Investigating extremotolerant microbes in non-extreme environments and altering the salinity growth limits of halophiles

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Abstract

In this thesis three key questions were addressed: 1) what are the physiological limits of extremophilic/extremotolerant microbes enriched from non-extreme environments; 2) how does the source environment (permanently hypersaline versus variable salinity) influence the development of a community enriched at different salinities; and 3) to what extent can populations adapt to grow at higher or lower salinity in a laboratory-evolution experiment. Methods included high-throughput microbial culturing, determination of microbial growth windows, microbial community analysis using Illumina MiSeq analysis of phylogenetically informative genes, and comparative genomics of evolved versus original strains. In addressing question 1, it was found that freshwater- and marine-derived microbes enriched in extreme conditions (e.g. with partially inhibitory concentrations of $ZnCl_2$, $CuSO_4$, NaCl, $MgCl_2$, sorbitol or HCl) had a significantly wider tolerance to stressors than microbes from the same samples enriched in control conditions with no stressor. However, the growth rate of nearly all stressor-enriched microbial communities was lower than the growth rate of the control-enriched communities. This suggests that being able to grow both in extreme and in non-extreme conditions comes with a trade-off for these extremotolerant microbes. For question 2, All enrichments were almost entirely overtaken by one genus by the end of the experiment. The haloarchaeon *Halorhabdus* sp. outcompeted every other microbe in enrichments from the permanently high-salt environment, while in the more variable salt-spring samples the bacterium *Halomonas* sp. dominated the low-salt enrichments, and the haloarchaeon *Haloferax* sp. outgrew other species in the mid-, and high-salt enrichments. In the laboratory evolution experiment, the bacterium *Halomonas elongata* 1H9 acquired more mutations in its genome in sub-optimal and super-optimal salinity cultures than the archaeon *Halobacterium salinarum* 91-R6, in part due to its faster growth rate and shorter generation time, and hence, higher probability of mutations. *Halomonas elongata* 1H9 also demonstrated changes in growth and adaptation to the salinity of the medium in which it was cultured.

However, further research needs to be done to identify a connection between the mutations and the evolved growth patterns. These findings support the theory that extremophiles and extremotolerants can be found far outside of their physiologically optimal habitat, are able to evolve reasonably fast to changes in environmental conditions, and verifies the ubiquitous dispersal of halophilic and halotolerant ‘microbial weed’ species.

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Abbreviations

% (w/v)	Percent of weight of solution in the total volume of solution
°C	Degree Celsius
ANOVA	Analysis of Variance
ASV	Amplicon Sequent Variant
ATP	Adenosine triphosphate
a_w	Water activity
bp	Base pair
CO_2	Carbon dioxide
$CuSO_4$	Copper sulphate
DHABs	Deep Hypersaline Anoxic Basins
DMSP	Dimethylsulfoniopropionate
DNA	deoxyribonucleic acid
$FeCl_2.4H_2O$	Iron (II) Chloride Tetrahydrate
HCl	Hydrochloric acid
ICoMM	International Census of Marine Microbes
ITS	Internal transcribed spacer region
KCl	Potassium chloride
log P_{ow}	The logarithm of the partition coefficient of the compound in an equimolar mixture of n-octanol and water, i.e. the toxicity of a solvent
M	Molar (mol/L) concentration
$MgCl_2$	Magnesium chloride
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
$MgSO_4$	Magnesium sulphate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MIC	Minimum Inhibitory Concentration

$MnCl_2.4H_2O$	Manganese (II) Chloride Tetrahydrate
MPN	Most Probable Number
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
nm	Nanometer
nMDS	Non-metric multidimensional scaling
OD	Optical density
OTU	Operational Taxonomic Unit
Pa	Pascal
PATRIC	Pathosystems Resource Integration Center
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
PGFams	PATRIC global protein families
pH	negative log10 of the hydrogen ion concentration expressed in mol/L
RAPDs	Random amplified polymorphic DNA
RI	Refractive Index
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
TAR	Taxa-area relationship
UV	Ultraviolet radiation
YE	Yeast extract
$ZnCl_2$	Zinc chloride

Declaration

This thesis is submitted to the University of Essex in support of my application for the degree of Doctor of Philosophy. I declare that the work presented in this thesis was conducted by me, under the supervision of Professor Terry McGenity, Dr Boyd McKew, and Dr Etienne Low-Decarie, with the exception of those instances where the contribution of others has been specifically acknowledged. The work presented, including data generated and data analysis, was carried out by the author except in the cases outlined below:

- The initial measurements of growth for Chapter 2 were performed by Sara Barreto and Greta Colombo
- Sample collection for Chapter 2 was performed by Russel Smart and Dr Etienne Low-Decarie
- Sample collection for Chapter 4 was carried out by Prof Terry McGenity, with the help of Thomas Edwards, Senior Exploration Geologist

Chapter 1: Introduction

1.1 Preamble

Life is thought to have originated at deep-sea hydrothermal vents on Earth (Martin *et al.*, 2008). However, when it comes to potential extraterrestrial life, the icy moons of Jupiter are quite good candidates, with their hypersaline seas having some similarities with Earth's hydrothermal vents, deep-sea brines and subglacial lakes (Peretó, 2005; Rampelotto, 2010; Dodd *et al.*, 2017; Antunes, Olsson-Francis and McGenity, 2020). For example, one of the best-known moons of Jupiter is Europa, which has a hypersaline ocean underneath its 20-30 km thick surface of ice and it is hypothesised that tidal forces and hydrothermal circulation may provide energy to sustain life in the subsurface water (Rampelotto, 2010; Antunes, Olsson-Francis and McGenity, 2020). The red planet Mars, on the other hand, is a closer and easier target for extraterrestrial research of life. More than three billion years ago, Mars had surface water and was thus habitable (Grotzinger *et al.*, 2014). Carbon, hydrogen, sulphur, nitrogen, and phosphorous were measured in sedimentary rocks in the Gale Crater on Mars, suggesting the biological viability of an ancient lake environment in the planet's past (Grotzinger *et al.*, 2014). Could the planet have sustained life on its surface in ice, inside salt crystals, in possible freezing and thawing brines, or in subsurface hypersaline aquifers for such a long time (Laye and DasSarma, 2017; Lauro *et al.*, 2021)? Are extreme microbes capable of surviving the various stress conditions that come with a planet with a very thin atmosphere, sub-zero temperatures, and high ultraviolet and ionising radiation? Some Earth-analogue haloarchaeal species would be a good candidates as potentially inhabiting Mars due to their resistance to many conditions detected on the red planet: anaerobic conditions, presence of chaotropic and oxidising minerals like perchlorates, along with more kosmotropic chloride and sulphate salts, toxic ions, and low water activity (DasSarma and DasSarma, 2017; DasSarma *et al.*, 2020; Matarredona *et al.*, 2020). There are many questions still

unanswered about potential life on Mars, and testing the viability is not yet possible on the planet itself, while it is also not easy to perfectly imitate Martian conditions (de la Vega, Rettberg and Reitz, 2007). On Earth however, many extreme environments are bursting with life, with a lot of research focused on organisms tolerating the harshest conditions, the underlying mechanisms by which they grow and survive, and how this relates to potential life outside Earth. These organisms are called extremophiles and they are found within Archaea, Bacteria and Eukarya. Two categories of organisms that live in extreme conditions can be distinguished: obligate extremophiles that require particular extreme conditions for their growth, being restricted to extreme environments, and facultative extremotolerants that do not require extreme conditions for growth but they can thrive in both extreme and in non-extreme environments (Rothschild and Mancinelli, 2001; Pikuta *et al.*, 2007; Oarga, 2009; Rampelotto, 2013). There is a lot of debate over the terms used for microbes living in extreme environments, especially the upper and/or lower values on any particular scale (e.g. salinity. pH, temperature, pressure) that are considered ‘extreme’, and not all organisms fall neatly into one category or another. Therefore, the term ‘extreme’ needs to be considered in the context in which it is used. Extreme conditions limit most organisms to function via their ‘normal’ metabolism and biochemistry, however, current extremophiles may have been the first ones to adapt to the harsh conditions, which were the ‘normal’ conditions in the planet’s past (Merino *et al.*, 2019). The harsh environments that many extremophiles and extremotolerants live in will inhibit the growth of most other organisms. These extreme environments are defined by usually more than one geochemical (salinity, pH, reactive oxygen species, high heavy metal concentration, etc.) or physical (temperature, radiation, pressure) parameter, collectively called physicochemical factors (Table 1). Common examples of extreme environments are: solar salterns, salt mines, deep-sea hypersaline anoxic brines (DHABs), acid and soda lakes, hot and cold deserts, deep-sea hydrothermal vents, ice, hot springs, evaporites, heavy metal-polluted environments, the atmosphere,

space and many more (Rothschild and Mancinelli, 2001; Oren, 2015). Whether considering life on Earth, on Mars, on icy moons of Jupiter, or elsewhere, extremophiles and extremotolerants are often the first candidates considered.

Table 1.1 ***Classification and examples of extremophiles based on physicochemical parameters***

Environmental parameter	Type	Definition	Example	Reference
Temperature	Psychrophile	Grows between -20°C and ~37°C but usually prefers colder temperatures of 0 – 20°C	*Planococcus halocryophilus* strain Or1, growth: -15 to 37°C; opt.: 25°C[a]	Clarke *et* al., 2013; Cavicchioli, 2016
	Thermophile	Grows between 60 – 80°C	*Thermus aquaticus*, growth: 70 – 75°C	Brock and Freeze, 1969
	Hyperthermophile	Grows between 80 – 122°C	*Methanopyrus kandleri* strain 116, growth: 116 – 122°C	Takai *et al.*, 2008
Radiation	Radiation-resistant	Resistant to ionizing radiation	*Deinococcus* sp., resistant to 30 kGy	Rainey *et al.*, 2005
Pressure	Piezophile/ Barophile	Grows optimally at high pressure: 50 – 100 Mpa[b]	*Shewanella benthica* strain DB21MT-5, opt. growth: 80 MPa	Kato *et al.*, 1998
	Hypobarotolerant	Grows at low pressure: ~ 1-2 kPa	*Serratia liquefaciens*, growth: 0.7 – 2.5 kPa	Schuerger *et al.*, 2013
pH	Acidophile	Grows optimally under pH 3	*Ferroplasma acidarmanus*, growth: pH 0 – 1.7; opt.: pH 1.0 – 1.5	Dopson *et al.*, 2004
	Alkaliphile	Grows optimally above pH 9	*Alkaliphilus transvaalensis*, growth: pH 8.5 – 12.5; opt.: pH 10.0	Takai *et al.*, 2001
Salinity	Non-halophile	Optimum growth: <0.2 NaCl	Most freshwater bacteria	
	Slight halophile	Optimum growth: 0.2 – 0.5 M NaCl	Most marine bacteria	Oren, 2006
	Moderate halophile	Optimum growth: 0.5 – 2.5 M NaCl	*Salinivibrio costicola*, growth: 0.1 – 3 M NaCl	Oren, 2006
	Extreme halophile	Optimum growth: 2.5 - 5.2 M NaCl	*Halobacterium salinarum,* growth: 2.4 – 5.2 M NaCl	Oren, 2006
	Halotolerant	Does not require high salt but can grow in it. Above 2.5 M NaCl: extremely halotolerant	*Halomonas elongata*, growth: 0.4 – 4 M NaCl	Oren, 2006
Osmotic pressure	Osmophile	Requires/grows in high osmotic pressure. Osmotic aspects: turgor pressure, cellular dehydration, desiccation	*Saccharomyces rouxii*, 20-60% (w/v) sucrose	Koh, 1975
Desiccation	Xerophile	Able to grow at water activity (a_w) below ~ 0.800	*Aspergillus penicillioides*, lowest limit: a_w = 0.585	Stevenson *et al.*, 2016
Chemical extremes	Metallotolerant	Resistant to one or more heavy metals	*Ralstonia (Alcaligenes)* sp. strain CH34, Zn, Co, Cd, Hg, Pb)	Mergeay *et al.*, 1985

[a]Observed growth and cell division at -15°C and metabolically active at -25°C. [b]some barophiles can grow at atmospheric pressure but obligate barophiles cannot grow at pressure less than 50 Mpa.

1.2 Extreme environments as model systems for investigating microbial biogeography

Extremophiles within extreme environments have been studied for a long time because of their innate interest and their unique properties that are valuable for humanity. These organisms thrive in physically and/or geochemically extreme conditions previously considered being lethal to life (Charlesworth 2016). However, there is no unified concept on what is considered extreme. As humanity is the one examining and writing about nature, something being extreme can be defined as those physicochemical conditions not supporting mammalian life or not considered being normal (Torsvik 2007). Of course, after the long-term observation of extreme environments, this concept is not very applicable for microorganisms considering what is normal and what is extreme for them. The traditional classification of 'extremes' defines physical and geochemical extreme conditions. In this thesis, any kind of extreme condition will be referred to as 'stressor'. The main physical stressors are temperature, radiation and pressure while geochemical stressors include salinity, osmotic changes, pH, reactive oxygen species (ROS), and desiccation. Some authors also include biological stressors (nutritional extremes, extreme population density, parasites, prey and predators, etc.) into this classification (Rothschild and Mancinelli, 2001; Matarredona *et al.*, 2020). Other stressors include anthropogenic pollution such as heavy metals, chemical waste, insecticides, antibiotics (Rothschild and Mancinelli, 2001; Matarredona *et al.*, 2020). However, when considering extra-terrestrial conditions, many more stressors come into place, e.g. vacuum, hypervelocity and radiation (Rampelotto, 2010; DasSarma *et al.*, 2020). Certain microorganisms are capable of enduring almost any type of the extreme environments listed Table 1. Therefore, it is essential to categorise these environments and their limiting parameters more precisely to get a view of the distribution, living conditions and life strategies of extremophiles. This overview will focus on the physicochemical parameters that are central to this thesis: water activity, salinity, chaotropicity, heavy metals and pH.

1.3 Physicochemical parameters limiting life and adaptation mechanisms of extremophiles

1.3.1 Water activity

Water is one of the most important compounds that determines the functionality of a cell, regardless of that cell being Archaea, Bacteria or Eukarya. It is often said that where there is liquid water, life can exist. Hence, it is crucial to better understand the minimum water availability that is able to sustain life. Water activity (a_w) is the ratio of the partial vapour pressure of a substance to the partial vapour pressure of pure distilled water at the same temperature. Pure water has an a_w of 1, while seawater for example has an a_w of ~0.986. It determines the ratio of available water in soil, food products or liquids, and it is commonly used in microbiology when one wants to adjust the concentration of salts and sugars in a microbial medium. Organisms with an optimal growth at low water activity can be halophiles (requiring high salt levels), osmophiles (requiring high osmotic pressure) and/or xerophiles (adapted to desiccation). The majority of microbes are only capable of thriving above 0.900 a_w (Stevenson *et al.*, 2014). Stevenson and colleagues demonstrated that microorganisms can live and multiply much below the water activity of saturated NaCl (0.755 a_w). They observed germination of *Aspergillus penicillioides* spores at a_w = 0.585 and a hypothetical a_w minimum of 0.565 was suggested for germination of *A. penicillioides* (Stevenson *et al.*, 2016). Interestingly, high rates of sulphate reduction, an indication of microbial activity, were found in a deep-sea, anaerobic hypersaline brine named “Kryos”, nearly saturated with $MgCl_2$, at an a_w of 0.4 (Steinle *et al.*, 2018). Discrepancies in the limits of activity or growth are sometimes seen between pure cultures and in-situ communities, and further verification would be required to confirm the result of Steinle *et al.,* 2018).

Low water activity can manifest in desiccation or in high osmotic pressure caused by high concentrations of a solute (sugars, salts, etc.). Changes in water activity in the cytoplasm can cause cell lysis under hypotonic, and dehydration under hypertonic, conditions. A cell must maintain a similar intracellular water potential to that outside of the cell (isotonic condition) using highly soluble small molecules (Torsvik and Øvreås, 2008). Some xerophilic fungi and some Actinomycetes species can enter into the state of anhydrobiosis where metabolic activity ceases and the amount of intracellular water is highly reduced, allowing them to survive long-term desiccation periods (Oarga, 2009). Others, like *Aspergillus* species, synthesize and accumulate glycerol when exposed to low water activity (Stevenson *et al.*, 2016).

1.3.2 Salinity

Osmotic aspects of life at high solute concentration and turgor pressure are characteristics of osmophilic organisms (osmophiles). Halophilic microorganisms require high osmolarity and salinity in high-salinity environments (Oarga, 2009). Adaptation to living at high salinity must be considered alongside adaption to low water activity due to the effect of salts on water activity. Every halophilic/halotolerant microorganism must cope with both high salinity by maintaining its intracellular osmotic equilibrium. Two strategies evolved to cope with this: the "salt-in" and the "compatible-solute" strategies (Torsvik and Øvreås, 2008) McGenity and Oren, 2012).

The salt-in strategy involves the accumulation of inorganic ions, mainly K^+ and Cl^- in high concentrations (~ 4.5 M KCl) into the cell and excluding toxic Na^+ ions from the cell to a high degree. As a result, all macromolecules and metabolic processes are adapted to work in this highly saline environment inside the cell. This strategy is energetically effective, although it

is highly sensitive to reductions in the external salt concentration (Oren, 2006; McGenity and Oren, 2012). Only a few groups of Archaea and Bacteria are using this strategy: the six families of the class Halobacteria (e.g. *Halobacterium salinarum*), the bacterial aerobe genus *Salinibacter* and the bacterial order of the anaerobic fermentative *Halanaerobiales* (McGenity and Oren, 2012).

The other, much more widespread strategy to tolerate high salt is the compatible-solute strategy. As its name suggests, it incorporates the use of organic compatible solutes or organic osmolytes in order to maintain metabolic activities in the cytoplasm by keeping intracellular salt concentrations low. Excess salt is expelled from the cell using active transport, and compatible solutes must be biosynthesised, or if available in the environment, imported into the cell. This mechanism is found in most halophilic and halotolerant Bacteria (e.g. *Halomonas elongata*), halophilic eukaryotic algae (e.g. *Dunaliella* sp.) and fungi (e.g. *Hortaea werneckii*), and also in the halophilic methanogenic Achaea (*Methanohalophilus portucalensis*) (Gunde-Cimerman *et al.*, 2018). The process to biosynthesise compatible solutes is energetically more demanding than the salt-in strategy, but it also gives more flexibility against changing environmental conditions, enabling microorganisms to grow in a broad salt concentration range (Oren, 2008a). Many compatible solutes are based on amino acids (e.g. proline) and their derivatives, sugars and sugar alcohols (Hoffmann *et al.*, 2012). Some of the most widely used organic osmolytes are glycerol, glycine betaine, ectoine, DMSP, sucrose and trehalose (McGenity and Oren, 2012; Gregory and Boyd, 2021).

Roberts (2005) identified that many halophile Bacteria and Archaea use more than one solute to reach osmotic balance unless one solute is provided within the medium. Moreover, these solutes can be a combination of anions and zwitterions or often several solutes with the same net charge (Roberts, 2005). Microorganisms can also form resistant structures such as spores to avoid osmotic stress. A filamentous cyanobacterium, *Microcoleus chtonoplastes* migrated to the surface or into the sediment depending solely on the changes of salt

concentrations. Kohl and colleagues named this rapid-response system halotaxis – movement in response to salinity (Kohls *et al.*, 2010; McGenity and Oren, 2012). A psychrophylic marine bacterium, *Colwellia psychrerythraea* 34H, demonstrated the ability to respond to salinity gradients, showing halotactic response towards lower and higher salinities than seawater at different temperatures. This could be an adaptation of this cold-adapted microbe to colonize freezing/thawing ice, depending on seasonal changes (Showalter and Deming, 2018).

As per the general broad definition from Oren (2008), halophilic microorganisms grow at a minimum 50 g/l salinity (0.85 M NaCl) and are able to tolerate at least 100 g/l salinity (1.7 M NaCl), while extreme halophiles require 2.5-5.2 M NaCl (Oren, 2008b). They are a metabolically diverse group, consisting of heterotrophic, phototrophic and methanogenic Archaea, photosynthetic, lithotrophic and heterotrophic Bacteria, and photosynthetic and heterotrophic Eukaryotes (DasSarma and DasSarma, 2017).

1.3.3 Chaotropicity

When speaking of microorganisms living in high salinity, usually the first salt coming into mind is NaCl. However, there are habitats where other salts are present at high concentrations. Magnesium chloride ($MgCl_2$) is one of the main components of several deep-sea hypersaline anoxic basins (DHABs) and of the Dead Sea (Hallsworth *et al.*, 2007). $MgCl_2$ is highly soluble in water and its concentration in a DHAB called “Discovery” varies between 0.05 (seawater) and 5.05 M (in the body of the brine). While NaCl is a mildly kosmotropic agent (e.g. most compatible solutes) that strengthens electrostatic interactions and stabilizes macromolecules, $MgCl_2$ is chaotropic, i.e. it weakens electrostatic interactions and destabilizes biological macromolecules (Hallsworth *et al.*, 2007). The growth of microbial isolates from Discovery Basin was entirely inhibited above the concentration of 1.26 M $MgCl_2$.

However, mRNA was recovered from the upper part of the chemocline at 2.3 M $MgCl_2$, which is an indicator of active microbes. The study stated that a few Archaea were able to grow at 2.5 M $MgCl_2$ concentration in the presence of a counteracting kosmotrope, such as NaCl. Therefore, the currently known upper limit of life in magnesium-rich environments is 2.3 M $MgCl_2$ concentration without a kosmotrope and is a maximum of 2.5 M $MgCl_2$ concentration with the presence of significant concentrations of NaCl (Hallsworth *et al.*, 2007; Zajc *et al.*, 2014). As mentioned earlier, potential microbial life was found in another DHAB called Kryos, with nearly saturated $MgCl_2$ concentrations, pushing the limits of life even further (Steinle *et al.*, 2018).

