Haloplasmataceae* *Haloplasma contractile* (EF999972), type sp., >84%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 137	*Firmicutes, Bacilli, Carnobacteriaceae* *Marinilactibacillus piezotolerans* (AB247277), >93%	0.00	0.00	0.00	0.04	0.00	0.00	0.00
OPU 138	*Firmicutes, Bacilli, Staphylococcaceae* *Stapylococcus lugdunensis* (AB009941), >99%	0.05	0.00	0.00	0.00	0.00	0.00	0.00
OPU 139	*Firmicutes, Bacilli, Staphylococcaceae* *Salinicoccus roseus* (X94559), *type sp.*, >99%	0.03	0.00	0.03	0.00	0.00	0.00	0.00
OPU 140	*Firmicutes, Bacilli, Bacillaceae* *Marinococcus halophilus* (X90835), type sp., >87%	0.01	0.04	0.00	0.00	0.00	0.00	0.00
OPU 141	*Firmicutes, Clostridia, Clostridiales* *Peptoniphilus coxii* (GU938836), >99%	0.01	0.00	0.00	0.00	0.00	0.00	0.00
OPU 142	*Cyanobacteria, Cyanobacteria, Family I* *Synechococcus sp.* (FJ497755), >93-99%	6.53	2.40	3.47	0.09	0.05	0.00	0.00
OPU 143	*Cyanobacteria, Cyanobacteria, ** *Uncultured bacterium* (EF092175), >98%	0.00	0.00	0.03	0.00	0.00	0.00	0.00