$MgCl_2$ is just one of the chaotropic agents; there are many more including ethanol, urea, and perchlorate salts (Hallsworth *et al.*, 2007; Laye and DasSarma, 2017). The latter were found in high abundance on Mars (Laye and DasSarma, 2017; Lauro *et al.*, 2021). Glycerol acts as a stress protectant against chaotropic molecules such as $MgCl_2$, ethanol or benzene and can also mitigate the effects of high turgor pressure or other mechanistically diverse stresses (de Lima Alves *et al.*, 2015). Perchlorate is a chaotropic anion and a strong denaturing agent, therefore it inhibits the growth of even most extremophiles (Laye and DasSarma, 2017). However, freezing temperatures on Mars and the presence of other salts could potentially sustain halophilic/chaophilic microbial life. For instance, *Halorubrum lacusprofundi*, a cold-adapted extreme halophile was able to grow anaerobically on 0.04 M sodium perchlorate, and aerobically with a half-maximal growth rate on 0.3 M sodium perchlorate and 0.1 M magnesium perchlorate; less than the estimated concentrations on Mars (Laye and DasSarma, 2017). On the other hand, the halotolerant yeast, *Debaryomyces hansenii*, was observed to grow at much higher perchlorate concentrations: on 2.4 M sodium perchlorate, which further supports the speculation that microbial life could very well be sustainable in Martian perchlorate brines (Heinz, Krahn and Schulze-Makuch, 2020).

1.3.4 pH

Most biological processes usually work around neutral pH as a cell mainly consists of water which is pH 7 (freshwater pH 6-8.5, seawater pH 7.5-8.4). By contrast, acidophiles can grow between pH values range from 0 and 5, while alkaliphiles grow above pH 9 (Pikuta, 2007). Proteins denature at low pH, therefore acidophilic organisms must possess suitable adaptations to survive. One of their common features is the presence of acidophilic lipids with cyclic rings and alkyl side chains in their fatty acids. They can also have unusual tetraether lipids in the cell membrane or a low content of fatty acids in their lipopolysaccharides (Torsvik and Øvreås, 2008). Most acidophiles keep the pH of their cytoplasm close to neutral (pH 5-7) (Pikuta, 2007). Other organisms, for example the bacterium *Acetobacter aceti*, has an acidified cytoplasm with proteins adapted to low pH (Menzel and Gottschalk, 1985). Acidophiles are often polyextremophiles and grow at high temperature and/or a high concentration of heavy metals (Pikuta, 2007). Therefore, bioleaching processes are widely used in the extraction of certain metals of low-grade ores. The two most acidophilic organisms known are both Archaea in the order Thermoplasmatales, namely *Picrophilus torridus* and *Ferroplasma acidarmanus*, capable of growing around pH 0 (Schleper *et al.*, 1995; Dopson *et al.*, 2004). They possess the aforementioned tetraether lipids which make the membrane impermeable to protons while proton pumps transport protons outside of their cytoplasm (Torsvik and Øvreås, 2008). The red algae *Cyanidium caldarium* is able to grow near pH 0 (Pikuta, 2007). The fact that acidophiles constitute a highly diverse group of organisms having different mechanisms of resistance suggests that they do not have a monophyletic ancestral root (Pikuta, 2007).

Alkaliphiles are organisms that grow well at pH higher than 8.5. Three groups of alkaliphiles can be distinguished: facultative alkaliphiles are capable of growing in basic conditions but their optimum is near neutral pH; obligate alkaliphiles can only survive above pH 6.5 and have an optimum growth above pH 9, often growing between pH 10 and 12, while

haloalkaliphiles not only need high pH for survival but the presence of NaCl as well (often in high concentrations) (Horikoshi, 1999). They have three different strategies to cope with high pH: having extracellular protection, the internal pH is at least two units lower than the external pH; the use of the Na^+/H^+ antiporter is used by facultative alkaliphiles, while obligate alkaliphiles have a unique mechanism of ATP synthesis and proton extrusion (Horikoshi, 1999; Hirabayashi *et al.*, 2012). The cell wall of alkaliphilic Bacteria contains a unique composition of peptidoglycans (high level of hexosamines and amino acids) and certain negatively charged acidic polymers, which can adsorb sodium and hydroxonium ions and repel hydroxide ions, helping to protect the plasmamembrane and the cytoplasm. In the case of the Na^+/H^+ antiporters, first H^+ ions are extruded to the extracellular space through respiration, which induces the antiporter to extrude Na^+ ions in exchange for H^+ ions into the cytoplasm. Then the Na^+ is uptaken by a symporter. This way the cytoplasm will be acidified and Na^+ will be kept for cellular mechanisms; this is the reason why facultative alkaliphiles need NaCl in their environment. The last strategy occurs during respiration: as alkaliphiles have a reversed pH gradient, a higher transmembrane electric potential is generated which contributes to the retention of protons from the outer membrane and a higher ATP production (Hirabayashi *et al.*, 2012). Alkaliphiles are widely researched due to their high variety of extracellular hydrolytic enzymes (proteases, amylases, cellulases, lipases, xylanases, pectinases, chitinases, etc.) functioning in alkaline conditions (Horikoshi, 1999).

1.3.5 Pollutants

1.3.5.1 Heavy metals

Extremophiles either maintain their intracellular milieu at normal conditions while only the extracellular space is extreme or they have protecting and repairing mechanisms in their cytoplasm and a physiological mechanisms to cope with extreme conditions within the cell

(Pikuta, 2007). Toxicity of heavy metal cations to a cell can be categorized based on their minimum inhibitory concentration (MIC) in the order Zn^{2+} > Ag^{+} > Ni^{2+} >> Cu^{2+} > Hg^{2+} >> UO_2^{2-} > Co^{2+} > CrO_4^{2-} > Au^{3+} > Cd^{2+} > Mn^{2+} >>> Pb^{2+} (Nies, 2000). Several mechanisms enable heavy metal-resistant microorganisms (metallotolerants) to live in harsh environments with high concentrations of metals. Metallotolerants have inducible efflux systems that export heavy metal ions to the extracellular space by active transport. This way they are able to detoxify their cytoplasm (Nies, 2000). If the heavy-metal ions are retained in the cytoplasm (bioaccumulation), these metal ions are transformed to less toxic metal species or are made less bioavailable. Another process is stable-complex binding (chelation) with organic ligands (extracellular or intracellular sequestering) (Torsvik and Øvreås, 2008). Biosorption is an energy-independent passive uptake of heavy metals by living or dead cells (biomass) (Hassan *et al.*, 2010). This process of metal exclusion prevents metals from entering into the cytoplasm by binding them to the cell wall. Both bioaccumulation and biosorption are used to remove toxic heavy metals from the environment. The development of microorganisms to act as a biosensor is very useful to determine ecotoxicity (based on the absolute/chemical concentration of a metal) of a heavy-metal polluted environment (Nies, 2000). A study observing uranium-mining effluent pools found rapid adaptation to uranium by certain microalgal species. It was revealed that random mutations of only one gene can cause uranium-resistance in a relatively short time (50 years) compared to long-term evolutionary changes (García-Balboa *et al.*, 2013).

1.3.5.2 Organic solvents and hydrocarbons

Organic solvents such as alcohols, aromatic compounds and phenols are known antimicrobial agents and are widely used in the food industry (Isken and de Bont, 1998). Although some of these solvents are naturally present in the environment, they are solely produced at low concentrations. However, due to human activities, organic solvents are

released to the environment at high concentrations enough to be lethal to microorganisms (Isken and de Bont, 1998). These solvents are toxic because firstly, they accumulate in the cell membrane, creating a permeabilization process which leads to a leakage of ATP, potassium ions or macromolecules (RNA, phospholipids, proteins, etc.) and secondly, an ion flux created by the presence of the solvent disrupts the proton-motive force, affecting both the proton gradient and the electrical potential (Isken and de Bont, 1998). Microbes resistant to organic solvents have many adaptations in their physiology to withstand such harsh conditions: 1) Structural changes in the cell membrane to increase rigidity and decrease permeability; 2) Increased production of membrane repair enzymes and solvent-inactivating enzymes; 3) Active export of solvents by solvent efflux pumps; 4) Releasing membrane vesicles with solvent molecules attached to them; 5) Production of phage shock proteins (Sardessai and Bhosle, 2002). These mechanisms are known to be present at Gram-negative Bacteria (mainly *Pseudomonas* strains and *E. coli* mutants), however, Gram-positive Bacteria were found to be resistant to certain solvents too (strains of *Bacillus, Rhodococcus* and *Arthrobacter*).

1.3.6 Temperature

According to our current understanding, life could only exist where liquid water is available. Temperature is an essential limiting factor to consider when looking for life elsewhere in the universe or simply trying to find the limits of life on Earth. Life can be broadly defined either as the completion of the life cycle or, in case of microorganisms, having some sort of active metabolism (Clarke *et al.*, 2013; Clarke, 2014). The currently known upper thermal limit to life is ~122°C, a temperature which can be found in hydrothermal vents where water under high pressures is superheated or in the deep subsurface (Marion *et al.*, 2003; Clarke, 2014). Microorganisms that are active in these environments are called thermophiles, with unique thermostable enzymes and cell membranes that allow them to live at very high temperatures

(Clarke, 2014). Specific adaptations to high temperatures include an increased number of weak interactions (e.g. Van der Walls- and hydrophobic interactions, and hydrogen- and ionic bonds) between macromolecules to increase stability, higher thermostability of membrane proteins, higher turnover rates of energy-transducing enzymes, and/or the sole use of sodium-ions rather than protons as coupling ions in energy transduction (Tolner, Poolman and Konings, 1997; Clarke, 2014).

The lower thermal limit of life where microbial growth was observed is ~-20°C (Clarke *et al.*, 2013; Clarke, 2014). Archaea are not the record holders here as microbial respiration was observed at such low temperatures of Bacteria in permafrost cores and dark respiration of lichens and yeasts of eukaryotes (Rivkina *et al.*, 2000; Clarke *et al.*, 2013; Clarke, 2014). It is an important difference that while the higher thermal limits of life are always reached, the lower thermal limits may never reach such a low temperature where life ceases to exist (Clarke *et al.*, 2013). It is only the metabolism that slows down considerably or ceases only to start again after temperature increases (Clarke *et al.*, 2013). Where extracellular ice is present, cells undergo desiccation and enter into a glass transition (vitrification) state where metabolism ceases, while where ice is not present, cells do not vitrify and are able to continue their slow metabolism (Clarke *et al.*, 2013). The molecular adaptations to low temperatures are often simply the reverse of the mechanisms involved in high-temperature adapted organisms. Chaperons (dehydrins and late embryogenesis abundant proteins), cryoprotectants (polyols), and thermal hysteresis (antifreeze) proteins could also ensure the safe transitions between normal fluid and vitrified state of the cell (Clarke, 2014).

1.3.7 Pressure

Pressure on Earth can be categorized to two main types: in air and in water. High pressures occur both in deep-sea and deep-earth environments, with two main differences: while deep-

sea hydrostatic pressure is easily calculated by depth, deep earth has either atmospheric pressure in air pockets or combined hydrostatic and lithostatic pressure in brine pockets (Marion *et al.*, 2003). Additionally, temperature is a highly limiting factor: it decreases with increasing depth in the deep sea while it increases with increasing depth in the deep earth (Marion *et al.*, 2003). Bacteria have been isolated from the sediment of Mariana Trench at a depth of 10898 m that has a pressure of ~110 MPa (Kato *et al.*, 1998; Marion *et al.*, 2003). In the deep earth, active microbial life was found 4.2 km below the surface at a temperature of 110°C (Marion *et al.*, 2003). Apparently, the ability to grow at the lowest and highest viable pressure is limited to Bacteria: various strains showed growth on solid medium at as low as 700 Pa , a pressure to consider when looking at potential life on Mars (Verseux, 2020).

1.3.8 Low energy

Many of the aforementioned limiting factors (desiccation, high salinity, chaotropicity, low temperature and high pressure) often also come with an environment lacking nutrients. These environments are called oligotrophic, and only organisms that are adapted to them can be competitive (Gray *et al.*, 2019). Some bacterial genera (e.g. *Bacillus, Streptomyces, Clostridia*) form dormant spores, with no metabolism, as survival structures, in order to wait for a period with higher nutrients (Gray *et al.*, 2019). However, not all cells become dormant, but according to Gray and colleagues (2019), *Bacillus subtilis* cells enter into an oligotrophic growth state where they stay active but their growth rate slows down significantly. In this state, cells are more resistant to environmental stresses but also able to resume normal growth when nutrient levels increase (Gray *et al.*, 2019). A truly oligotrophic environment is the open ocean itself, with a low nutrient flux and low ambient nutrient levels that are suboptimal for biological production. From picocyanobacteria to eukaryotic phytoplankton, all nutrient-starved communities can quickly react to the occasional nutrient influx, which once it is depleted, picocyanobacterial communities re-establish their dominance over other taxa (Hayakawa, Huggett and Rappé, 2011). Besides the most abundant bacterium in the ocean,

the oligotrophic *Candidatus* Pelagibacter ubique, other environments host various oligotrophic planktonic bacterial communities too, such as lakes in the McMurdo Dry Valleys of Antarctica (Stingl *et al.*, 2008).

1.3.9 Radiation

Two types of radiation can be distinguished when taking the limits of life into account: ionizing and UV radiation (Marion *et al.*, 2003). Several members of the domains Archaea and Bacteria have been observed to be resistant to either or both types of radiation (Rainey *et al.*, 2005). However, the most well-known radiation resistant microorganism is *Deinococcus radiodurans* that is capable of surviving 600 J/m^2 UV and 6000 Gy ionizing radiation (Marion *et al.*, 2003; Rainey *et al.*, 2005; de la Vega, Rettberg and Reitz, 2007; Gaboyer *et al.*, 2017). Interestingly, when subjected to multiple stressors (vacuum/low pressure, anoxic atmosphere, diurnal cycles of temperature and relative humidity) simulating a Martian environment, UV radiation affected *D. radiodurans* cells the most (de la Vega, Rettberg and Reitz, 2007). However, non-direct exposure facilitated shielding from UV radiation and survival using nano-sized hematite (de la Vega, Rettberg and Reitz, 2007). Because such high level of radiation resistance evolved in several microbial groups, especially haloarchaea, it has been suggested that the genes responsible for DNA repair may have initially evolved to counter desiccation not radiation (Rainey *et al.*, 2005). Haloarchaea can survive in brine pockets of salt crystals for millions of years, where salt not only creates an enclosed system but provides shielding protection against high radiation (Jaakkola *et al.*, 2016; Gaboyer *et al.*, 2017; DasSarma *et al.*, 2020; Matarredona *et al.*, 2020). Hence, haloarchaea are ideal models for astrobiology to observe how life may have survived/evolved on early Earth, Mars, Europa, or other planetary bodies (DasSarma *et al.*, 2020; Matarredona *et al.*, 2020).

1.3.10 Time and multiple extremes

Perhaps the most intriguing limiting factor of life is time. Even though vertebrates are the latest products of evolution, the longest-living example, the Greenland shark (*Somniosus microcephalus*) still lives only for a few hundred years (Nielsen *et al.*, 2016). On the other hand, microorganisms are on an entirely different level when it comes to surviving for a long time. There are many theories on when and how life first evolved on Earth, however, another important question from the present-day is raised by science: how long can life survive in a dormant state on another planet like Mars (Marion *et al.*, 2003)? Are there any organisms on Earth that could survive current Mars conditions? As mentioned in the previous paragraph, haloarchaea are polyextremophiles: they cannot only survive for millions of years inside fluid inclusions, but are also capable of withstanding various Martian conditions: saturating salinity, anoxic conditions, high levels of UV and ionizing radiation, subzero temperatures, desiccation, toxic ions and perchlorates (Jaakkola *et al.*, 2016; DasSarma *et al.*, 2020). Albeit there are many other polyextremophiles found on Earth, they do not have many of the advantages for such hostile conditions than what haloarchaea have, making them an intriguing target for astrobiology research (Harrison *et al.*, 2013; Mesbah and Wiegel, 2014; Poli *et al.*, 2017; DasSarma *et al.*, 2020).

1.4 Extremophiles capable of growing in non-extreme conditions

Recent studies revealed that microorganism usually living in extreme environments can be found in non-extreme environments. Using endospore germination experiments, a thermophilic, metabolically and phylogenetically diverse member of the *Firmicutes* group was found in the cold arctic seabed (Hubert *et al.*, 2009). These spores did not show any activity until reaching 50°C temperature where they mineralised or fermented organic matter and started reducing sulphate. It was considered that these spores were carried to the seabed

from warm subsurface petroleum reservoirs and ocean crust ecosystems. Low-Decarie and colleagues (2016) enriched microbial communities from a freshwater lake in laboratory conditions that were extreme compared with the lake habitat: highly acidic (pH 2), highly basic (pH 12) or saline (40 g/L NaCl, 0.68 M). These algal and bacterial communities are considered extremotolerants, as they were able to grow in both extreme and non-extreme conditions, which suggests that they were not necessarily transported to this environment from another. In another study, haloarchaea earlier considered to be restricted to extremely high salinities are found in salt marsh sediments (Purdy *et al.*, 2004). These were capable of slow growth in seawater salinity (2.5% w/v NaCl) while haloarchaea were thought to require at least 9% w/v NaCl concentration to grow. Many studies focused on how microorganisms can adapt to organic solvents such as toluene or benzene in solvent-contaminated sites. However, five strains belonging to the *Bacillus* genus are found to be tolerant to toluene despite the fact that they were isolated from soil containing no organic solvents (Isken and de Bont, 1998). Interestingly, quite the opposite of these findings occur as well. Two novel non-extremophile sulphate-reducing Bacteria were isolated in the genus *Desulfovibrio* from heavy metal contaminated sediments (Sass *et al.*, 2009). All of these findings bring into question the distribution of extremotolerant and extremophile microorganisms. As Whitaker said (2006), “with the combination of comparative analysis of population characteristics (e.g. spatial structure of the environment), the examination of biogeographic population structure, and new methods to measure recombination, selection and mutation rates will give new insights into our current knowledge of microbial allopatry and into microbial biogeography”.

1.5 The concept of microbial biogeography

Microbial biogeography is the study of the distribution of microbial biodiversity over space and time. It aims to reveal where organisms live, at what abundance, and how (Brown and

Lomolino, 2000). To answer these questions, one has to examine the background processes that drive biodiversity, namely speciation, extinction, dispersal and species interaction (Martiny *et al.*, 2006). High-throughput DNA sequencing now enables the comparative study of microbial communities at a global level. Microorganisms in the environment vary in abundance, distribution and diversity, thus they exhibit biogeographical patterns (Martiny *et al.*, 2006). Therefore, we can think of Baas Becking's hypothesis "Everything is everywhere but the environment selects" in a way that it suggests the spatial variation of microorganisms (Baas Becking, 1934). The existence of biogeographic patterns among microbes supports that environmental selection depends on both geographical isolation and historical events. Also, passive dispersal rate, population density and range size is not restricted by the size of microorganisms (which occurs in the case of macroorganisms). Microbial biogeography is crucial to understand how microbial composition affects ecosystem processes such as CO_2 respiration, decomposition, autotrophic and heterotrophic production, and nitrogen cycling (Martiny *et al.*, 2006).

1.5.1 Biogeographic patterns of microorganisms

1.5.1.1 Biogeographic patterns

Microbial life is nearly ubiquitous on Earth where there are several factors limiting biotic activity along with available water, chaotropicity, pH and temperature (Harrison *et al.*, 2013; Stevenson *et al.*, 2014). Microorganisms have a crucial role in a community and in ecosystem functioning such as driving biogeochemical cycles and biodegradation. Microorganisms, due to their size, abundance, reproducibility and other biological characteristics, were considered to be cosmopolitan: where dispersal limitation is basically non-existent (Green and Bohannan, 2006). However, recent studies identified that microorganisms vary in abundance, distribution and diversity over different spatial scales (Martiny *et al.*, 2006). Genetic distance

was shown to be related to geographic distance, which suggests that microbial community composition varies between different geographical areas within an environment (Martiny *et al.*, 2006). Beyond culture-based assays, molecular genetic methods advanced our understanding of microbial diversity through higher taxonomic resolution and depth of analysis. Several studies identified various biogeographic patterns in the microbial world and some of them also addressed the processes that shape them (Hanson *et al.*, 2012). Three spatial patterns include: the distance-decay relationship (the decrease in compositional similarity between communities with increasing geographical distance), the taxa-area relationship (TAR; the relationship between number of species in an area and the size of that area) and the local:global taxa richness ratio. There are more species in a larger area compared to a smaller one but the similarity of microbial communities decreases with the increase of an area (Green and Bohannan, 2006).

The first parameter to describe species distribution is the distance-decay relationship, which is the decline of similarity between different communities as a function of geographic distance separating them (Barreto *et al.*, 2014). An important difference between this and the taxa-area relationship is that the distance-decay relationship takes into consideration the relative abundance of the species too (Barreto *et al.*, 2014). The pattern of declining similarity with increasing geographical distance can be generated by two mechanisms: local adaptation and dispersal limitation (Bell, 2010). On one hand, if the conditions are highly variable in an environment, community similarity tends to be lower within two niches as each community can be locally adapted to that specific niche. On the other hand, if species are dispersal limited, they will presumably populate adjacent sites over sites further away. This will cause communities closer to each other to be more similar than communities apart from each other. Thus, a distance-decay relationship will be created even without differences in environmental conditions or without locally adapted communities (Bell, 2010). Nevertheless, one has to take into consideration the scale of geographical distance when studying community similarity. It

has been proposed that variable environmental conditions have a more important role at smaller spatial scales while dispersal limitation can act both at local and at larger spatial scales (Meyer *et al.*, 2018).

The taxa-area relationship (TAR) is a commonly studied biogeographical pattern in ecology mainly focusing on macroorganisms so far (Barreto *et al.*, 2014). It is described by the equation of a power-law relationship, $S \propto cA^z$, where *S* is species richness, *A* is the sampled area, c is a constant derived from the taxa and location assessed and *z* is the power-law index, representing the rate of the increasing species richness along the increasing sampling area (Barreto *et al.*, 2014). The *z*-value can be interpreted as a scaling factor describing how fast the response of species richness to area changes, with a higher number representing strong evidence of biogeographic distribution of a taxon. After many studies of macroorganisms, the advancement of molecular methods allowed observation of this pattern in microorganisms. However, one has to take into consideration the difficulty of simultaneously detecting common and rare taxa in a microbial community. Therefore, environments where there are significant shifts in community structure correlating with area are the most ideal places to observe TAR (Woodcock *et al.*, 2006). As an example, a taxa-area relationship was described for Bacteria in salt marshes, where bacterial community composition was more similar in closer communities than in communities further apart from each other (Horner-Devine *et al.*, 2004).

Local-global taxa richness ratio is based on the hypothesis that microorganisms are cosmopolitan and do not have dispersal limitation: microbial species present in a local habitat can represent a large part of the phylogenetic diversity identified in similar habitats across the globe (Green and Bohannan, 2006; Gibbons *et al.*, 2013). Regarding local-global taxa richness ratio, local-global overlap was discovered to be much higher in human-associated microbial habitats than in soil, lake or ocean communities (Livermore and Jones, 2015). This suggests that the general dispersal amongst human ecosystems is greater due to global

travel, whereas microorganisms in the environment on one continent will be less likely to disperse to another one, therefore local selection is relatively strong in comparison with dispersal (Livermore and Jones, 2015).

1.5.1.2 Processes that drive biogeographical patterns

Speciation in microbial communities is a relatively rarely mentioned topic because studies rather focused on species definitions. Unfortunately, the mechanisms that drive divergence between microorganisms are excluded this way. Whitaker (2006) created a good summary of the differentiation of biogeographic patterns between microorganisms. Biogeographic differentiation partitions variation into unique groups that can arise from environmental factors (ecological speciation) or physical barriers to dispersal (allopatric speciation). Ecological speciation occurs when biogeographic patterns are driven by environmental selection, while allopatric speciation indicates that these patterns result from the local evolution in geographically isolated populations. The most common way to identify biogeographic patterns is the well-known analysis of phylogenetic relationships of microbial sequences from different locations. Even though 16S rRNA is the most commonly used phylogenetic marker, it is not specific enough to reveal recent evolutionary changes. This requires the use of more rapidly evolving loci such as multilocus sequence typing of protein-coding genes. However, the most effective method is the genome fingerprinting metrics (RAPDs, box-PCR, rep-PCR) as this allows the identification of more recent differentiations. Whether biogeographic patterns are created by geographical barriers or environmental selection,it is determined by the local *in situ* evolution (neutral mutation, drift and adaptation) and dispersal (migration). High migration rates prevent local adaptation. However, when these rates are low, divergence is created mainly by local adaptation. The latter can be caused by random mutation, genetic exchange among one population and horizontal gene transfer. Regarding the dispersal of microbes, there are several cases when one species is more likely to be

ubiquitous than another: microorganisms possessing flagella or cilia, shorter generation time, bigger population size, and heterotrophic growth instead of a highly specific metabolism. Whitaker described allopatric divergence in microorganisms which is another step to differentiate between biogeographic patterns of microorganisms (Whitaker, 2006). In the past few years, several studies demonstrated that microorganisms display biogeographic patterns and do not exhibit ubiquitous dispersal. Another study also identified that the four evolutionary processes driving biogeographic patterns are speciation, selection, dispersal and ecological drift (Hanson *et al.*, 2012).

1.5.2 Examples of biogeographic segregation

While most environments harbour a high diversity of microbes, there are some, more unique environments (such as acid mine drainage), which are the home for relatively fewer taxa (Baker and Banfield, 2003). Pichel et al. (2013) identified a clear pattern of biogeographic segregation between two cyanobacterial species: colder and warmer habitats were dominated by *Microcoleus vaginatus* and *Microcolues steenstrupii*, respectively. Both Papke et al. (2003) and Whitaker et al. (2003) revealed the existence of endemism and geographical isolation between global hot spring microbial communities, verifying that these communities can diverge through local adaptation or random genetic drift. Takacs-Verbach et al. (2008) also examined hot springs, however, her group did not find correlation between geographic distance and geochemistry of springs but historical events instead. These findings support the study of Martiny et al. (2006) regarding the existence of both contemporary and historical events determining biogeographic patterns. Clark et al. (2017) aimed to examine biogeographic regionalisation within halophilic microbial communities in halite crystals. Communities were grouped into biogeographical clusters based on the community turnover rate. As a result, turnover at regional spatial scales did not show correlation with geographical distance; rather it was bigger at subregional spatial scales (< 500 km). Although this study

did not verify the existence of biogeographic regions of halite communities, it revealed that the processes creating biogeographic regions function differently on individual microbial taxa compared to on entire communities (Clark *et al.*, 2017). All these results confirm that the dispersal of microorganisms is not ubiquitous. Albeit, a study seems to partially support the first part of Baas Becking's statement: „Everything is everywhere" (Gibbons *et al.*, 2013). The study isolated a bacterial community from only one site of the English Channel and compared it to datasets of the International Census of Marine Microbes (ICoMM). Surprisingle, 31.7-66.2% of the operational taxonomic units (OTUs) overlapped with the ICoMM biome. These findings do not say that microorganisms have got no biogeographic patterns but that the marine biosphere possesses a persistent microbial seed bank in the global ocean (Gibbons *et al.*, 2013). Another study examined hyperthermophilic Bacteria *Thermotoga* sp. at the genome level, where recombination occurred even within species and ecotypes (Nesbø *et al.*, 2006). They hypothesised that no single archaeal or bacterial species concept can conform such disconnection of ecotypic and genetic aspects of cohesion and diversity, and the ubiquitousness of Archaea and Bacteria cannot be addressed without one appropriate concept. Hence, they suggest that microbial biogeographical questions about distribution and dispersal must be addressed at the level of genes and alleles (Nesbø *et al.*, 2006).

1.6 Evolution of microorganisms

1.6.1 The concept of experimental evolution

In order to do experimental evolution, one has to consider the definition itself: evolution is the change of gene frequency in a gene pool (van Ditmarsch and Xavier, 2014). Gene frequencies can change through four mechanisms: neutral mutation, natural selection (adaptation), genetic drift and dispersal (migration) (van Ditmarsch and Xavier, 2014). Mutation is a process that creates genetic diversity while selection acts on this diversity and

favours alleles with increased reproductive success (van Ditmarsch and Xavier, 2014). High migration rates prevent local adaptation and vice versa. However, when these rates are low, divergence is created mainly by local adaptation. Local adaptation can be caused by random mutation, genetic exchange among one population, and horizontal gene transfer. Regarding the dispersal of microbes, there are several cases when one species is more likely to be ubiquitous than another: microorganisms possessing flagella or cilia, bigger population size or heterotrophic growth instead of a specific metabolism. As Lenski described in his evolution experiment with *Escherichia coli* (Lenski *et al.*, 1991), the divergence of populations (descendants of the same clonal ancestor or even mixed microbial communities) may simply reflect their adaptation to different environments. On the other hand, genetic differences between populations can appear in the same environment due to subsequent evolution (Lenski *et al.*, 1991).

Evolutionary changes in an organism may occur gradually or suddenly (Barrick and Lenski, 2013). Contrary to evolutionary experiments of macroorganisms, changes in the genome or even in a community of microorganisms are easier to observe directly due to their short generation times, their sustainable large populations and the fact that samples of evolving populations can be kept frozen for later analysis. This way, different time periods of a population can be compared to their ancestor and to each other (Zeyl, 2006; Barrick and Lenski, 2013). The route of the evolution of experimental populations will respond to is determined by the culture conditions, such as a type of stress (e.g. high osmolarity or temperature) or competition for a limiting nutrient. Rare mutations that increase reproductive success (natural selection) will appear owing to the selective pressure (Zeyl, 2006). Then, adaptation can be measured either as a shift in growth rate or observations of the changes in the genome/microbial community with advanced, fast and cost-effective molecular techniques such as whole genome sequencing or next-generation sequencing of a taxonomic marker gene.

The main aim of this research was to better understand the interplay between connectivity and dispersal and the environmental filtering of extremophilic and extremotolerant microbial communities, and to observe their dispersion and adaptability to changing physicochemical conditions. To achieve this, first, Chapter 2 was focusing on enriching microbes in different stressor media (salts, metals, and sorbitol) from non-extreme marine- and freshwater habitats to find and isolate potential generalist extremophiles or extremotolerants. Then, growth was assessed of each enrichment on a range of each stressor. This was followed by 16S rRNA and ITS metagenetic sequencing in order to identify the most abundant Bacteria and Fungi, and to compare them across environments. The second aim was to determine how is community composition influenced based on the source environment, but only examining one stressor: NaCl. Samples were collected from salt springs which are highly variable in salinity and water activity, and from a salt mine with little to no variability in the environmental conditions. Samples were enriched in low, medium, and high salinities, and were cultured in the same salinity for 20 months. Following this, 16S and 18S metagenetic sequencing was performed to identify dominant taxa, and to see how community composition changes over a relatively long time in selective media. The third was to achieve evolution in three laboratory cultures of a generalist halotolerant bacterium *Halomonas elongata* 1H9, and two high-salt specialist haloarchaea, *Hbt. salinarum* 91-R6 and NRC-1. The strains were subjected to salinities at their limits of growth for 20 months, and growth was assessed throughout the evolution experiment. Following this, whole-genome sequencing was performed on the evolved cultures and their genomes were compared to the genome of the original cultures to identify mutations with a role in osmoadaptation. Collectively, the aim of this research was to enhance current understanding on the limits, resilience, and evolution of microbial life and how dominant taxa can outgrow their neighbours when conditions become favourable.

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Chapter 2: Microbial biogeography: Enrichment with different stressors reveals the presence of extremotolerant microorganisms in freshwater and marine systems

2.1 Introduction

Microbial life is widespread on Earth, but several physical, chemical and biological factors limit microbial activity, and define microbial community composition and ultimately the extent of the biosphere. These factors include, but are not limited to: availability of water (water activity, a_w), salinity, chaotropicity, pH, temperature, pressure and radiation (Rothschild and Mancinelli, 2001; Hallsworth *et al.*, 2007; Dartnell, 2011; Harrison *et al.*, 2013; Stevenson *et al.*, 2014). A low diversity of organisms tends to be found at the physicochemical extremes (Benlloch *et al.*, 2002; J. Liu *et al.*, 2014; Sharp *et al.*, 2014). In these extreme conditions, microbes demonstrate specialised adaptations to their metabolism, enzymatic activity and cell structure. Thus, extremophiles are often specialists with an obligate requirement for a particular stressor (obligate extremophiles), such as low water activity (a_w), low or high pH, low or high temperature, high salinity (Takai *et al.*, 2001; Dopson *et al.*, 2004; Clarke, 2014; Stevenson *et al.*, 2015; Gunde-Cimerman *et al.*, 2018). For instance, one of the most extremely halophilic members of Bacteria is *Salinibacter ruber* M31^T (Figure 2.1), which requires a minimum of 2.5 M NaCl and shows optimum for growth of between 3.4 and 5.1 M NaCl (Antón *et al.*, 2002). On the other hand, extremotolerant microbes are able to grow in extreme environments, but do not require high concentrations of a stressor for growth and often grow optimally in less extreme conditions, i.e. they are generalists (Torsvik and Øvreås, 2008). For example, a typical extremely halotolerant member of Bacteria is *Halomonas elongata* 1H9 (Figure 2.1), which grows optimally between 0.6 and 1.4 M NaCl but is able to grow in very low (0.05 M NaCl, seawater is 0.6 M NaCl) and in very high (4.5 M NaCl) salinities (Vreeland *et al.*, 1980).

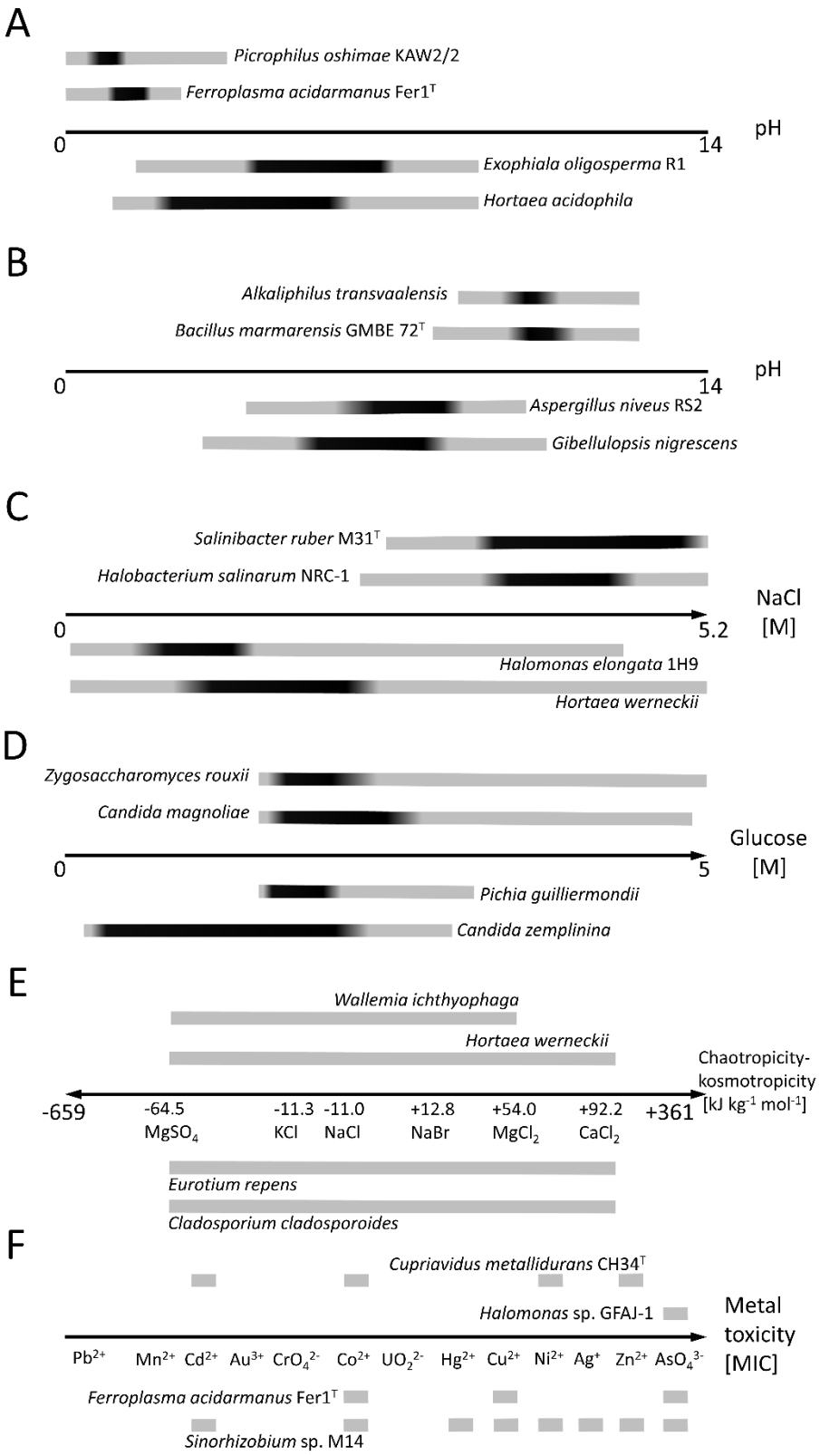

Figure 2.1 Schematics of growth range of extremophiles (upper two on each scale A to F) and extremotolerants (lower two on each scale A to F) in different stress conditions. Black: optimum, grey: outside of optimum growth conditions. A: Acidic conditions – acidophiles and acidotolerants; B: Alkaline conditions – alkaliphiles and alkalitolerants; C: Saline conditions – halophiles and halotolerants; D: High osmotic pressure – osmophiles (grow in > 60% total sugar/salt concentration) and osmotolerants (grow in ≤ 60% total sugar/salt concentration); E: Chaotropic conditions – chaotolerants (all four); F: Resistance to metals (heavy metals, metalloids) based on MIC (minimum inhibitory concentration) – metallotolerants (all four). For detailed information see Table S2.1.

Extremophiles and extremotolerants are good models for examining microbial biogeography, because the conditions that constrain their growth allow their extreme habitats to be considered as islands that are distinct from the surrounding environment (Clark *et al.*, 2017). In contrast to the view of extremophiles constrained to islands of extreme habitat, extremotolerant organisms can be found in a non-extreme environment such as a freshwater lake (Low-Decarie *et al.*, 2016). The presence of extremotolerant microbes in non-extreme environments may be explained in part by dispersal from extreme environments and dormancy. Some extremophiles can be dispersed to, and survive in non-extreme conditions, for instance with highly resistant spores that stay dormant until the conditions improve again (Hubert *et al.*, 2009; O'Sullivan *et al.*, 2015). The microbial "seed bank", the non-growing forms of microbes that are tolerant to conditions that may otherwise limit dispersal, represents a large proportion of the microbes in many environments (Lennon and Jones, 2011; Gibbons *et al.*, 2013) and may contain numerous extremotolerant organisms.

Seemingly out-of-place extremophiles can help to address fundamental questions about the biogeography of extremophiles. Can extremophilic and extremotolerant microbes be found in any non-extreme habitat? Are they dormant or actively growing? If they are growing, is their growth dependent on the environmental conditions? Can a microbial seed bank be established independently of habitat? We were interested in whether it was possible to enrich microbial communities under extreme conditions from non-extreme habitats and how the growth of the enriched communities was affected by the level of stressor. Moreover, the strategy adopted here of cultivating extremophilic and extremotolerant microbes from non-extreme environments opens up opportunities for local bioprospecting for microbes/activities with potential industrial applications.

Specifically, our hypotheses were that: 1) If environments contain organisms well adapted to a diversity of conditions not found in that environment, it may be possible to enrich extremotolerant microbes from non-extreme environments. 2) If extremotolerant microbes

from non-extreme environments actively grow in the non-extreme environment, they would collectively grow over a wider range of stressor concentrations (extremotolerant generalists) than microbial communities enriched in the absence of a stressor (non-extreme specialists). 3) If dispersal and microbial seed banks maintain a larger diversity of microbes in all environments, then stressor type and connectivity would be major determinant of bacterial and fungal community composition rather than source environment.

The experimental design allowed the factors shaping microbial community composition to be tested, including connectivity and type of ecosystem. We took water samples from three rivers and their connected marine sites across Southeast England. The river systems were the Colne river/estuary, which has served as a long-term study site for investigating microbial processes (Nedwell *et al.*, 2016), the Thames and its estuary, the longest river in the UK and one of the most impacted and studied rivers in the EU (McKew *et al.*, 2007), and the River Great Ouse and its estuary, the fourth longest river in the UK. The catchments of the Great Ouse and the Thames have received waste from diverse heavy industries, including major agricultural works, leaving a legacy of polluted sediments and waters (O'Reilly Wiese, MacLeod and Lester, 1997; Pinder *et al.*, 1997; Neal *et al.*, 2000; Neal, 2003; Bowes *et al.*, 2018). On the other hand, the Colne is a much smaller, hypernutrified river that has one main input from sewage treatment works (Nedwell *et al.*, 2016). Although metal mining is not a characteristic of the East of England, all sites likely receive metal pollution from other sources: sewage treatment plants, boats (antifouling paints and zinc-based sacrificial anodes), brickworks, steel manufacturers, battery recycling centres, car manufacturers and gas works.

Samples were enriched for at least 30 generations in stressor media, focusing on salinity, chaotropicity, low water activity, acidity, alkalinity, heavy metals and a no-stressor control (Figure 2.2). Then, we measured the growth of the enriched communities in a gradient of the same stressor and compared stressor- and control-enriched communities.

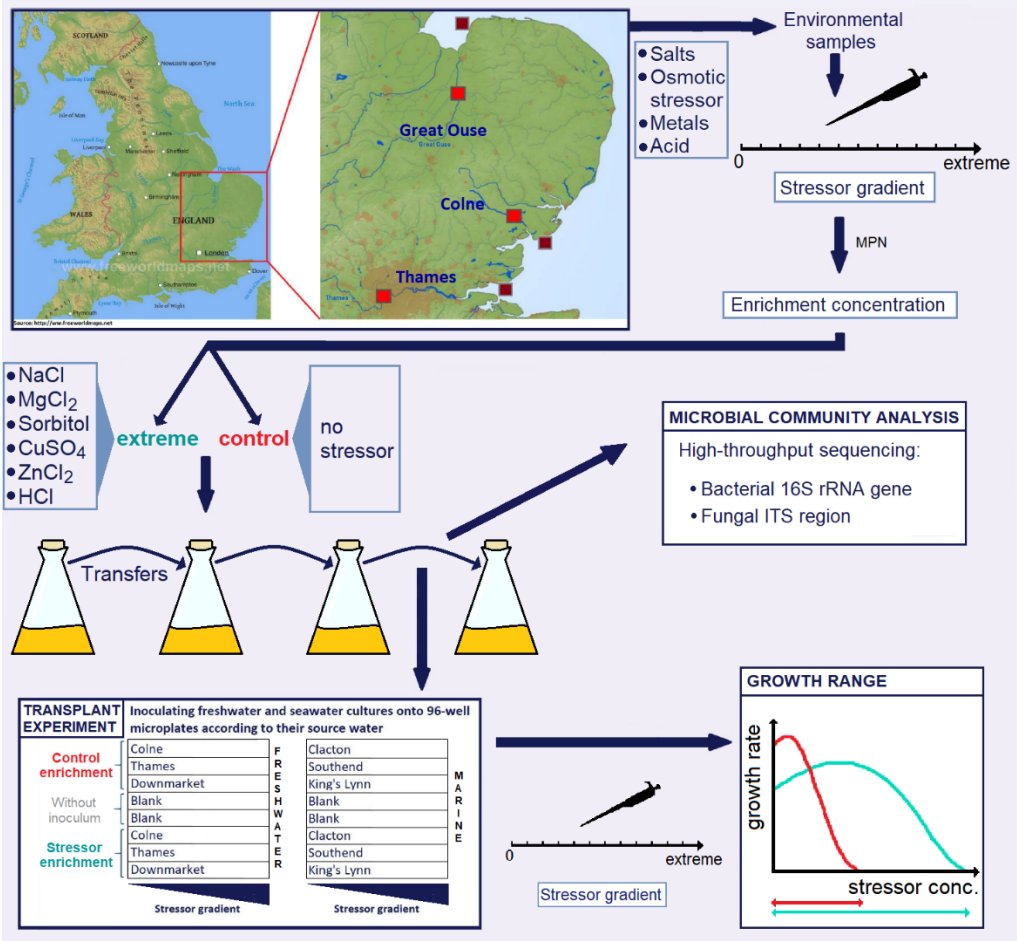

Figure 2.2 Schematic representation of the experimental design –and location of the sampling sites in the UK. Every sampled freshwater river (red square) was paired with seawater (dark red square) from the same estuary, and samples were taken from each paired locations on the same day. Location of sampling sites based on rivers: Colne (08/02/2017): freshwater: Hythe, Colchester (lat 51.891 long 0.915) – marine: Clacton (lat 51.784 long 1.158); Thames (06/03/2017): freshwater: Teddington Lock, London (lat 51.429 long -0.318) – marine: Southend (lat 51.515 long 0.722); Great Ouse (22/02/2017): freshwater: Silt Fen Farm, Downham Market (lat 52.584 long 0.345) – marine: Hunstanton, near King's Lynn (lat 52.951 long 0.495). Using an extinction dilution series (MPN, Most Probable Number) from no stressor to extreme levels, the enrichment concentrations for each stressor were extrapolated, where microbial growth was detected at the highest permissive stressor level that is considered extreme (see

Table S2.2.). Samples were cultured and transferred in each stressor media along with control samples without stressor. For the culture media 1% w/v yeast extract was provided, and MilliQ water was used for freshwater samples, while artificial seawater was used for marine samples. The carbon source was yeast extract. A transplant experiment was done after multiple generations in the enrichment, assessing the growth of each microbial community in a stressor gradient from no-stressor to extreme stressor levels. Growth rate, then stressor range of growth was calculated from the growth data.