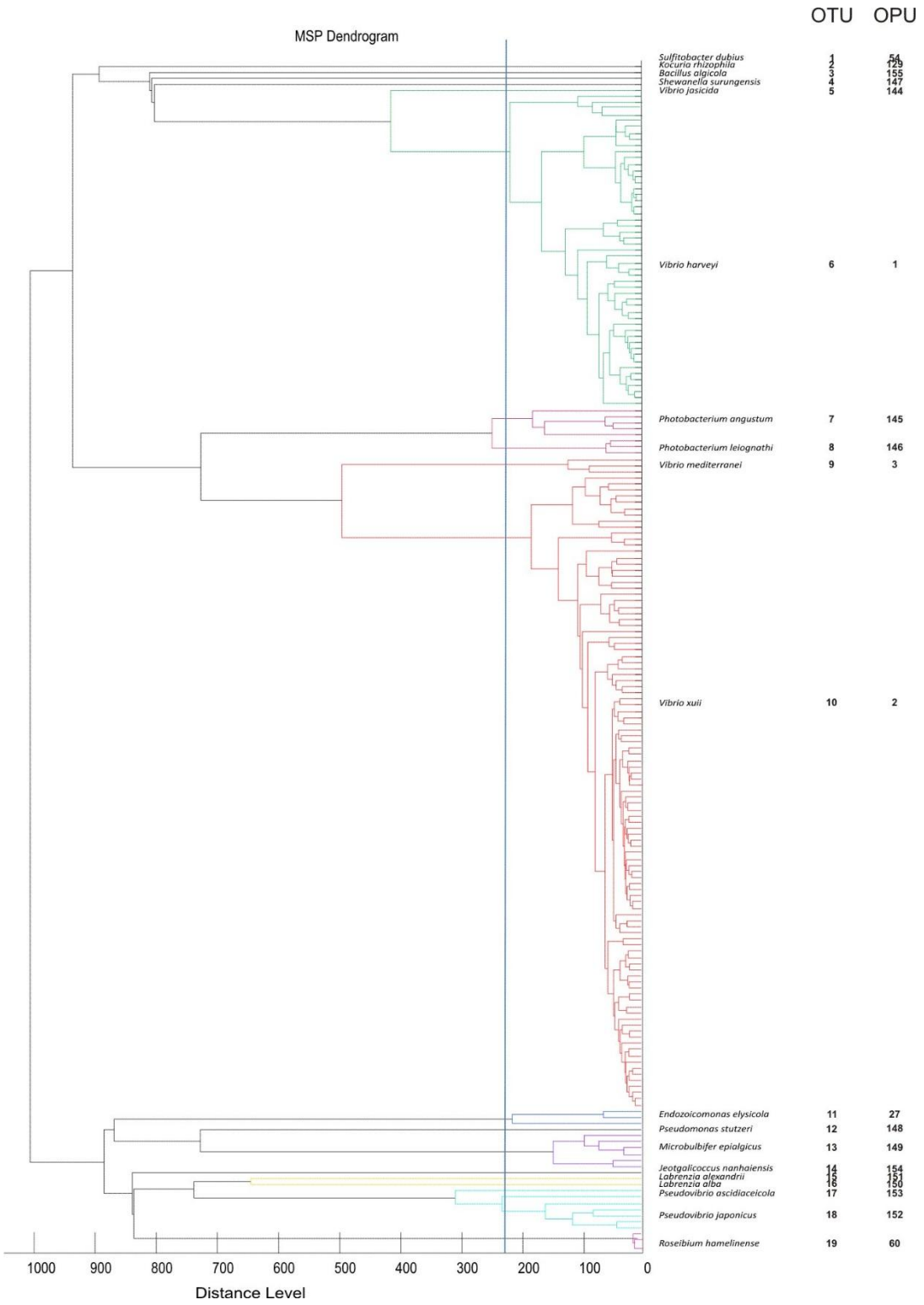

Figure A 7.2 Dendrogram based on the 190 MALDI-TOF MS spectra generated. The identification of each cluster is given in the left column, the OTU numbering given in this study in the middle column and the corresponding OPU in the main tree in the left column.

Table A 7.3 Diversity indices

		Dominance (D)	Shannon (H')	Chao-1
Pyrosequencing	M1BT	0.59	1.05	117.2
	M1BF	0.83	0.35	27.2
	M2BT	0.35	1.36	60.43
	M2BF	0.50	0.86	25.5
	M3BF	0.63	0.74	14
	M4BT	0.71	0.76	51.75
	M4BF	0.95	0.14	21.5
cultures	M1	0.53	1.04	13
	M2	0.43	1.39	10.6
	M3	0.4	1.4	31
	M4	0.65	0.88	15.5

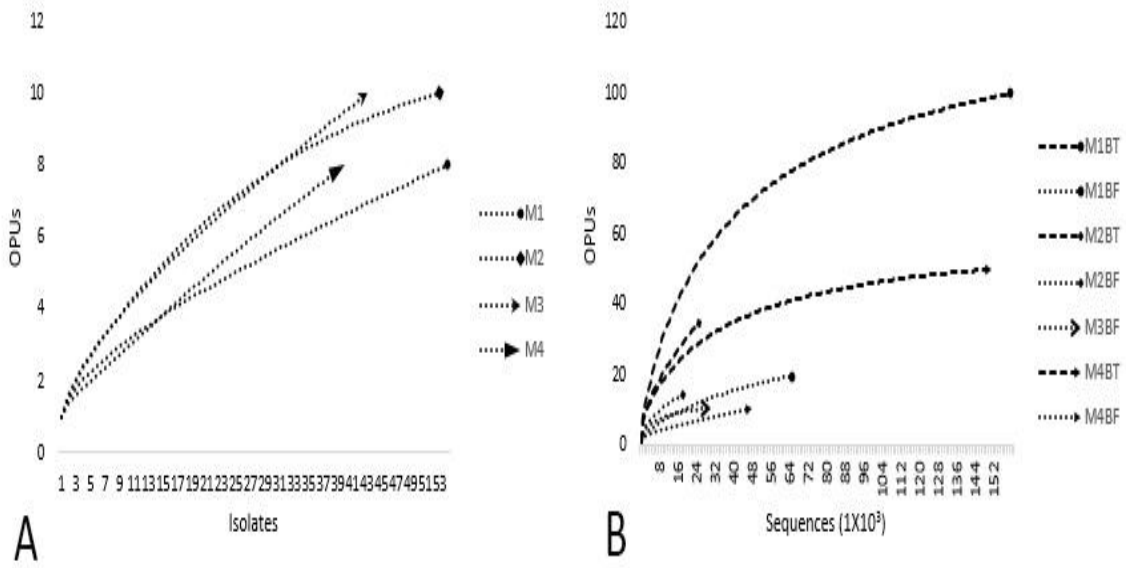

Figure A 7.3 Rarefaction curves of the culturable fraction (A) and pyrosequencing data (B) for the distinct samples studied.