2.2 Methodology

2.2.1 Sample collection and growth assessment of the microbial communities

For this study, three rivers and three connected marine sites were sampled across the UK (Figure 2.2). Water samples were taken in sterile 20-litre carboys and kept in a cooler filled with ice until processing on the same day. Processing of field samples included the determination of abundance of microbes as a function of stressor concentration through an

MPN assay, the enrichment in large volume culture and the extraction of microbial DNA for sequencing.

2.2.2 Enrichment and assay media

Each medium was made using MilliQ water and artificial seawater (ASW) for freshwater and for marine samples, respectively (Berges, Franklin and Harrison, 2002), with 1% (w/v) Oxoid yeast extract (YE) as a source of carbon, energy and nutrients.

2.2.3 MPN assay across a stressor gradient

We measured the abundance of microbes in the samples in 96-well microtiter plates across a gradient of each target stressor ($ZnCl_2$, NaCl, sorbitol, HCl, $MgCl_2$ and $CuSO_4$) based on most probable number (MPN) approach.

Log_5 dilution series with seven steps were made leading to concentrations of microorganisms ranging from five times (concentrated source samples) to 0.00032 times (diluted source sample) the source concentration of microorganisms. A blank (filter-sterilised source water) was also included as an 8th concentration of zero. To obtain concentrations of five fold (5x), duplicates of 50 mL of samples in Falcon tubes were centrifuged at 4000 rpm (2222 rcf) for 10 minutes at 4°C. After centrifugation, 45 mL of supernatant from each sample was removed with a syringe and filter sterilized through a 0.22 µm filter to act as dilution media. Then, samples were resuspended in the remaining 5 mL in the tube by vortexing for two minutes to form the 5x concentrated samples compared to the source concentration. The original source sample, served as the second step of the dilution series of 1x. To create the remaining five steps of the log_5 dilution series (0.2x, 0.04x, 0.008x, 0.0016x, 0.00032x), we performed a five-step serial dilution of 2 mL source sample into 8 mL filter-sterilized dilution media. In total of 100 µL of each sample was used to inoculate 110 µL of stressor media.

Concentration of stressors varied linearly from maximum stressor concentration to zero across the 12 columns of the microplate (

Table S2.2). Each stressor by sample culture was replicated four times. Cultures in microtiter plates were sealed with air-permeable seals (StarLab) and incubated at 20°C for 48 hours. Growth was measured by optical density (OD_{600}) using a plate reader before and after incubation and growth was established as a change significantly larger ($p < 0.05$) than any change observed in the blanks. At each stressor concentration, MPN values were calculated based on the number of replicates in which growth was detected across the range in sample volume. MPN varied log-linearly as a function of stressor concentration (Figure S2.1) and the log-linear relationship was used to extrapolate higher stressor concentrations requiring larger sample volumes for growth detection.

2.2.4 Enrichments of microbial communities

Enrichment concentration was chosen as the concentration at which MPN would be 100 cells/L so that one litre of sample would have a high likelihood of yielding growth. For this initial enrichment of the environmental microbial community samples, one litre of sample was mixed with one litre of media at 2x the target stressor concentration. For sorbitol, saturating concentrations of sorbitol were used to reach MPN of 100 cells/L as the predicted concentrations required exceeded the solubility of sorbitol in the concentrated media. Stressor media were made using MilliQ water and artificial seawater (ASW) for freshwater and for marine samples, respectively (Berges, Franklin and Harrison, 2002). The final concentrations of each stressor medium were the following: 1.72 M NaCl, 1.05 M $MgCl_2$, 12 mM $ZnCl_2$, 1.5 mM $CuSO_4$, 2.52 M sorbitol and 18 mM HCl, amended with 1% (w/v) Oxoid yeast extract (YE). Control media were amended with 1% (w/v) Oxoid YE without any stressor added. After the initial enrichment yielded growth, 1.5 mL of each of the enriched cultures was inoculated into 150 mL of control- and stressor media for each stressor. The enrichments were kept in 200 mL Erlenmeyer flasks sealed with foam bungs and aluminium foil (allowing gas exchange) and were incubated at 20°C for one month.

Stressor-enriched and control-enriched microbial communities were subcultured every month. 100 µL was inoculated from the one-month-old cultures into 10 mL of the respective media containing the same base (1% (w/v) YE in either MilliQ water or ASW for the control-enriched cultures, plus the respective stressor. New enrichments were kept in glass Universal bottles sealed with foam bungs and aluminium foil and were incubated at 20°C.

2.2.5 Growth assay of enriched communities across stressor gradient

Transplant experiments were carried out to test the growth response to each stressor after being repeatedly subcultured in either control or in stressor media for a longer time period (minimum four months). Both control- and stressor-enriched cultures were inoculated into 96-well microtiter plates. Media were prepared to a final volume of 200 µl, with a maximum (100%) concentration of each stressor: 1.72 M NaCl, 1.05 M $MgCl_2$, 12 mM $ZnCl_2$, 1.5 mM $CuSO_4$, 2.52 M sorbitol and 18 mM HCl (the concentration of NaCl was increased 1.5 times to 2.58 M and the concentration of $CuSO_4$ was doubled to 3 mM in order to be able to distinguish the growths of stressor- and control-enriched cultures). A gradient was generated for each stressor in 11 steps, such that their concentration was 91%, 82%, 72.5%, 63.5%, 54.4%, 45.5%, 36.5%, 27.5%, 18%, 9% of the maximum concentration, with a no-stressor (0%) included. Each 200 µL medium had 1% YE (Oxoid) and was inoculated with 10 µL of inoculum from the enriched microbial cultures in 96-well microtiter plates (rows A to C), except rows D and E which were blanks without inoculum. The microtiter plates were sealed with air-permeable films (StarLab) and were incubated at 20°C. Optical density (OD) was measured twice daily for 48 hours (λ = 600 nm) using a plate reader with initial shaking (200 rpm, 10 s). The wavelength of 600 nm was used because a high growth rate was expected from the control-enriched cultures in control media, reaching an OD too high to be reliable on lower wavelengths. Additionally, measuring at this wavelength enabled to minimize interference from the yellow-orange-coloured media.

There was no growth seen in the plastic microplates of the sorbitol-enriched cultures after several attempts, even though the cultures showed visible growth in the glass Universals bottles during the transferring process. Therefore, an additional analysis was conducted in glass test tubes to reveal whether the sorbitol-enriched cultures are affected by plastic. A three-scale stressor gradient was used (instead of the 12-scale gradient used for the microplates) with three concentrations equivalent to the dilutions in the third, seventh and tenth well of the microtiter plates (low (0.45 M), medium (1.37 M) and high (2.07 M) level of stressor concentration). Stressor NaCl was used additionally to check whether growth differs in glass test tubes compared to plastic microplates with stressors other than sorbitol. Cultures were incubated on a shaker (95 rpm) at 20°C. OD was measured at λ = 600 nm daily using a spectrophotometer for three days instead of two because the sorbitol-enriched cultures grew slower than other stressor-enriched cultures.

2.2.6 Microbial community composition analysis

Next generation sequencing (MiSeq) was conducted on the source samples taken directly from the environment and on the enrichment communities after three transfers, with primers targeting the Bacterial 16S rRNA gene (F: 341F – TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, R: 805R – GACTACCAGGGTATCTAATCC) and the Fungal ITS gene (F: ITS3_KYO2 - GATGAAGAACGYAGYRAA, R: ITS4 - TCCTCCGCTTATTGATATGC)(Toju *et al.*, 2012; Granzow *et al.*, 2017; Li *et al.*, 2017). DNA was extracted from the source upon arriving at the lab (within six hours of collection) and from the enrichment cultures after three transfers (six weeks). Environmental source samples were concentrated using Sterivex filters (Gilbert, Jansson and Knight, 2014) with water volume being filtered until the filter saturated (180 mL ± 5 mL for marine, and 240 mL ± 5 mL for freshwater samples). Enrichment cultures were concentrated by centrifuging 50 mL of each at 4000 rpm (2222 rcf) for 10 minutes, using the

precipitate. DNA extraction was done using the MoBio Sterivex and MoBio Power Water Kits. Amplicon libraries were prepared following the Illumina MiSeq Library preparation protocol, with primary PCR adapted for Bacterial 16S (Li *et al.*, 2017) and Fungal ITS (Toju *et al.*, 2012) genes using 25 cycles. The following PCR reaction conditions were used for Bacteria: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and final extension at 72°C for 5 min (Li *et al.*, 2017). The following PCR reaction conditions were used for Fungi: initial denaturation at 98°C for 30 s, followed by 6 cycles of denaturation at 98°C for 15 s, annealing at 53°C for 30 s decreasing 0.5°C in each cycle, followed by extension at 72°C for 30 s and 19 cycles of denaturation at 98°C for 15 s, annealing at 50°C for 30 s, followed by extension at 72°C for 30 s, with a final extension at 72°C for 2 min (Granzow *et al.*, 2017). V3 MiSeq reagents were used for sequencing. Quality trimming was done using Sickle (Fass and Joshi, 2011), error correction within SPAdes (Bankevich *et al.*, 2012), using the BayesHammer algorithm (Nikolenko, Korobeynikov and Alekseyev, 2013) and pair-end aligned with PANDASeq (Masella *et al.*, 2012). VSEARCH (Rognes *et al.*, 2016) was then used to remove singleton OTUs, along with all chimeric sequences using denovo chimera checking, to depreplicat, sort by their abundance, and pick OTU centroids using at the 97% level. Taxonomy assignment was performed with the RDP Classifier (Cole *et al.*, 2014) against the RDP 16S and Warcup ITS training sets and against the UNITE database (Abarenkov *et al.*, 2010; Deshpande *et al.*, 2016). The R software (versions between 3.3.2 and 4.0.4) was used for all statistical analysis (R Core Team, 2018).

2.3 Results

2.3.1 Determining a suitable stressor concentration for subsequent experiments by measuring microbial growth as a function of stressor concentration

We determined the concentration of each stressor that would allow growth yet drive community evolution in the enriched river/marine samples (Figure 2.2). Water samples were obtained from three rivers and their connected marine sites, and their growth was measured over a concentration gradient of each stressor (NaCl, $MgCl_2$, $CuSO_4$, $ZnCl_2$, sorbitol, HCl; Figure 2.2). Growth was observed from all environmental samples, including stressor concentrations at which only extremotolerant microbes would be expected to grow. As stressor concentrations increased, the abundance of microorganisms capable of growing decreased log-linearly (Figure S2.1). The stressor concentrations to be used in the subsequent enrichments were calculated using the log-linear relation between MPN (most probable number) and stressor concentration to identify a concentration at which MPN was 100 cells/L (see Experimental Procedures 'Sample collection and growth assessment of the microbial communities'). On this basis, the concentrations of each stressor that was used in the repeated enrichment cultures were: 2.58 M NaCl, 1.05 M $MgCl_2$, 2.52 M sorbitol, 18 mM HCl (~pH 2.5 – 3.8), 12 mM $ZnCl_2$, and 3 mM $CuSO_4$, along with a no-stressor control.

2.3.2 Repeated enrichment of riverine and marine samples in extreme conditions

The environmental samples were enriched in media with the stressor concentrations given above, and subcultured every 14-30 days in the same media for at least four months. Growth was observed in all cultures except for the HCl-enrichment inoculated with a sample from the River Colne, which was therefore excluded from further analysis. Then, the growth rate of

cultures was measured using a concentration gradient of the same stressor in which they were enriched, comparing with no-stressor control enrichments. In nearly all cases, stressor-enriched cultures grew across a wide range of the stressor gradient, and grew better at higher stressor concentrations than communities from the no-stressor control enrichment (Figure 2.3). In contrast, for no-stressor-control-enriched cultures there was a marked decrease in growth rate as the stressor concentrations increased (Figure 2.3). No-stressor control cultures had an average higher growth rate at low stressor concentrations, while stressor-enriched cultures were generally able to grow at the highest stressor levels (Figure 2.3).

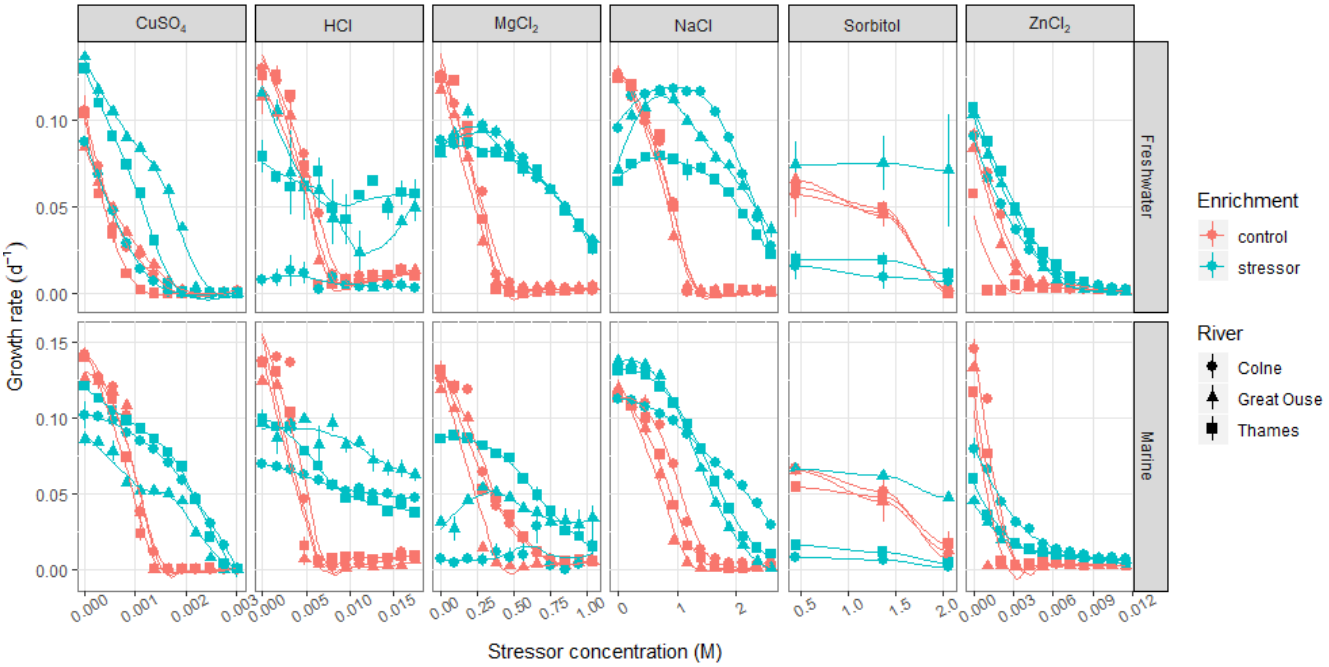

Figure 2.3 Growth rate (μ; d^{-1}) of no-stressor-control-enriched and stressor-enriched microbial communities at different concentrations of the stressors: $CuSO_4$, HCl, $MgCl_2$, NaCl, sorbitol and $ZnCl_2$. Control media: 1% (w/v) yeast extract; stressor media: 1% (w/v) yeast extract plus a range of concentrations of the following stressors (up to the maximum concentration): $CuSO_4$ (0.003 M), HCl (0.018 M), $MgCl_2$ (1.05 M); NaCl (2.58 M), sorbitol (2.07 M), or $ZnCl_2$ (0.012 M). Media were made with MilliQ water or artificial seawater for freshwater or marine environmental samples, respectively. Control-enriched and six stressor-enriched microbial communities were exposed to a stressor gradient from 0 to maximum stressor concentration in 11 increments in 96-well plates. Owing to difficulty reading some of the sorbitol-grown cultures with a plate reader, sorbitol-enriched cultures were grown in glass test tubes at three concentrations. Growth was measured as OD at 600 nm. Error bars indicate standard errors. N = 4, except for sorbitol-enriched cultures which where N = 3.

We investigated the impact on growth of the biogeographical region of the different river systems (Colne, Great Ouse or Thames) and habitat type (freshwater or marine). Growth rate did not vary consistently as a function of river and habitat type from which the communities were enriched (Figure 2.3). Stressor-enriched cultures consistently grew better than control cultures at higher stressor concentrations (Figure 2.3 --- averaged across freshwater and

marine), with the exception of marine cultures enriched in sorbitol. Control-enriched culture consistently grew better than stressor-enriched cultures at lower stressor concentrations (Figure 2.3 --- averaged across freshwater and marine), with the exception of marine culture tested at low NaCl and freshwater culture tested at low Zn for which no difference could be identified.

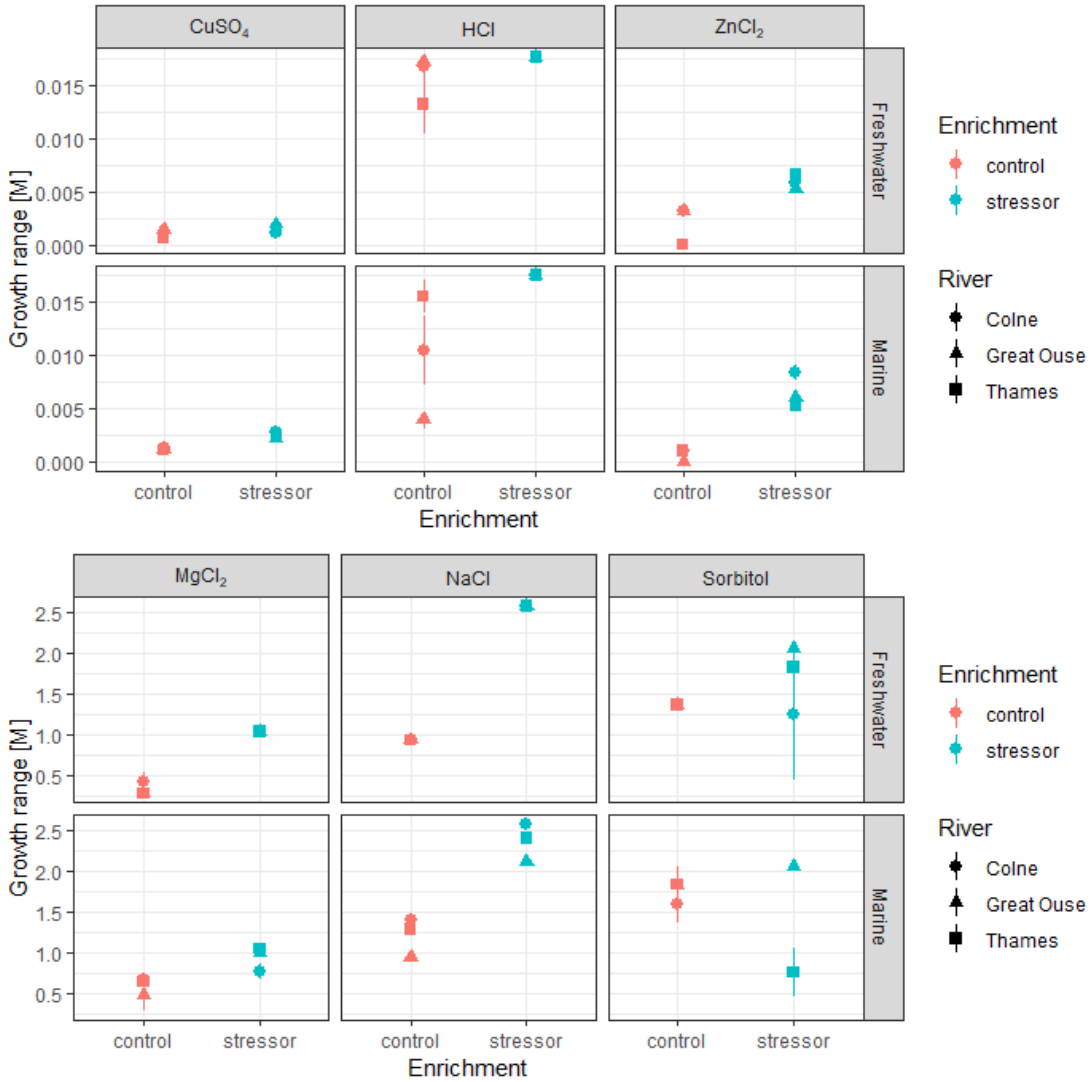

Figure 2.4 Stressor range of growth of the microbial communities comparing control- and stressor-enriched microbial communities in different stressors: $CuSO_4$, HCl, $MgCl_2$, NaCl, sorbitol and $ZnCl_2$. Growth range was defined as the range of stressor concentration where the growth rate (μ) was positive: growth range = max. stressor conc. (M) – min. stressor conc. (M), where μ > 0.01. The average growth range of the stressor-enriched cultures was significantly higher than the control-enriched cultures except for the sorbitol-enriched communities from the marine sites of Colne and Thames. Error bars indicate standard error. N = 4, except for sorbitol-enriched cultures which where N = 3.

The stressor range of growth of the microbial communities (Figure 2.4) was calculated using the growth rates and the stressor concentrations from Figure 2.3, focusing on the comparison of control-enriched and stressor-enriched microbial communities. The river and the source

type did not have an effect on the stressor range, therefore data were combined for this analysis. We investigated the interaction between enrichment type (control versus stressor) and growth range using Welch's t-test. The growth range of the stressor-enriched cultures was higher than the control-enriched cultures in all stressors except sorbitol, where there was no significant difference (Table S2.4).

2.3.3 Impact of stressors on microbial community composition and OTU richness

Amplicon sequencing of the bacterial 16S rRNA gene and the fungal ITS region was performed on the enrichment cultures after three transfers in order to examine the community composition and richness of the stressor-enriched, control-enriched cultures and source communities (original riverine and marine samples). Out of all the factors, enrichment type (comparing control versus stressor enrichments) had the most significant effect on both bacterial and fungal community composition (Figure 2.5, Figure 2.6, Table S2.5). The river sampled (Colne, Great Ouse, Thames) had a significant effect only on the fungal community composition (Figure 2.6, Table S2.5), while the water type (freshwater/marine) had a significant effect only on the bacterial community composition (Figure 2.5, Table S2.5).

For both Bacteria and Fungi, source communities had much higher OTU richness than control-enriched communities (mean of freshwater and marine combined ± standard error) - Bacteria (472.5 ± 210.69) and Fungi (68.17 ± 9.32). The next most OTU-rich were the control-enriched communities (Bacteria (43.5 ± 4.74) and Fungi (17.5 ± 4.5), while most of the stressor-enriched communities had relatively low OTU richness (six stressors combined: Bacteria (14.03 ± 2.3) and Fungi (9.33 ± 1.05) (Table 2.1, Table 2.2, Figure 2.5.D, Figure

2.6.D, Figure S2.2). Factorial analysis of variance (ANOVA) supported this clear difference between the control-enriched and stressor-enriched community OTU richness (Table S2.6 and Table S2.7). There were some differences in OTU richness in stressor-enriched communities (Table S2.6 and Table S2.7). For example, for the Bacteria, freshwater samples enriched in $MgCl_2$ had the lowest value, while HCl-enriched marine communities had a surprisingle high OTU richness (Figure 2.5.D); while, for the Fungi, both freshwater and marine cultures enriched in HCl had the lowest, while NaCl-enriched marine communities had a relatively high OTU richness (Figure 2.6.D).

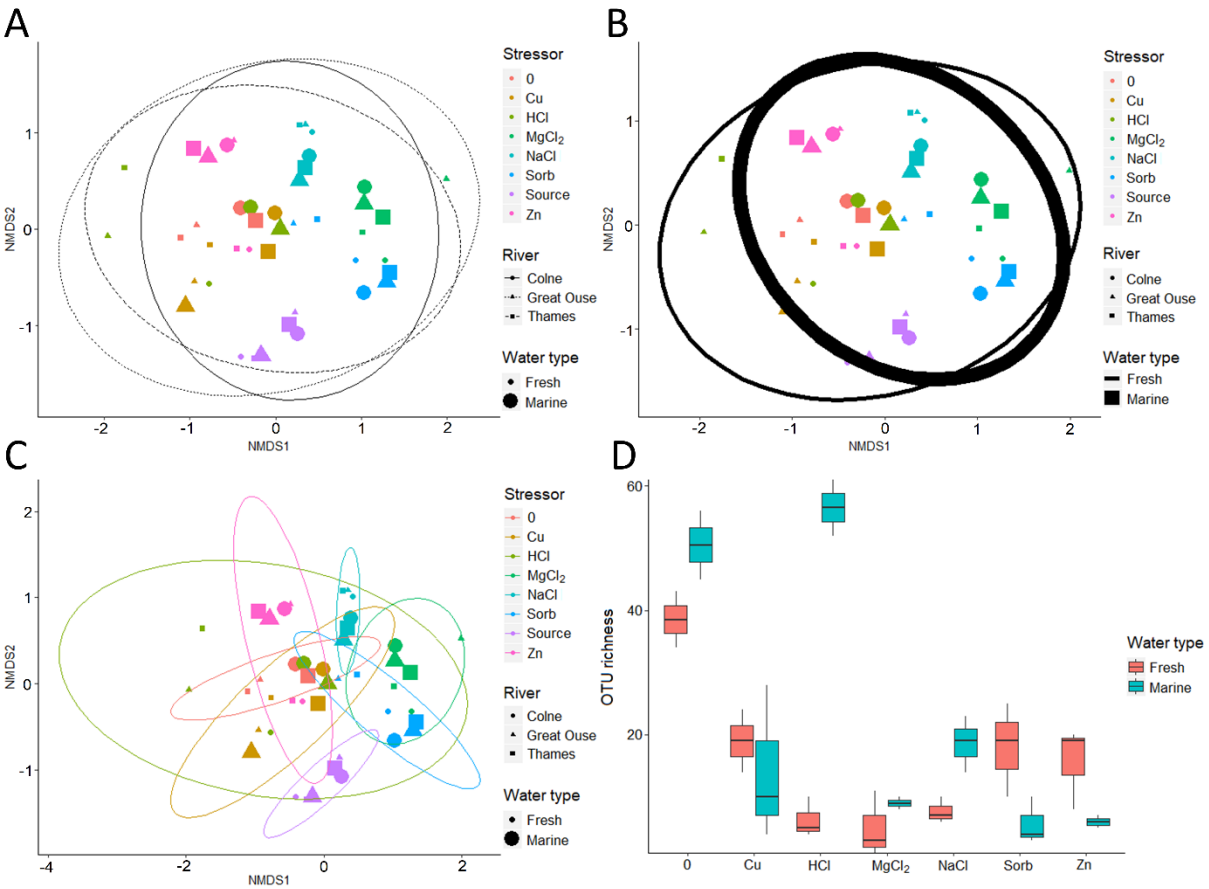

Figure 2.5 Analyses of bacterial communities based on 16S rRNA gene sequence analysis and clustering sequences into operational taxonomic units (OTUs) at the 97% identity threshold. Panels A – C show community composition based on Non-metric multidimensional scaling (NMDS, Bray-Curtis dissimilarity, 5000 iterations) in the different treatments with 95% confidence ellipses for A: River, B: Water type and C: Stressor. 0: control-enriched communities; Source: source communities. Panel D is a box plot with whiskers showing OTU richness of the control-enriched and stressor-enriched communities (horizontal line = median, lower part of the box = 25th Percentile, upper part of the box = 75th Percentile; lower and upper vertical lines/whiskers = remaining 25% of the data on upper and lower part). OTU richness of the source communities (mean of three freshwater samples = 357, mean of three marine samples = 588) is not shown as it would distort the scaling (see Figure S2.2). Stressor abbreviations are Cu = CuSO4, Sorb = Sorbitol, Zn = ZnCl2, and the control-enriched community is indicated by "0".

2.3.3.1 Bacteria enriched with different stressors

Dominant bacterial genera selected with a given stressor were largely similar in the freshwater and in the marine enrichments. Community composition was somewhat similar between the $MgCl_2$- and the sorbitol-enriched communities and between the Cu-, Zn- and HCl-enriched communities (Table 2.1). The dominant taxa were: *Halomonas* (NaCl enrichments), *Halobacillus* ($MgCl_2$ enrichments), *Staphylococcus* (sorbitol enrichments, and co-dominant in $MgCl_2$ freshwater enrichments), *Acetobacterium* (Cu freshwater enrichments), *Acetobacter* (Cu marine enrichments, HCl enrichments), *Alkanindiges* (freshwater Zn enrichments), *Acinetobacter* (marine Zn enrichments) (Table 2.1). With the exception of *Acetobacterium*, none of these genera was dominant in no-stressor-enriched communities that were sampled at the same time (Table 2.1).

Table 2.1 Mean relative abundances (%) of the dominant Bacteria (genera or families) in the stressor enrichments. Percentage contribution of taxa was calculated with respect to the total Bacterial community in each enrichment.[1]

Stressor	**Bacteria**			
	Freshwater		Marine	
	Genus/Family	Rel. abund. (%)	Genus/Family	Rel. abund. (%)
NaCl	*Halomonas*	86.1	*Halomonas*	36.5
	Marinilactibacillus	11.4	Halomonadaceae[2]	24.8
			Idiomarina	21.8
			Pseudoalteromonas	5.9
			Vibrionaceae[2]	5.3
$MgCl_2$	*Halobacillus*	37.4	*Halobacillus*	80.7
	Staphylococcus	31.7	*Staphylococcus*	18.2
	Salinicola	21.8		
	Virgibacillus	7.9		
Sorbitol	*Staphylococcus*	64.6	*Staphylococcus*	67.1
	Enterobacteriaceae[2]	24.4	*Salinicola*	16.2
	Serratia	4.8	*Kushneria*	11.1
	Weissella	4.3	*Tetragenococcus*	5.3
Cu	*Acetobacterium*	78.1	*Acetobacter*	51
	Arcobacter	4.7	*Algoriphagus*	32.5
	Acholeplasma	3.8	*Alistipes*	8.1
	Aureispira	3.5	*Arcobacter*	2.7
HCl	*Acetobacter*	32.5	*Acetobacter*	23.4

	Amphritea	25.2	*Arenimonas*	15.5
	Bacteroides	11.4	*Arcobacter*	11.7
	Arcobacter	11.2	*Aeromonas*	7.5
	Arenimonas	1.7	*Acetoanaerobium*	6.1
			Alcaligenes	3.4
			Acinetobacter	3.1
Zn	*Alkanindiges*	31.4	*Acinetobacter*	63.1
	Acholeplasma	29.9	*Arcobacter*	12.4
	Arcobacter	13.3	*Aquabacterium*	12.2
	Arenimonas	6.5	*Arenimonas*	6.3

[1] The relative abundance (%) of the most abundant genera/families shown in this table in the no-stressor control enrichments are: Freshwater: *Acetobacterium* (30.9), *Aquabacterium* (24.9), *Colwellia* (3.4), *Bdellovibrio* (3.4), *Bacteroides* (2.9), *Acinetobacter* (2.9), *Bacteriovorax* (2.8), *Carnobacterium* (1.8), *Cellvibrio* (1.4), *Desulfopila* (1.5), *Comamonas* (1.0); Marine: *Anderseniella* (25.3), *Arcobacter* (20.9), *Arenimonas* (10.9), *Bacteriovorax* (7.2), *Acetobacter* (6.4), *Alistipes* (5.5), *Acinetobacter* (3.1), *Brevundimonas* (1.4), *Clostridium* (1.3).

[2] Taxa could be confidently assigned only to family level. Genera stated in the table but belonging to these families are excluded from the relative abundance shown for the families.

Given the abundance of *Staphylococcus* in both $MgCl_2$ and sorbitol enrichments the phylogeny of their OTUs was assessed in order to determine whether these stressors enriched for different OTUs, and to determine similarity to known *Staphylococcus* species, including pathogens. OTU1, which was 99.14% similar to *S. equorum* PA 231 based on 16S rRNA sequences, was by far the most abundant out of all the *Staphylococcus* OTUs in all investigated enrichments. The other OTUs were mostly present in the freshwater sorbitol enrichments, and in much lower abundance (Figure S2.3).

2.3.3.2 Fungi enriched with different stressors

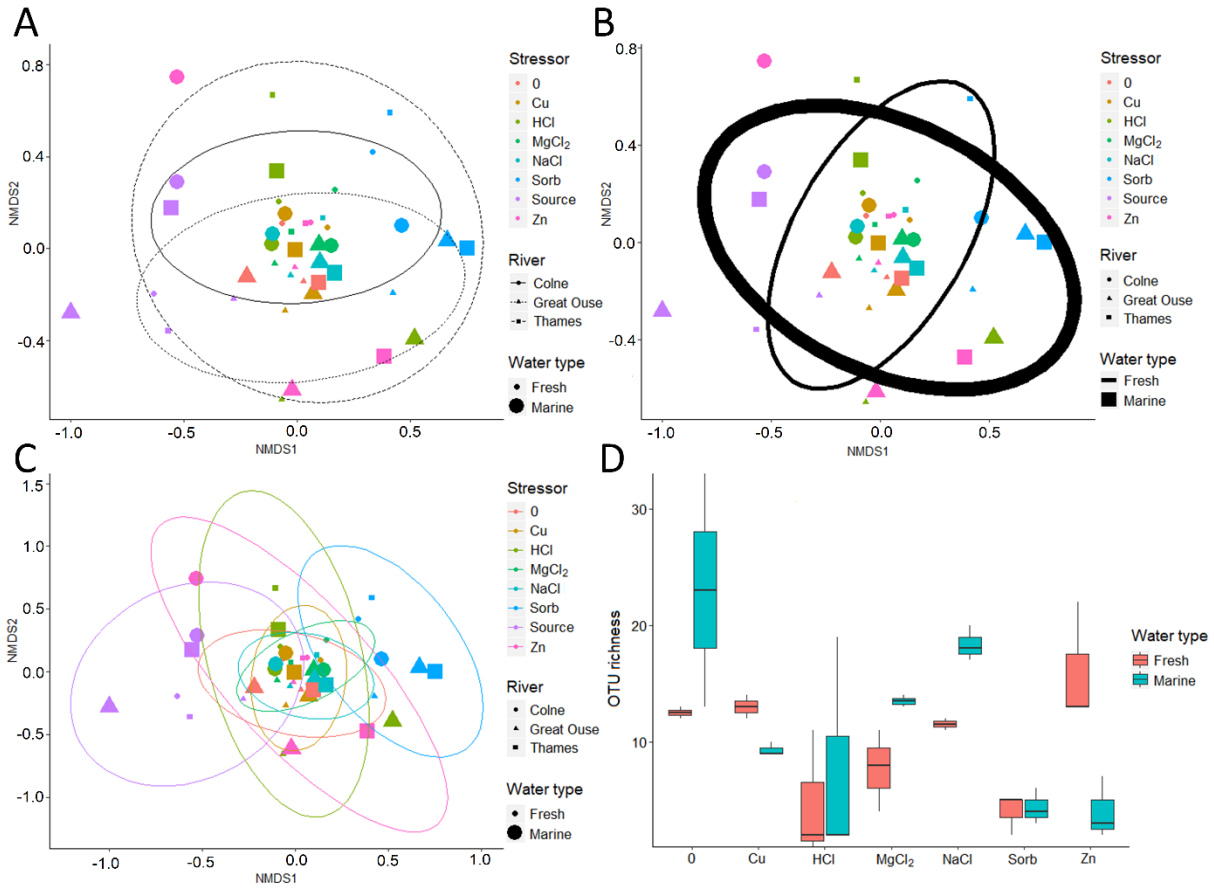

Figure 2.6 Analyses of fungal communities based on the ITS region sequence analysis and clustering sequences into operational taxonomic units (OTUs) at the 97% identity threshold. Panels A – C show community composition based on Non-metric multidimensional scaling (NMDS, Bray-Curtis dissimilarity, 5000 iterations) in the different treatments with 95% confidence ellipses for A: River, B: Water type and C: Stressor. 0: control-enriched communities; Source: source communities. Panel D is a box plot with whiskers showing OTU richness of the control-enriched and stressor-enriched communities (horizontal line = median, lower part of the box = 25th Percentile, upper part of the box = 75th Percentile; lower and upper vertical lines/whiskers = remaining 25% of the data on upper and lower parts). OTU richness of the source communities (mean of three freshwater samples = 73.7, mean of three marine samples = 62.7) is not shown as it would distort the scaling (see Figure S2.2). Stressor abbreviations are Cu = $CuSO_4$, Sorb = Sorbitol, Zn = $ZnCl_2$, and the control-enriched community is indicated by "0".

While enrichment type clearly altered the community composition, compared with the bacterial enrichments, more similar fungal genera were detected across a range of stressors in relatively high abundance (Table 2.2). The dominant taxa were: *Geotrichum* (NaCl freshwater and HCl enrichments), *Penicillium* ($MgCl_2$ and Zn freshwater enrichments and NaCl, $MgCl_2$, sorbitol and Cu marine enrichments), *Metschnikowia* (sorbitol freshwater enrichment), *Debaryomyces* (sorbitol marine enrichment), *Candida* (Cu and HCl freshwater enrichments and Cu, HCl and Zn marine enrichments), *Cladosporium* (NaCl and $MgCl_2$ freshwater enrichments and $MgCl_2$ and Cu marine enrichments) (Table 2.2). However,

several of the stressor-enriched genera, *Penicillium, Cladosporium* and *Candida,* were also in relatively high abundance in the no-stressor control (Table 2.2), and so more detailed analysis was performed to determine whether there was distinction at the OTU level between stressor- and control-enriched communities.

Table 2.2 Mean relative abundances (%) of the dominant fungal taxa (genera or families) in the stressor enrichments. Percentage contribution of taxa was calculated with respect to the total fungal community in each enrichment.[1]

Stressor	**Fungi**			
	Freshwater		Marine	
	Genus/Family	Rel. abund. (%)	Genus/Family	Rel. abund. (%)
NaCl	*Geotrichum*	23.9	*Penicillium*	29.2
	Cladosporium	23.2	*Cladosporium*	12.0
	Kluyveromyces	17.9	*Candida*	11.9
	Penicillium	9.3	*Leucosporidiella*	6.2
	Candida	6.1	*Ilyonectria*	3.0
$MgCl_2$	*Penicillium*	54.5	*Penicillium*	39.7
	Cladosporium	38.8	*Cladosporium*	25.3
	Geotrichum	2.1	*Ustilago*	7.6
	Metschnikowia	1.1	*Cladosporium*	7.2
			Sporobolomyces	3.5
Sorbitol	*Metschnikowia*	64.9	*Debaryomyces*	31.5
	Candida	19.2	*Leucosporidiella*	29.5
	Guehomyces	8.7	*Penicillium*	28.3
	Penicillium	7	Cystofilobasidiaceae[2]	10.2
Cu	*Candida*	40.1	*Penicillium*	29.3
	Penicillium	22.5	*Cladosporium*	28
	Metschnikowia	16.6	*Candida*	27.7
	Cladosporium	7.1	*Geotrichum*	4.3
HCl	*Geotrichum*	35.1	*Geotrichum*	35.6
	Candida	30.7	*Candida*	33.9
	Penicillium	12	*Penicillium*	10.3
	Cladosporium	2.7	*Rhodotorula*	5
	Metschnikowia	1.7	*Cladosporium*	4.4
			Aspergillus	2.7
Zn	*Penicillium*	49.8	*Candida*	60.1
	Cladosporium	14.4	*Rhodotorula*	33.3
	Candida	6.3	*Pichia*	5.5
	Metschnikowia	4.4	*Kluyveromyces*	0.5
	Tilletiopsis	2.6		

[1] The relative abundance (%) of the most abundant genera/families shown in this table in the no-stressor control enrichments are: Freshwater: *Penicillium* (32.4), *Cladosporium* (12.0), *Candida* (7.3), *Metschnikowia* (6.3), *Guehomyces* (6.2), *Geotrichum* (5.4), *Devriesia* (1.1);

Marine: *Candida* (37.9), *Penicillium* (28.7), *Cladosporium* (6.5), *Geotrichum* (2.5), *Mycena* (2.3), *Debaryomyces* (1.5), *Cystofilobasidium* (1.3).

[2] Taxa could be confidently assigned only to family level. Genera stated in the table but belonging to these families are excluded from the relative abundance shown for the families.

2.4 Discussion

The aim of the study was to identify the physiological limits and stressor range of growth of microbial communities from non-extreme environments that were enriched with and without stressors. We enriched microorganisms capable of growing in several extreme conditions despite sampling from non-extreme marine and freshwater sites across three geographically and physicochemically distinct river systems. This finding suggests that microorganisms capable of growth in extreme conditions may be ubiquitous. Even specific genera maybe ubiquitous, as generally, for a given stressor, very similar genera dominated each enrichment independent of their source environment.

2.4.1 Extremotolerants/extremophiles in non-extreme environments

Many of the genera identified in the communities enriched in extreme conditions have been reported previously as containing species that are facultative extremotolerants or obligate extremophiles. The presence of extremotolerant microbial communities in seemingly non-extreme riverine and marine environments can be explained by three scenarios that are not mutually exclusive: 1) the microbes are periodically confronted by these stressors in their environments and so have evolved mechanisms to resist them as a means of being competitive in rivers and seawater; 2) they are inherently tolerant of other stressors present in their environment that fortuitously protect against the stressors added here; 3) they derive from environments (along a spectrum of nearby to remote) where they are subjected to the stressors used in this study.

Scenario 3 can be invoked in all cases, while scenarios 1 and 2 are more likely to be the case for heavy metals than for the other stressors, as the high levels of salts and sorbitol used in this experiment would not occur in these riverine or marine waters, while HCl additions would be buffered and there are no reports of any of these environments reaching a pH of ~ 3.5, which would have led to catastrophic death of wildlife.

2.4.2 Why heavy metal resistance may be a common trait

Metal-resistant microorganisms are common in aquatic environments (Squadrone, 2020), and the selected rivers in this study may be contaminated with relatively high concentrations of heavy metals. The microbes enriched from the water column may have resided in sediment where metal loads are higher, but were resuspended when sampled. One of several metal-resistance mechanisms (Hobman and Crossman, 2015), efflux pumps, can provide protection against other environmental challenges and lead to antibiotic resistance (Bazzi *et al.*, 2020; Squadrone, 2020). Equally, heavy-metal resistance can be caused by the presence of other contaminants, including antibiotics, conferred by co-resistance (i.e. resistance genes for heavy metals and, for example, antimicrobial agents, being in close proximity on mobile genetic elements which then could be transferred easily) or cross-resistance (e.g. via efflux pumps that can remove toxic organics and metals from the cell) (Bazzi *et al.*, 2020). The reassurance of such resistance mechanisms in a microbial community can be enhanced by lateral gene transfer (Romaniuk *et al.*, 2016).