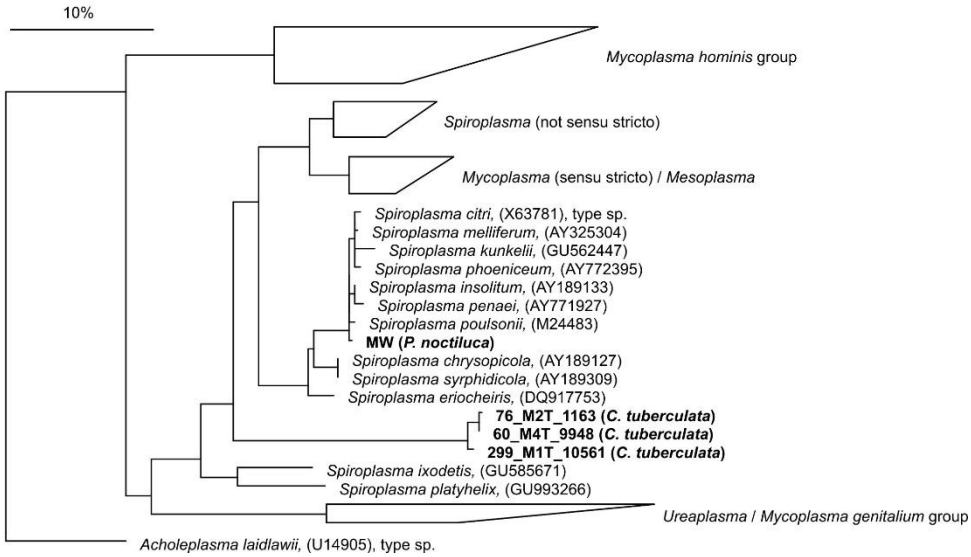

Figure A 7.4 Phylogenetic tree based on neighbor joining algorithm with all members of the *Mollicutes* class. The representative sequences (about 800 nuc) of the *Cothyloriza. tuberculata* corresponding to the *Spiroplasma* had been inserted using the parsimony tool implemented in ARB software.

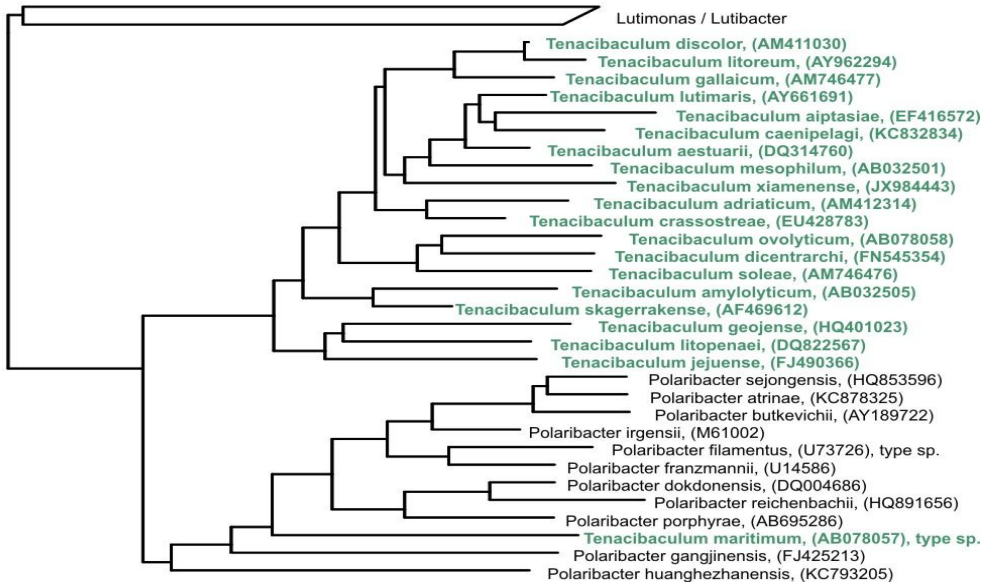

Figure A 7.5 Phylogenetic reconstruction showing the members of the genus *Tenacibaculum* and *Polaribacter*, and especially showing a paraphyletic nature of the genus *Tenacibaculum* in where the type species affiliates differently from the rest of species.