The selection of *Acinetobacter* from marine waters in enrichments with high concentrations of zinc is noteworthy, although it does not reach the currently known limits of zinc resistance (*Ralstonia* isolate TAK1, a cadmium-resistant bacterium isolated from a zinc mine (0.4 M) (Mergeay *et al.*, 1985; Prapagdee and Watcharamusik, 2009)).This genus is found in various environments (Jung and Park, 2015; Al Atrouni *et al.*, 2016), and has extremotolerant and

pathogenic strains, including multi-drug resistant human pathogens (Jung and Park, 2015; Al Atrouni *et al.*, 2016), and strains exhibiting heavy-metal resistance (Veress *et al.*, 2020). Indeed, it has been proposed that heavy-metal toxicity enhances antimicrobial resistance (AMR) in *Acinetobacter baumanii* during armed conflicts (Bazzi *et al.*, 2020). However, both dominant *Acinetobacter* OTUs in our study were distinct from *A. baumanii* (< 97.2% 16S rRNA sequence identity). OTU24880 had 98.3% 16S rRNA identity to *A. tjernbergiae* DSM 14971, but was much more similar (99.4-99.8%) to uncultivated sequences detected in diverse wastewater treatment plants. OTU30122 had 99.6% identity to *A. movanagherensis* strain Movanagher 4 and 99.8-100% identity to heavy-metal resistant isolates (Abbas *et al.*, 2014) and both sequences and isolates from various water bodies including wastewater treatment systems and rain water. Thus, *Acinetobacter* strains may have been sourced from wastewater treatment plants, where the combination of high concentrations of metals and other pollutants (Chipasa, 2003), along with high concentrations of organics and aeration, would provide an ideal “breading ground” for members of this genus (Zhang *et al.*, 2009).

2.4.3 Halotolerant/osmotolerant Bacteria may derive from nearby environments with a relatively low water activity and/or are part of the globally dispersed seed bank

As mentioned above, Scenario 3, in which enriched microbes derive from environments beyond the rivers and seawater, is most likely to have led to selection of some halotolerant and many osmotolerant microbes. Each of the estuaries and coastal areas associated with the marine sites has mudflats and salt marshes, which are subjected to desiccation and extreme salinities due to varying tides and rainfall that can select for halotolerant and haloversatile microbes (McKew *et al.*, 2011), which can be washed into the estuary and the sea. However, up-river there are no such mudflats and salt marshes, yet, for a given stressor

very similar taxa dominated each enrichment, irrespective of whether the source was a river or seawater (*Halomonas* with NaCl, *Halobacillus* with $MgCl_2$ and *Staphylococcus* with sorbitol). Thus the presence of these enriched taxa in other surrounding environments and in the rivers and seawater itself must also be considered. Indeed, *Halomonas*, which dominated NaCl enrichments, is a cosmopolitan species, distributed from pole-to-pole (Staley and Gosink, 1999), shallow to deep sea (Kaye *et al.*, 2011), and arid/saline soils (de la Haba *et al.*, 2014), and species can collectively grow in media from nearly zero to saturated NaCl (de la Haba *et al.*, 2014). Chaotropic $MgCl_2$ enrichments were dominated by spore-forming *Halobacillus,* which is also relatively ubiquitous (Staley and Gosink, 1999; Sass *et al.*, 2008; McKew *et al.*, 2011). A similar result – *Halobacillus* enriched with $MgCl_2$ and *Halomonas* with NaCl – were found when agricultural soil was inoculated into yeast-extract media (Patel, Low-Decarie, McGenity, unpublished). However, there have been no mechanistic studies to explain why *Halobacillus* would be more competitive in chaotropic enrichments. Large-scale chaotropic environments are relatively uncommon (Hallsworth *et al.*, 2007), but bacterial spores are readily dispersed by wind, birds and water, and are abundant, especially in soils and sediments where they can survive for many years (Lennon and Jones, 2011; Ellegaard *et al.*, 2020), thus providing a large microbial seed bank.

Staphylococcus was very abundant in both freshwater and marine enrichments with sorbitol (Table 2.1). Sorbitol is a sugar alcohol that is a major end-product of photosynthesis, and, like NaCl, is kosmotropic and exerts osmotic stress but without the associated ionic effects (de Lima Alves *et al.*, 2015). While NaCl did not select for *Staphylococcus*, the chaotropic salt, $MgCl_2$, led to its enrichment as the second-most abundant genus (Table 2.1). Species such as *S. aureus* grow down to a water activity of 0.860 (Grant, 2004), which is lower than the water activity of the enrichment media supplemented with sorbitol (freshwater: 0.928, marine: 0.912) or $MgCl_2$ (freshwater: 0.915, marine: 0.905). The latter is consistent with the capacity of *S. aureus* to grow in media with up to 0.77 M Mg^{2+} (Armitano *et al.*, 2016).

However, the OTUs identified were not closely related to *S. aureus*, (two OTUs were similar but not identical in 16S rRNA sequence to pathogenic species of *S. haemolyticus* and *S. warneri;* Figure S2.3). In fact, in three of the enrichments OTU1 with 99.14% 16S rRNA sequence identity to *S. equorum* constituted >99% of the staphylococci; the exception was the freshwater sorbitol enrichment, which had an even distribution of five OTUs shown in Figure S2.3, plus low abundance of two others. Staphylococci are found ubiquitously in the environment, and *S. equorum* is common on the skin of animals and cheese (Kastman *et al.*, 2016). The skin environment of mammals may represent a local source of *S. equorum*-like microbes in the freshwater and marine environments. This species has been shown to be a slow coloniser and a poor competitor of other non-coagulase staphylococci in the cheese environment, yet its widespread dominance on cheese rinds was aided by interactions with fungi, including *Penicillium* species (Kastman *et al.*, 2016), which were also abundant in the three enrichments where OTU1 exceeded 99% relative abundance of *Staphyloccus* species (Table 2.1 and Table 2.2). Thus, we speculate whether similar biotic interactions may also be at play in our enrichments.

2.4.4 Acidic microenvironments as a likely source of acidotolerant microbes

The enrichment of acidotolerant microbes from rivers and seawater in this study and from a lake (Low-Decarie *et al.*, 2016) may be due to their growing in locally acidic micro-environments in riverine, lacustrine or marine sediments and/or nearby soils, or even in the acidic gastro-intestinal tracts of aquatic organisms. Bulk pH measurements within environments often disguise large variation on a micro-scale. For example, microbial communities within biofilms in Antarctic granite rocks formed a much more acidic microhabitat (pH 3) compared to the outer surface (pH 6) (Los Ríos *et al.*, 2003). This activity allows acidotolerants to outcompete other microbes that cannot tolerate or grow in acidic conditions (Johnson and Schippers, 2017).

The main bacterial genus enriched at low pH from both rivers and seawater was *Acetobacter*, which aerobically converts ethanol to acetic acid, thus they are adapted to the low pH environment that they generate (Kersters *et al.*, 2006). Although *Acetobacter* is typically associated with terrestrial, sugar-rich environments, and with vinegar manufacture, the genus is widespread, including in coastal environments (Küsel *et al.*, 1999). *Acetobacter* was also enriched from marine waters in the presence of high concentrations of copper. Although *Acetobacter* is neither an extreme acidophile (e.g. pH 0 for *Ferroplasma acidarmanus* (Dopson *et al.*, 2004)), nor is highly resistant to copper (e.g. 0.8 M Cu for *Acidithiobacillus ferrooxidans* (Orell *et al.*, 2010), it was abundant in both stressor enrichments. Given that metals generally increase solubility in acidic conditions, it is perhaps unsurprising that they should be metallotolerant, e.g. by sorption of the metal (Gupta and Diwan, 2017).

2.4.5 Most abundant fungal species in stressed and in non-stressed conditions

The role, abundance and function of Fungi in aquatic systems are not as well-studied compared to Bacteria (Gadd *et al.*, 2012; Taube *et al.*, 2018). However, recent studies showed that fungi can constitute up to 50% of eukaryotic abundance in freshwater and marine environments (Grossart *et al.*, 2019). However, even in this study, while the bacterial OTU richness was between 300 and 600, fungal OTU richness was only between 60 and 80 in the source communities (Fig S2). Fungi are found in various natural and artificial aquatic environments such as wastewater treatment plants and sewage systems (Grossart *et al.*, 2019). They have a major role in decomposition but also in the transformation and accumulation of metals (Gadd *et al.*, 2012). *Candida*, *Penicillium* and *Cladosporium* were the most abundant taxa, forming between 40 – 80% of most of the enrichments (Table 2.2). Interestingly, OTU715 (99.34% similar to *Penicillium scabrosum*, *P. swiecickii* and *P. kojigenum*) and OTU259 (99.34% similar to *Cladosporium angustiherbarum*, *C. aggregatocicatricatum, C. cladosporioides* and *C. rhusicola*) dominated every enrichment

over other *Penicillium* and *Cladosporium* species. *P. scabrosum* and similar taxa are general spoilage/decomposing moulds present in soil and air but can also be essential endophytes to crops or even plant pathogens, resistant to fungicides (Frisvad, Samson and Stolk, 1990; Kanetis, Förster and Adaskaveg, 2010; Visagie *et al.*, 2014; Waqas, Khan and Lee, 2014). *C. angustiherbarum* and similar taxa mostly cause plant diseases but are also present in lakes, soil and in halophilic plants such as *Pistacia* spp. (Abdel-Motaal *et al.*, 2009; Bensch *et al.*, 2015). However, being the most abundant OTUs in the control enrichments too, they seem to possess an inherent stress tolerance against sudden changes in the environmental conditions.

In contrast to *Penicillium* and *Cladosporium*, the relative abundance of *Candida* OTUs varied between different stressor and water types and between the control enrichments. The three dominant OTUs were OTU2 (99.3% similar to *C. sake*), OTU4 (99.45% similar to *Yarrowia deformans*), and OTU45 (98.6% similar to *Teunomyces cretensis*). These species are widespread in nature as spoilage-causing moulds, present in decomposing soil, normal part of the human microbiome or causing plant and fish diseases (Hoegl *et al.*, 1998; Middelhoven and Kurtzman, 2007; Boyd *et al.*, 2017). The only potential human pathogen found was OTU19 (99.3% similar to *C. parapsilosis*), which was 14.9% abundant in the Zn-marine enrichment only. As many infectious microbe require essential trace micronutrients, including zinc, an environment with high amounts of zinc could have induced growth of the marine yeast *C. parapsilosis*, that was previously reported to be tolerant to high concentrations of this metal (Jayasree and Saramma, 1996; Malavia *et al.*, 2017). In this study, the enrichment concentration was 12 mM $ZnCl_2$ compared to a previous study where the minimum inhibitory concentration of $ZnCl_2$ was 14.7 mM (Jayasree and Saramma, 1996).

Members of the genera *Cladosporium, Penicillium, Candida, Yarrowia*, *Rhodotorula, Debaryomces* and *Metschnikowia* were found to grow at low water activity isolated from various environments: salt flats, salterns, the Dead Sea and the Great Salt Lake but also in

salted food products, freshwater and soil, explaining the existence of these genera in the oceans (Butinar *et al.*, 2005; Jaouani *et al.*, 2014). Fungi are seemingly better at coping with osmotic stress than prokaryotes possibly due to their ability to sense and respond to salinity changes using the high-osmolarity glycerol (HOG) pathway, enabling more flexible adaptations (Zajc *et al.*, 2014). The stressor-enriched cultures grew well at the lowest NaCl levels as well as at highest, so they might be capable of growing at even higher salinities (2.6 – 5.2 M NaCl), which suggests that these communities can include extreme halotolerant taxa (Oren, 2006). On the other hand, the concentration of magnesium chloride (1.05 M) in the enriched media was only half of the currently known concentration supporting life without added kosmotropes (2.1 M) (Hallsworth *et al.*, 2007; Zajc *et al.*, 2014; Gunde-Cimerman, Plemenitaš and Oren, 2018). Considering that NaCl is a kosmotropic while $MgCl_2$ is a chaotropic agent, the communities withstanding the $MgCl_2$ enrichment concentrations can be considered chaotolerants. Zajc and colleagues managed to enrich several *Cladosporium* and *Penicillium* species from magnesium bitterns at ≥1.5 M $MgCl_2$, including *C. cladosporoides* and *P. brevicompactum* which was highly abundant in the freshwater $MgCl_2$ enrichment in this study as well, which might suggest some of these taxa capable of growing at the limits of $MgCl_2$ concentrations supporting life (Zajc *et al.*, 2014).

Candida species were found to be relatively tolerant to high concentrations of zinc and copper (Harrison *et al.*, 2006; Vadkertiová and Sláviková, 2006), hence why they were found in high abundance in the metal enrichments as well. Acid mine drainages cause the environment to become acidic and high in heavy metals which requires the microbes to tolerate such conditions. The taxa *Penicillium, Candida, Geotrichum* and *Rhodotorula* were previously found to tolerate similar conditions, hence their abundance in the HCl- and zinc enrichments (Patel, Tipre and Dave, 2009). This can facilitate the emergence of polyextremophiles/polyextremotolerants that can enter the freshwater systems and then disperse in the sea (Dopson *et al.*, 2004; Glukhova *et al.*, 2018).

2.4.6 Cost of extremotolerance

We reasoned that the stressor-enriched communities could have contained facultative extremotolerants (generalists) and/or previously dormant extremophiles (specialists). The community data supports the idea that generalists were predominantly enriched. Local adaptation usually comes at a cost, i.e. an evolutionary trade-off: the balance between the benefits of adaptation to one condition and the associated costs in another condition (Hereford, 2009; Samani *et al.*, 2015). Exposing microbial communities to extreme conditions led to a wide range of growth rate (Figure 2.4). Thus, extremotolerant microbial communities are in an advantageous position over non-extreme specialists and can adapt faster to environmental perturbations (Sriswasdi, Yang and Iwasaki, 2017). The trade-off however is that the resultant community functions less well in the absence of the stressor in most cases, based on the lower growth rate at lower stressor concentrations, while the non-extreme specialist community has a higher growth rate and functions only at those concentrations (Figure 2.3). Stressor-enriched cultures also had different optimal stressor concentrations for different types of stressors. This raises further research questions on the resilience of the extremotolerant communities: how they would change and function after reverting to non-stressed conditions and whether tolerance to one stressor confers protection against another stressor (i.e. OTU715 and OTU259 as potential polyextremotolerants) (Gostinčar, Muggia and Grube, 2012; Gostinčar *et al.*, 2019).

2.4.7 Is everything everywhere? – The concept of microbial seed banks

Many previous studies have reported out-of-place microorganisms that are able to grow in conditions different from their surrounding environment (Purdy *et al.*, 2004; Hubert *et al.*, 2009; Gibbons *et al.*, 2013; Low-Decarie *et al.*, 2016). We need to focus on the second part

of the hypothesis of “Everything is everywhere, but the environment selects” (Baas Becking, 1934). Even though microbial seed banks contribute to a large portion of the uncultured microbes in any environment worldwide, biogeographic regionalisation does exist at a local scale. This is especially true for extreme environments. Extremophile microbes might survive normal conditions but it is not likely that mesophile microbes can do the same in extreme conditions. Extremotolerant microbes however, are able to survive in both, and might be capable of growth too. On the other hand, these microbes are not the dominant taxa when conditions are not extreme. There is a lack of research how these rare taxa are contributing to securing ecosystem functions (Kurm, Geisen and Gera Hol, 2019). Studying these microbes growing in non-extreme environments will facilitate our understanding of microbial biogeography and open up potentials for finding extreme-adapted enzymes for the field of biotechnology. For example, halophilic and halotolerant prokaryotes and fungi have many applications: biofuel, hydrolase, metabolite, pharmaceutical zinc nanoparticle production, bioremediation of pollutants (oils, heavy metals, hydrocarbons), development of salt- and drought-tolerant crops, or as genetic resources (Musa, Kasim and nagoor gunny, 2018). On the other hand, when serchin for extremotolerants locally for biotechnological use, one has to consider the disadvantages of finding potential opportunistic pathogens not only in rivers or the sea, but even inside the dishwasher (Raghupathi *et al.*, 2018; Zupančič *et al.*, 2019).

In conclusion, while the stressor-enriched microbial communities did not reach the currently known limits of the tested extreme conditions, community composition analysis verified the existence of extremotolerant microorganisms in these enrichments. This leads us to the conclusion that they do not contain previously dormant obligate extremophiles specialised to extreme environments but generalist facultative extremotolerants capable of withstanding extremes. In addition, the microbial community composition was mainly dependent on the stressor type, not the environment they were enriched from (three freshwater and three marine locations). This further supports the theory that microorganisms can be found

everywhere (microbial seed bank and high rates of dispersal), in varying abundances, defined by environmental conditions (Gibbons *et al.*, 2013).

2.5 References

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2.6 Supplementary material

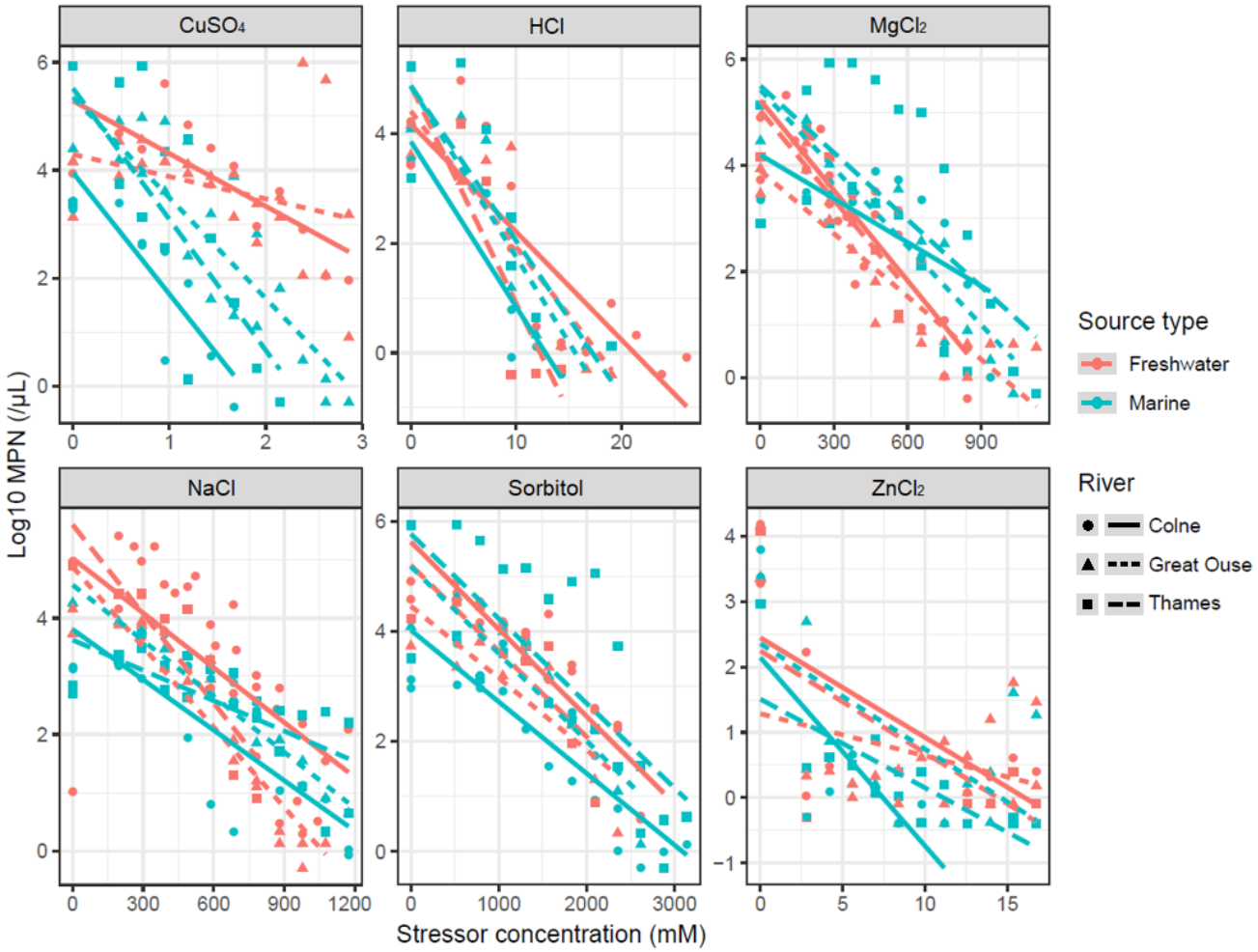

Figure S2.1 Abundance of microorganisms capable of growing at different concentrations of six stressors. The abundance declines log-linearly with stressor concentration and there is little difference between sampling sites for most stressors. Maximum concentrations in the gradient: $CuSO_4$ - 2.86 mM, HCl – 26.23 mM, $MgCl_2$ – 1130 mM, NaCl – 1170 mM, Sorbitol – 3140 mM, $ZnCl_2$ – 16.75 mM. The lines represent a linear regression fit to each sample from the three different rivers and from the two different locations (freshwater or marine) across each stressor gradient.

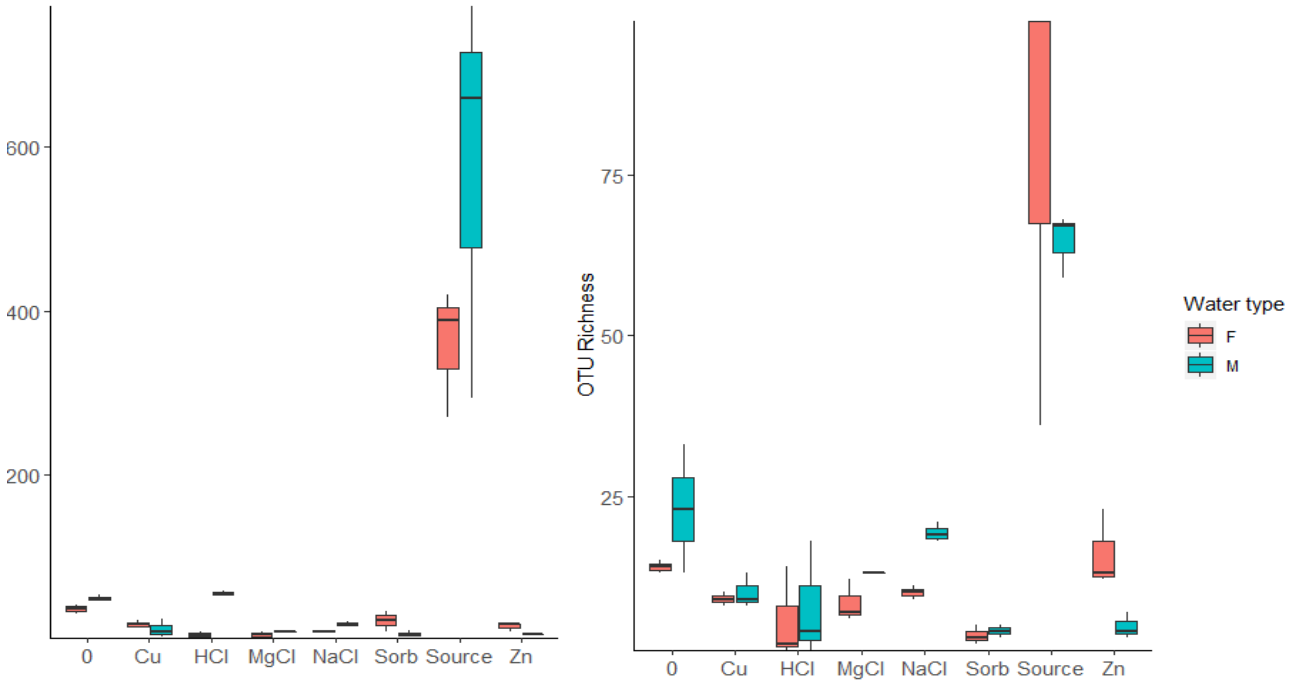

Figure S2.2 Bacterial (left panel) and fungal (right panel) OTU richness of the control-enriched, stressor-enriched and source microbial communities. Water type: F – Freshwater, M – Marine. 0 – Control enrichment with no stressor. The source communities had the highest OTU richness, followed by the control-enriched communities. Most of the stressor-enriched communities composed of very few OTUs.

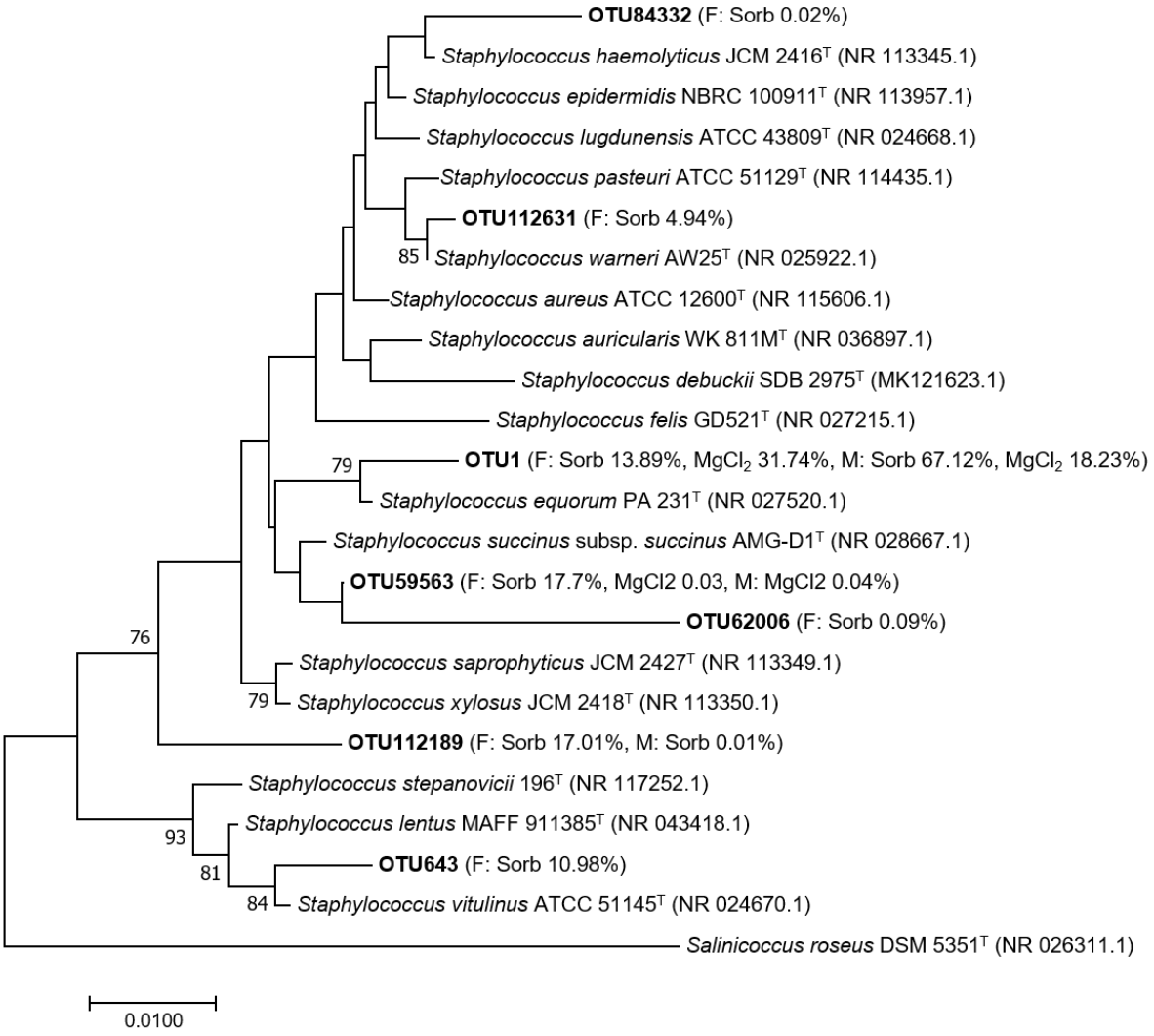

Figure S2.3 Neighbour-joining phylogenetic tree between *Staphylococcus* strains and OTUs based on 16S rRNA gene sequences. The phylogenetic tree was generated from a MUSCLE alignment of the type strain sequences, with *Salinicoccus roseus* DSM 5351T as an outgroup. The neighbour-joining method was used and the evolutionary distances were computed using the Kimura 2-parameter method in MEGA7 software (Kumar et al., 2016). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1500 replicates) are shown next to the branches (above 70%). The sequences were obtained from the National Center for Biotechnology Information (NCBI) with sequence accession numbers for each 16S rRNA sequence shown after each strain name. The relative abundances (%) are shown in brackets after the OTUs in each enrichment, rivers combined. The enrichments where an OTU was not present are not shown.

Table S2.1 Classification and examples of extremophiles and extremotolerants. Arch: Archaea, Bac: Bacterium, Euk: Eukaryote, opt: optimum growth.

Environmental parameter	Type	Description	Example	References
pH	Acidophile	Requires pH < 3	*Picrophilus oshimae* KAW2/2 (Arch), pH 0 – 3.5, opt: pH 0.4 – 1.2	Schleper *et al.*, 1995
			Ferroplasma acidarmanus Fer1^T (Arch), pH 0 – 2.5, opt: pH 1.0 – 1.7	Dopson *et al.*, 2004
	Acidotolerant	Able to grow in pH < 3	*Exophiala oligosperma* R1 (Euk), pH 1.5 – 9.0, opt: pH 4.0 – 7.0	Rustler and Stolz, 2007
			Hortaea acidophila (Euk), pH 1.0 – 9.0, opt: pH 2.0 – 6.0	Hölker *et al.*, 2004
	Alkaliphile	Requires pH > 9	*Alkaliphilus transvaalensis* (Bac), pH 8.5 - 12.5, opt: pH 10.0	Takai *et al.*, 2001
			Bacillus marmarensis GMBE 72 (Bac), pH 8.0 - 12.5, opt: pH 10.0	Denizci *et al.*, 2010
	Alkalitolerant	Able to grow in pH > 9	*Aspergillus niveus* RS2 (Euk), pH 4.0 – 10.0, opt: pH 8.0	Sudan and Bajaj, 2007
			Gibellulopsis nigrescens (Euk), pH 4.0 – 10.4, opt: pH 5.3 – 7.2	Bondarenko *et al*,, 2015; Zare *et al.*, 2007
Salinity	Borderline extreme halophile	Optimum growth: 1.4 - 4.0 M NaCl	*Halorhodospira halophila* (Bac)	Oren, 2006
	Extreme halophile	Optimum growth: 3.5 - 4.5 M NaCl	*Halobacterium salinarum* (Arch), 2.4 - 5.2 M NaCl, opt: 3.5 – 4.5 M NaCl	Oren, 2009
			Salinibacter ruber M31^T (Bac), growth: 2.5 - 5.2 M NaCl, opt: 3.4 – 5.13 M NaCl	Antón *et al.*, 2002
	Halotolerant	Able to grow in high salinity. If it grows above 2.5 M NaCl, it is considered extremely halotolerant	*Halomonas elongata* strain 1H9 (Bac), 0.05 – 4.5 M NaCl, opt: 0.6 – 1.4 M NaCl	Vreeland and Martin, 1980
			Hortaea werneckii (Euk), 0 – 5.2 M NaCl, opt: 1 – 1.7 M NaCl	Kogej *et al.*, 2005
Osmotic pressure	Osmophile	Grows in ≥ 60% total sugar/salt concentration and in a_w < 0.85.	*Zygosaccharomyces rouxii* (Euk), 27 - 90% (w/v)/ 1.5 – 5 M glucose, opt: 30% (w/v)/1.66 M glucose	Martorell *et al.*, 2007

			Candida magnoliae (Euk), 27 – 85.5% (w/v)/ 1.5 – 4.75 M glucose, opt: 30 - 50% (w/v)/1.66 – 2.7 M glucose	Martorell *et al.*, 2007
	Osmotolerant	Able to grow in high osmotic pressure, ≤ 60% total sugar/salt concentration	*Meyerozyma (Pichia) guilliermondii* (Euk), 20 – 57.6% (w/v)/ 1.1 – 3.2 M glucose, opt: 20 – 30% (w/v)/ 1.1 - 1.7 M glucose	Martorell *et al.*, 2007
			Candida zemplinina (Euk), growth: 2 – 60% (w/v)/0.1 – 3.3 M glucose, opt: 2 – 40% (w/v)/ 0.1 - 2.2 M glucose	Sipiczki, 2003
Chemical extremes	Metal-accumulating metallotolerant	Resistant and is able to accumulate/bioccumulate one or more metals	Microbial consortium *Ing5* (Bac), resistance and accumulation of Zn, Cd, Hg; bioaccumulation of Zn	Sprocati *et al.*, 2006
	Metallotolerant	Resistant to one or more metals	*Cupriavidus metallidurans* CH34 (Bac), resistant to Zn, Co, Cd, Ni	Mergeay *et al.*, 1984
			Halomonas sp. GFAJ-1 (Bac), resistant to AsO_4^{3-}	Erb *et al.*, 2012
			Ferroplasma acidarmanus $Fer1^T$ (Arch), resistant to Co, Cu, AsO_4^{3-}	Dopson *et al.*, 2004
			Sinorhizobium sp. M14 (Bac), resistant to AsO_4^{3-}, Cd, Co, Cu, Fe, Hg, Ni, Ag, Zn	Romaniuk *et al.*, 2017
Chaotropicity	Chaotolerant	Able to grow in chaotropic substances but grows better with additional kosmotropes	*Wallemia ichthyophaga* (Euk), Max: 2.1 M MgCl2 + 2 M NaCl; 4.0 M NaBr + 2 M NaCl	Zajc *et al*., 2014
			Hortaea werneckii (Euk), Max: 2.0 M MgCl2; 1.7 M CaCl2; 4.0 M NaBr	Zajc *et al*., 2014
			Eurotium repens (Euk), Max: 2.1 M MgCl2; 1.5 M CaCl2; 2.5 M NaBr	Zajc *et al*., 2014
			Cladosporium cladosporoides (Euk), Max: 2.0 M MgCl2; 1.7 M CaCl2; 3.0 M NaBr	Zajc *et al*., 2014

Table S2.2 Initial maximum stressor concentrations in the gradient for the measurement of abundance. Concentration of stressors varied linearly from maximum stressor concentration to zero across the 12 columns of the 96-well microplate.

Condition	Initial maximum concentration	Increment in the gradient
NaCl	1.17 M	0.106 M
$MgCl_2$	1.13 M	0.103 M
$ZnCl_2$	16.75 mM	1.523 mM
$CuSO_4$	2.86 mM	0.26 mM
Sorbitol	3.14 M	0.285 M
HCl	26.23 mM	2.385 mM

Table S2.3 Differences in the growth rate (μ (d^{-1})) of the stressor-enriched versus the control-enriched microbial communities sorted by concentration categories within the concentration gradient (zero to high stressor; Fig. 3): low (first quartile), mid-low (second quartile), mid-high (third quartile) and high (fourth quartile). Sorbitol was only assessed at 3 concentrations, so no binning was done.

Stressor	Concentration category	*t*-value	Degrees of Freedom	Control (μ (d^{-1}), mean ± SD)	Stressor (μ (d^{-1}), mean ± SD)	95% Confidence Intervals
NaCl	Low#	0.929	98	0.111 ± 0.011	0.109 ± 0.024	[-0.003, 0.009]
NaCl	Midlow***	-13.387	105	0.045 ± 0.033	0.103 ± 0.016	[-0.066, -0.049]
NaCl	Midhigh***	-26.401	75	0.003 ± 0.004	0.071 ± 0.022	[-0.073, -0.063]
NaCl	High***	-13.383	72	0.002 ± 0.002	0.032 ± 0.019	[-0.035, -0.026]
$CuSO_4$	Low#	-0.371	119	0.097 ± 0.036	0.099 ± 0.022	[-0.012, 0.008]
$CuSO_4$	Midlow***	-6.698	139	0.030 ± 0.032	0.064 ± 0.028	[-0.044, -0.024]
$CuSO_4$	Midhigh***	-9.769	72	0.001 ± 0.002	0.033 ± 0.028	[-0.039, -0.026]
$CuSO_4$	High***	-4.060	72	0.000 ± 0.001	0.004 ± 0.009	[-0.006, -0.002]
HCl	Low***	10.478	108	0.121 ± 0.016	0.084 ± 0.022	[0.030, 0.043]
HCl	Midlow***	-11.084	118	0.018 ± 0.024	0.067 ± 0.024	[-0.057, -0.040]
HCl	Midhigh***	-14.895	63	0.006 ± 0.004	0.051 ± 0.023	[-0.052, -0.039]
HCl	High***	-24.487	79	0.008 ± 0.005	0.051 ± 0.012	[-0.046, -0.039]
$MgCl_2$	Low***	10.296	99	0.111 ± 0.016	0.065 ± 0.034	[0.037, 0.054]
$MgCl_2$	Midlow***	-9.280	131	0.025 ± 0.022	0.066 ± 0.030	[-0.049, -0.032]
$MgCl_2$	Midhigh***	-15.993	83	0.006 ± 0.007	0.050 ± 0.023	[-0.050, -0.039]
$MgCl_2$	High***	-13.165	74	0.003 ± 0.002	0.029 ± 0.016	[-0.030, -0.022]
$ZnCl_2$	Low#	-0.678	108	0.058 ± 0.048	0.062 ± 0.026	[-0.017, 0.008]
$ZnCl_2$	Midlow***	-13.181	86	0.005 ± 0.004	0.024 ± 0.012	[-0.022, -0.016]
$ZnCl_2$	Midhigh***	-12.563	95	0.003 ± 0.001	0.009 ± 0.003	[-0.006, -0.005]
$ZnCl_2$	High***	-5.513	90	0.002 ± 0.001	0.004 ± 0.003	[-0.003, -0.001]
Sorbitol	Low***	3.885	21	0.061 ± 0.010	0.033 ± 0.029	[0.013, 0.043]
Sorbitol	Mid*	2.261	21	0.048 ± 0.011	0.030 ± 0.030	[0.001, 0.033]
Sorbitol	High#	-2.060	20	0.007 ± 0.009	0.024 ± 0.033	[-0.034, 0.000]

Significance (Welch's t test) is indicated in the 'concentration category' column: # is $p > 0.05$, *** is $p < 0.001$.

Table S2.4 Differences between the growth range (stressor concentration; Molar) of the stressor-enriched and the control-enriched microbial communities.

Stressor	*t*-value	Degrees of Freedom	Control conc. (M) , mean + SD	Stressor conc. (M) , mean + SD	95% Confidence Intervals
NaCl***	-25.059	45.327	1.077 ± 0.204	2.472 ± 0.181	[-1.508, -1.284]
Sorbitol#	0.110	18.550	1.563 ± 0.319	1.541 ± 0.719	[-0.398, 0.442]
$CuSO_4$***	-6.599	30.979	0.001 ± 0.000	0.002 ± 0.001	[-0.001, -0.001]
HCl***	-3.999	23.000	0.013 ± 0.006	0.018 ± 0.000	[-0.007, -0.002]
$MgCl_2$***	-9.945	37.274	0.464 ± 0.223	0.990 ± 0.132	[-0.633, -0.419]
$ZnCl_2$***	-12.210	45.984	0.001 ± 0.001	0.006 ± 0.001	[-0.006, -0.004]

Significance (Welch's t test) is indicated in the ''Stressor" column: # is $p > 0.05$, *** is $p < 0.001$.

Table S2.5 Results of PERMANOVA analysis in community composition among control and stressor enrichments, water type and river. The factors enrichment type and water type had a significant main effect on community composition and there was a significant interaction between them.

	Bacteria				Fungi			
Effects	F value	Degrees of Freedom	*p*-value	R^2	F value	Degrees of Freedom	*p* value	R^2
Enrichment	3.11	7	< 0.001	0.357	1.528	7	< 0.01	0.227
Water type	2.03	1	< 0.01	0.033	1.353	1	0.134	0.029
River	0.90	2	0.695	0.030	1.649	2	< 0.05	0.070
Enrichment x Water type	1.65	7	< 0.001	0.190	1.114	7	0.249	0.165
Enrichment x River	0.83	14	0.978	0.191	0.904	14	0.759	0.268
Water type x River	1.07	2	0.345	0.035	1.179	2	0.235	0.050
Residual	-	10	-	0.164	-	9	-	0.191
Total	-	43	-	1	-	42	-	1

Table S2.6 Results of Tukey HSD follow-up test comparing the bacterial OTU richness across enrichment types (control and stressor enrichments) and water types. All control-enriched communities had higher OTU richness than all stressor-enriched communities except the HCl marine enrichment. The HCl marine enrichment had much higher OTU richness than any other stressor enrichment and the control enrichments, making it an outlier, therefore it was excluded from the analysis. Water type does not have a significant effect on OTU richness without the HCl marine enrichment, thus there is no difference between the marine and freshwater enrichments within a stressor and only the enrichment type is affecting OTU richness.

Stressor type	**Difference between means**	**95% Confidence Intervals**	***p*-value**	**Stressor type**	**Difference between means**	**95% Confidence Intervals**	***p*-value**
Cu-0	-28	[-42.13],[-13.87]	$p < 0.001$	Cu-0	-28	[-42.13],[-13.87]	$p < 0.001$

HCl-0	-18.8	[-32.93],[-4.67]	$p < 0.01$	HCl-0	-38.67	[-54.76],[-22.57]	$p < 0.001$
MgCl-0	-37.17	[-50.76],[-23.57]	$p < 0.001$	MgCl-0	-37.17	[-50.77],[-23.57]	$p < 0.001$
NaCl-0	-30.67	[-44.26],[-17.07]	$p < 0.001$	NaCl-0	-30.67	[-44.27],[-17.07]	$p < 0.001$
Sorb-0	-31.67	[-45.26],[-18.07]	$p < 0.001$	Sorb-0	-31.67	[-45.27],[-18.07]	$p < 0.001$
Zn-0	-34.67	[-48.26],[-21.07]	$p < 0.001$	Zn-0	-34.67	[-48.27],[-21.07]	$p < 0.001$
M-F	6.54	[2.14],[10.93]	$p < 0.05$	M-F	0.28	[-4.24],[4.79]	$p = 0.9$
HCl:M-0:M	2.5	[-21.86],[26.86]	$p = 1$				
HCl:M-Cu:M	40	[17.76],[62.24]	$p < 0.001$				
MgCl:M-HCl:M	-47	[-69.24],[-24.76]	$p < 0.001$				
NaCl:M-HCl:M	-37	[-59.24],[-14.76]	$p < 0.001$				
Sorb:M-HCl:M	-49	[-71.24],[-26.76]	$p < 0.001$				
Zn:M-HCl:M	-49	[-71.24],[-26.76]	$p < 0.001$				

Left table: TukeyHSD results with the HCl marine enrichment included; Right: TukeyHSD results without the HCl marine enrichment. Cu = $CuSO_4$, 0 = Control, MgCl =: $MgCl_2$, Sorb = Sorbitol, Zn = $ZnCl_2$, M = Marine, F = Freshwater.

Table S2.7 Results of Tukey HSD follow-up test comparing the fungal OTU richness across enrichment types (control and stressor enrichments) and water types. The OTU richness varied across enrichment types and water types. Water type did not have a significant effect on OTU richness, thus there is no difference between the marine and freshwater enrichments within a stressor and only the enrichment type determines the differences between OTU richness. Cu = $CuSO_4$, 0 = Control, MgCl =: $MgCl_2$, Sorb = Sorbitol, Zn = $ZnCl_2$, M = Marine, F = Freshwater.