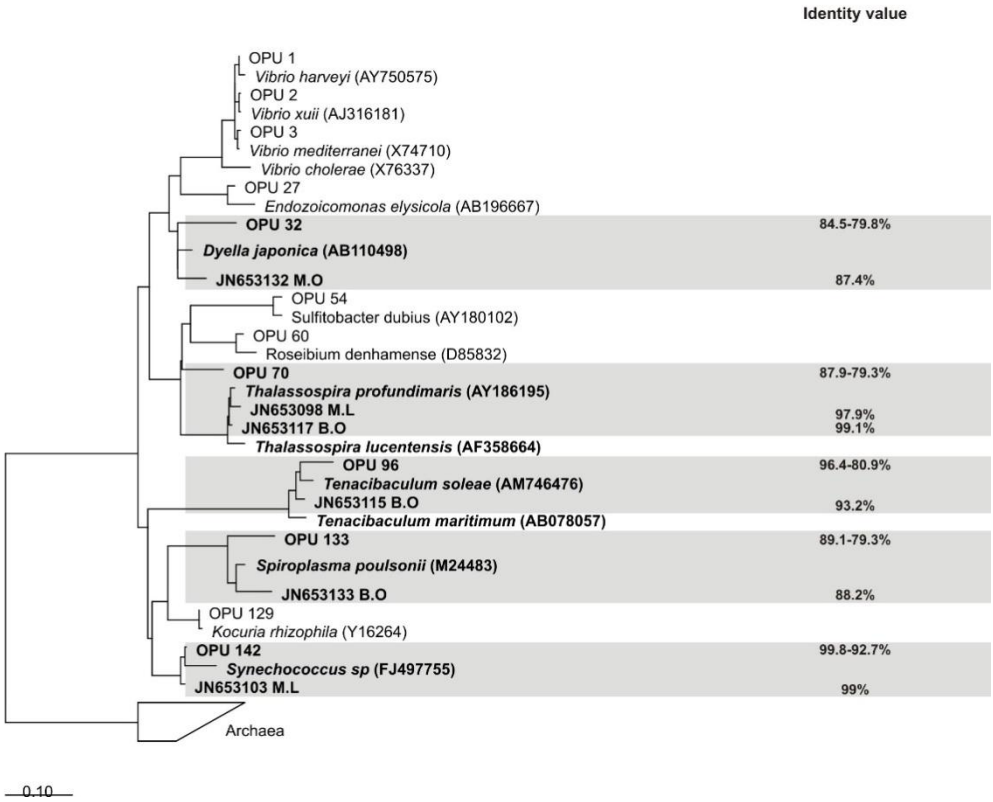

Figure A 7.6 Phylogenetic tree based on neighbor joining algorithm with sequences of the *Cothyloriza tuberculata* coincident by pyrosequencing and cultures. In addition, the sequences (about 400 nuc) affiliated with interest phyla of two ctenophores *M. leidyi* (M.L) and *B. ovata* (B.O). All partial sequences had been inserted using the parsimony tool implemented in ARB. Identity value (right column) were obtained to compare reference sequences (marked in grey) and sequences of interest.

Annex and appendices´ list of figures and tables

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Kol Alel

Mis primeros meses en Mallorca fueron sin duda alguna peculiares, desde llegar al laboratorio más mojada que una galleta de animalito nadando en café debido a una tormenta; descubrir que uno de mis objetos de estudio estaba lejos de ser una linda orquídea y pareciéndose más a lo que en un eufemismo diríamos "estéticamente diferente"; sufrir un par de actos xenófobos; mi nombre pocas veces bien pronunciado (todavía me enojo), no.. no me llamo M**é**rit, me llamo **Merit**; pasando por no entender por qué las personas dicen "i griega" y no "ye" (http://www.rae.es/consultas/un-solo-nombre-para-cada-letra); de preguntarme por qué se usa tanto la ironía (capacidad que tenía prácticamente anulada); ese café tan cargado; descubrir que necesito tener más cuidado con el pan (con tantas semillas) que con el *Heloderma* de mi *ransho*; ese frío abrumador de otoño-invierno y primavera (si, la única estación en el que no tengo frío es en verano) y decenas de cosas más. Sin embargo, con el paso de los meses y posteriormente de los años, esta isla, este país me dejó ver su hermosura. Gracias Mallorca y a cada persona que conocí aquí por las experiencias que me han dado, por los amigos, las risas (no, no soy de las que agradecen las lágrimas, a esas feas no les debo ningún gracias), por los paisajes que mis ojos han disfrutado, por la comida (integrante oficial del club: "defensores del pan moreno"), por haberme puesto en bandeja de plata el amor, ese que encontré en el edificio contiguo y que afortunadamente se quedó a mi lado (creo que le di T.D.C.). Y si bien, mis ratos poco agradables también suman en la lista, en el recuento de los daños, sin duda alguna, me siento afortunada...si, afortunada.

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Sin duda alguna, todo esto no hubiera sido posible sin las personas que me han acompañado desde siempre (ahh.. es que no se podían librar de mí): mami, últimamente me pregunto ¿no te agota quererme? tengo tres décadas y aún te necesito como el primer día. Longis, aún sin muchas palabras, los gritos de tu corazón cruzan el océano y los escucho tan claros como el mejor de tus discursos. Polito, no somos gemelos, pero créeme, mi alma está entretejida a la tuya desde que naciste, no podría pedir un mejor ángel. Gracias a los tres, no por hoy, no por este trabajo, sino por todo lo que hubo antes y lo que habrá en el futuro.

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Notes and errata