Stressor type	Difference between means	95% Confidence Intervals	*p*-value
Cu-0	-5.3	[-13.97],[3.37]	$p = 0.46$
HCl-0	-10.17	[-18.51],[-1.82]	$p < 0.05$
MgCl-0	-8.3	[-16.97],[0.37]	$p = 0.067$
NaCl-0	-0.7	[-9.37],[7.97]	$p = 1$
Sorb-0	-12.33	[-20.68],[-3.99]	$p < 0.01$
Zn-0	-5.67	[-14.01],[2.68]	$p = 0.34$
M-F	-0.69	[-3.42],[2.04]	$p = 0.61$

Chapter 3: *In vitro* evolution to extend the salinity growth range of halophilic and halotolerant microorganisms

3.1 Introduction

3.1.1 Extremophiles: halophiles and halotolerants

Extremophiles are mostly microorganisms that require one or more extreme condition for growth. Within this category, halophiles are organisms that require high salt concentrations for growth and primarily thrive in high salinity environments. Halotolerants grow in high salt concentrations but they do not require high salinity for growth, often growing better at lower salinities (Torsvik and Øvreås, 2008; DasSarma and DasSarma, 2017). This study will focus on one extremely halotolerant and two extremely halophilic microbial strains. Halophiles are a metabolically and taxonomically diverse group, distributed amongst the three domains of life (archaea, bacteria and eukaryotes). They can be characterized based on their requirement for salinity. Slight halophiles grow between 0.2 and 0.5 M NaCl (most marine bacteria), while moderate halophiles grow between 0.5 and 2.4 M NaCl (Oren, Arahal and Ventosa, 2009; McGenity and Oren, 2012; DasSarma and DasSarma, 2017). The last group of halophiles is called extreme halophiles and they can grow in salinities even above saturation: between 2.5 and 5.2 M NaCl. The three orders of Halobacteria (Halobacteriales, Haloferacales, and Natrialbales) consist entirely of halophiles, and predominantly extreme, obligate halophiles. Adaptations to living at high salt concentrations are often similar to adaptions to low water activity due to the effect of solutes on water activity. The key adaptation is osmoregulation and it includes the utilization of compatible solutes or osmoprotectants (osmolytes) by the cell (Torsvik and Øvreås, 2008). Every halophilic microorganism must cope with both high osmolarity and low water activity by maintaining intracellular osmotic equilibrium with the external environment. The two main strategies that

evolved for adaptation to high salt are the “salt-in” and the “compatible solute” strategy (described in section 1.3.2).

3.1.2 Experimental evolution in microbial populations

Evolution is the change of gene frequency in a gene pool. The divergence of populations (descendants of the same clonal ancestor or even mixed microbial communities) could reflect their adaptation to changes in environmental conditions (Lenski *et al.*, 1991). Evolutionary changes in an organism can occur gradually or suddenly (Barrick and Lenski, 2013). Changes in the genome of a single microbe or even in a whole microbial community are easier to observe in laboratory conditions due to their relatively short generation times, their sustainable large populations and the fact that samples of evolving populations can be kept frozen for later analysis. This allows the comparison of evolved cultures to their ancestors (Zeyl, 2006; Barrick and Lenski, 2013). The route of the evolution of experimental populations is determined by the culture conditions, such as a type of treatment (e.g. high osmolarity or temperature) or competition for a limiting nutrient. Rare mutations that increase reproductive success (natural selection) will appear owing to the selective pressure (Zeyl, 2006). Adaptation can be measured either as a shift in growth rate (e.g. towards a certain carbon source or salinity), sequencing taxonomic marker genes of communities, or through observations of genes that mutated in the genome. Mutations are changes in the nucleotide sequence of DNA that could be confined to a single base pair or involve multiple base pairs including deletion, insertion, duplication, frameshift and recombination. Mutation rate is the probability that a base pair or a larger DNA sequence changes with time (Balin and Cascalho, 2010). Mutations are usually detected by changes in phenotype per unit of time indicated as cell generations or days. Most bacteria have an average mutation rate of 10^{-7} to 10^{-10} base substitutions per nucleotide per generation (Westra *et al.*, 2017), while haloarchaea and some thermophiles and acidophiles were found to have an average genomic mutation rate

of 10^{-3} to 10^{-4} spontaneous mutations per genome per replication (Busch and DiRuggiero, 2010). However, we also need to account for generation time in each microbe: the halotolerant bacterium *H. elongata* has an average generation time of 1.16 (growth rate = 0.6 h^{-1}), while the obligate extreme halophile *Hbt. salinarum* has an average generation time of 6.93 (growth rate = 0.1 h^{-1}) at optimal conditions (calculated based on results shown in Figure 3.10).

For this study, the serial transfer method was chosen to carry out a laboratory-based evolution experiment (Figure 3.1). A fraction of the population was periodically transferred to fresh media and was allowed to regrow until it reached late exponential phase, prior to nutrient depletion, where it was subcultured again, allowing perpetual population growth (Barrick and Lenski, 2013). The serial transfer method was chosen for this study (and for Chapter 4 – Experimental evolution on halophilic and halotolerant microbial communities) as it leads to adaptive evolution, while genetic diversity is maintained through each transfer. The aim was to trigger mutations in a gentle way rather than using mutation accumulation, where populations are deliberately forced through a bottleneck of one or few individuals, as this

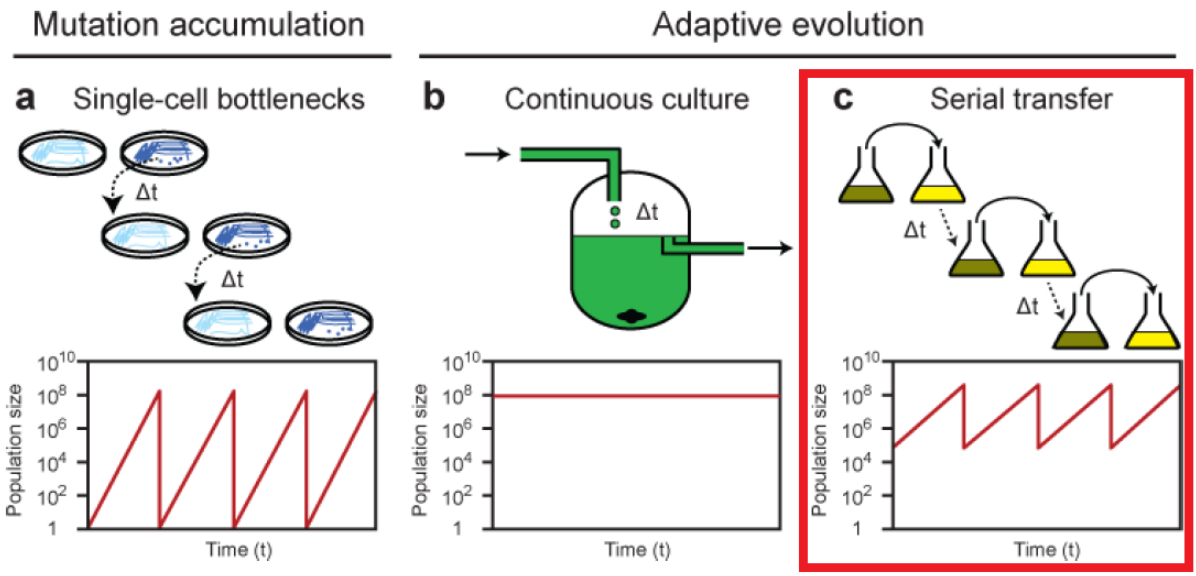

Figure 3.1 Types of evolution experiments. a: Mutation accumulation: one or a few randomly picked colonies of microorganisms that grow from single cells on agar *plates. Leads* to reduced genetic diversity and fixed arbitrary mutations. b: Continuous culture: chemostat with constant inflow of nutrients and constant outflow of leftover nutrients, metabolic waste and *microorganisms. Leads* to adaptive evolution and genetic diversity with nearly constant-sized populations. c: Serial transfer: Batch growth, where a fraction of the population is periodically transferred to fresh *media. Leads* to adaptive evolution with maintained genetic diversity through each transfer. Method c was used in this study. Figure adapted from Barrick and Lenski, 2013.

method reduces genetic diversity and does not account for natural selection (Barrick and Lenski, 2013). A continuous culture also leads to adaptive evolution but with constant instead of fluctuating selective pressure, however, this requires the addition of fresh nutrient medium and the removal of the same volume of cultures from the system, which was not feasible at the time becuase of the high number of cultures and lack of chemostats.

The experiment included three individual microbial strains: two haloarchaea and a halotolerant bacterium. *Hbt. salinarum* 91-R6 and *Hbt. salinarum* NRC-1 are extreme halophiles, growing in 2.4 – 5.2 M NaCl, while *H. elongata* 1H9 is an extremely halotolerant bacterium, growing in 0.4 – 4 M NaCl, however, it prefers low salinities (~2 M NaCl) over high salinities (Vreeland *et al.*, 1980; Oren *et al.* 2009). All of these strains have their own specific growth range, with the opaque-coloured, gram-negative rod-shaped *H. elongata* 1H9 being a generalist: having a wider growth range over salinity concentrations and synthesising mainly ectoine as a compatible solute (Vreeland *et al.*, 1980). The pink-pigmented, rod-shaped *Hbt. salinarum* 91-R6 and *Hbt. salinarum* NRC-1 use the “salt-in” strategy as adaptation against high salt, therefore they are able to grow only in relatively small ranges of salt concentrations, making them specialists to high salinities (Mohr and Larsen, 1963). The aim was to expose these strains to low, optimal and high salinities (0.4, 2.0 and 4.0 M NaCl for *H. elongata* 1H9 and 3.0, 4.0 and 4.7 M NaCl for *Hbt. salinarum* 91-R6 and *Hbt. salinarum* NRC-1, respectively) for two years, in order to assess how their salinity preference, genome, and morphology changes. Osmoadaptation requires genes that are involved in maintaining the intracellular ionic conditions suitable for growth in *Hbt. salinarum* species. Thus, based on previous studies, changes are expected in the genes that regulate the transport of potassium, chloride, sodium, phosphate and peptides, superoxide dismutase-encoding genes (role in response to oxidative stress), and transcription regulatory factors in *Hbt. salinarum* spp. (Coker *et al.*, 2007; Vauclare *et al.*, 2014). For *H. elongata* 1H9, changes are expected in the regulation of potassium and sodium transporter genes, transporter genes for

ectoine and other compatible solute accumulation, and genes having a role in the biosynthesis and degradation of ectoine (Kindzierski *et al.*, 2017; Gunde-Cimerman *et al.*, 2018). Sequencing the genome will shed light on how each genome of the strains have changed over time, and what mutations the cultures at different salinities acquired. The following hypotheses were established:

H1: Repeated culturing at high salinity will increase the capacity of all three strains to grow at high salinity and diminish their capacity to grow at low salinity.

H2: Repeated culturing at low salinity will increase the capacity of all three strains to grow at low salinity and diminish their capacity to grow at high salinity.

H3: Growth at optimal salinity will have no effect on the growth capacity of the evolved strains, meaning that if each strain is consequently subcultured at their optimum salinity conditions, it is expected that their salinity growth limits will not change compared to the salinity limits of the original strains.

H4: Beneficial mutations will primarily occur in genes with a role in osmoadaptation.

H5: More mutations will be seen in *Halomonas elongata* strain 1H9 owing to its shorter generation time than *Halobacterium* species.

3.2 Experimental procedures

3.2.1 Strain selection

One archaeal and one bacterial type strain was obtained from the Leibniz Institute's Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ): *Halobacterium salinarum* strain 91-R6 (DSM-3754) and *Halomonas elongata* strain 1H9 (DSM-2581), respectively. *Hbt. salinarum* 91-R6 was revived in 20% (3.4 M) NaCl Payne medium, while *H. elongata* 1H9 was revived in 15% (2.57 M) NaCl Payne medium (Payne *et al.*, 1960). *Hbt. salinarum* NRC-1 was revived in 20% Payne medium from the laboratory's culture collection.

3.2.2 Media testing and selection

The initial aim was to prepare media with a water activity (a_w) close to the currently known water activity limit of life (a_w = 0.585) (Stevenson *et al.*, 2016) that would have been used for diluting it to the required a_w and salinity for each medium, however, the low-water-activity media used in this study precipitated when cooling. A total of 52 different media were tested for water activity. Water activity values (a_w) were measured using Novasina AW SPRINT TH-500 instrument at 25°C. Various combinations of NaCl, $MgCl_2$, $MgSO_4$, sorbitol, glycerol and ethylene glycol were used in each medium (Table 3.1).

Table 3.1 Composition of tested initial media.

Medium No.	NaCl [M]	$MgCl_2.6H_2O$ [M]	$MgSO_4.7H_2O$ [M]	Sorbitol [M]	Glycerol [M]	Ethylene glycol [M]	Yeast extract [%]	Water activity (a_w)
1	2.57	0	0	0.27	0	0	1	0.877[a]
2	2.57	0	0	0.55	0	0	1	0.869[a]
3	2.57	0.25	0	0	0	0	1	0.871[a]
4	2.57	0.49	0	0	0	0	1	0.839[a]
5	2.57	0.25	0	0.27	0	0	1	0.857[a]
6	2.57	0.49	0	0.27	0	0	1	0.836[a]
7	2.57	0.25	0	0.55	0	0	1	0.857[a]
8	2.57	0.49	0	0.55	0	0	1	0.86[a]
9	3	1	0	0	0	0	1	0.795[a]
10	2	2	0	0	0	0	1	0.718[a]
11	1	3	0	0	0	0	1	0.65[a]
12	3	1	0	2	0	0	1	0.691[b]
13	2	2	0	2	0	0	1	0.658[c]
14	1	3	0	2	0	0	1	0.608[b]
15	3	1	0	3	0	0	1	0.658[b]
16	2	2	0	3	0	0	1	0.645[c]
17	1	3	0	3	0	0	1	0.559[b]
18	3	1	0	1.5	0	0	1	0.706[c]
19	2	2	0	1.5	0	0	1	0.625[b]
20	1	3	0	1.5	0	0	1	0.622[c]
21	3	2	0	1	0	0	1	0.649[b]
22	2	3	0	1	0	0	1	0.628[b]
23	3	2	0	0	0	0	1	0.696[b]
24	2	3	0	0	0	0	1	0.639[b]
25	3	1.5	0	1	0	0	1	0.7[b]
26	1.5	3	0	1	0	0	1	0.612[b]
27	2.5	2	0	1	0	0	1	0.671[b]
28	2	2.5	0	1	0	0	1	0.629[b]
29	3	1.5	0	0	0	0	1	0.76[d]
30	1.5	3	0	0	0	0	1	0.639[b]
31	2.5	2	0	0	0	0	1	0.695[b]
32	2	2.5	0	0	0	0	1	0.669[b]
33	3	1	0	1	0	0	1	0.726[a]
34	1	3	0	1	0	0	1	0.579[b]
35	2	2	0	1	0	0	1	0.668[b]
36	2	1.75	0	1	0	0	1	0.68[c]
37	2	1.75	0	1.5	0	0	1	0.662[b]
38	1.5	2.5	0	0	0	0	1	0.671[a]
39	2	1.5	0	1	0	0	1	0.69[a]
40	2	1.5	0	0	1	0	1	0.716[b]
41	2	1.5	0	0	0	1	1	0.702[b]
42	2.5	1	0	1	0	0	1	0.732[a]
43	2.5	1	0	0	1	0	1	0.754[d]
44	2.5	1	0	0	0	1	1	0.765[b]
45	0.4	0	0.04	0	0	0	1	0.982[a]
46	2	0	0.08	0	0	0	1	0.918[a]
47	2.4	0	0.04	0	0	0	1	0.904[a]
48	4	0	0.08	0	0	0	1	0.791[a]
49	4.9	0	0.08	0	0	0	1	0.749[c]
50	4.8	0	0.08	0	0	0	1	0.753[b]
51	4.7	0	0.08	0	0	0	1	0.765[b]
52	4.6	0	0.08	0	0	0	1	0.774[b]

[a]No precipitation; [b]Precipitation, salts did not dissolve; [c]Precipitation after cooling; [d]Opalescent-looking precipitation. Media 45, 46, 47, 48, 50 and 51 were chosen for further modification to make up more complex, Payne-based media (see Table 3.2). The hydrated salts $MgCl_2.6H_2O$ and $MgSO_4.7H_2O$ were used.

Based on previous studies on salinity growth range (Cánovas *et al.*, 1996; Aharon Oren, 2002; Oren, Arahal and Ventosa, 2009), three media were designed for *Hbt. salinarum* 91-

R6, *Hbt. salinarum* NRC-1 and for *H. elongata* 1H9: one close to the lower growth limits, one that supports optimal growth and one close to the higher limit of growth (Table 3.2).

Table 3.2 Finalised Payne-based media used in the laboratory evolution experiments

Component/Medium No.	M45[a]	M46[a]	M47b[b]	M48[ab]	M51[b]
NaCl [g/l]	23.376 (0.4 M)	116.88 (2.0 M)	175.32 (3.0 M)	233.76 (4.0 M)	274.668 (4.7 M)
$MgSO_4.7H_2O$ [g/l]	9.859 (0.04 M)	19.718 (0.08 M)	19.718 (0.08 M)	19.718 (0.08 M)	19.718 (0.08 M)
KCl [g/l]	2	2	2	2	2
Na_3Citrate [g/l]	3	3	3	3	3
Yeast extract (Difco) [g/l]	10	10	10	10	10
Casamino acids (Difco) [g/l]	7.5	7.5	7.5	7.5	7.5
$FeCl_2.4H_2O$ [mg/l]	36	36	36	36	36
$MnCl_2.4H_2O$ [mg/l]	0.36	0.36	0.36	0.36	0.36
Agar [g/l]	15	15	15	15	15
Water activity (a_w)	0.979	0.908	0.858	0.795	0.746

[a]Used for *H. elongata* 1H9; [b]Used for *Hbt. salinarum* strains 91-R6 and NRC-1.

3.2.3 Growth curve of the pre-cultures of *Halobacterium salinarum* 91-R6, *Halobacterium salinarum* NRC-1 and *Halomonas elongata* 1H9

Pre-cultures were made in triplicates of each strain with 150 µL inoculum in 15 mL liquid medium (Table 3.2) in 50 mL sterile Falcon tubes and were incubated at 30°C on a shaker of 200 rpm in the dark (Table 3.2). Optical density (OD_{600}) of *Hbt. salinarum* 91-R6, *Hbt.*

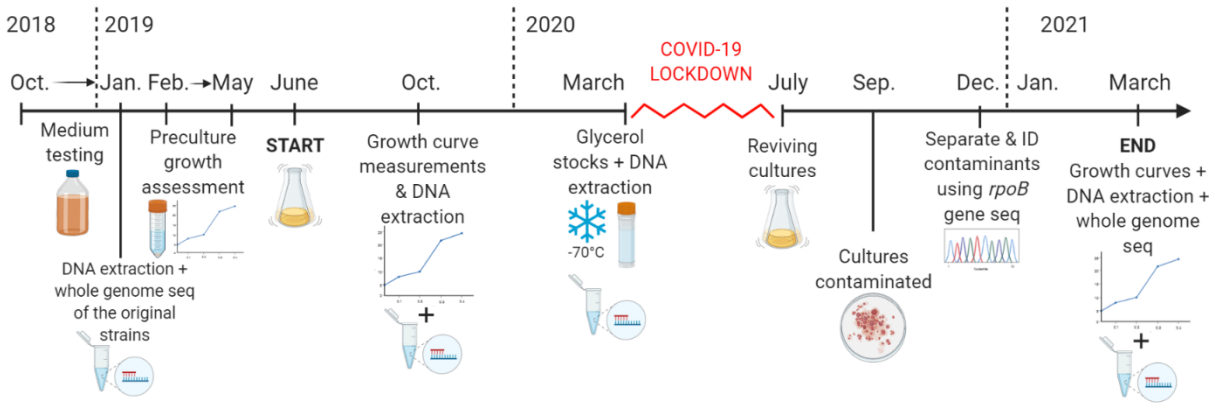

Figure 3.2 Schematic representation of the experiment design showing the experiment. Two halophilic (*Hbt. salinarum* NRC-1, *Hbt. salinarum* 91-R6) and one halotolerant strain (*H. elongata* 1H9) were subjected to experimental evolution to investigate how the genome and growth range change with different salinities.

salinarum NRC-1 and *H. elongata* 1H9 was measured with a spectrophotometer every 2 - 9 days and 8 - 48 hours, respectively, until cultures reached mid-stationary phase.

3.2.4 Culturing *Hbt. salinarum* 91-R6, *Hbt. salinarum* NRC-1 and *H. elongata* 1H9 at low, optimal and high salinities

After confirming that all pre-cultures were growing in all media, new cultures were set up for the long-term evolution experiment. A fresh inoculum (in late exponential phase) of 100 µL was used in 10 mL of medium for each strain at each salinity. *Hbt. salinarum* 91-R6 and NRC-1 were inoculated into media M47b (3 M NaCl), M48 (4 M NaCl) and M51 (4.7 M NaCl) in sterile Universal bottles. *H. elongata* 1H9 was inoculated into media M45 (0.4 M NaCl), M46 (2 M NaCl) and M48 (4 M NaCl) in sterile Universals. Based on the growth curve of the pre-cultures, the two haloarchaeal strains were continuously transferred every seven days, while *H. elongata* 1H9 was transferred every three to four days, after they reached exponential phase. All samples were incubated at 30°C on a shaker at 100 rpm in the dark. After four months and 20 months, the growth capacity of cultures was assessed over a range of NaCl concentrations: the two haloarchaeal strains were inoculated into six media of different salinities, while *H. elongata* 1H9 was inoculated into seven different salinity media, all in triplicate (Table 3.3). Optical density (OD_{600}) was measured every one to three days, until the cultures reached stationary phase. Growth rate was calculated based on the exponential phase of the growth curves. R statistical software was used to compare growth rate results between samples, based on salinity medium (R Core Team, 2021).

Table 3.3 Payne-based media used for the growth assessment of [b]Hbt. salinarum 91-R6, [b]Hbt. salinarum NRC-1 and [a]H. elongata 1H9 after six months of continuous subculturing. Labels [a] and [b] incdicate which medium was used for which strain.

Component/ Medium no.	M52[a]	M53[a]	M54[a]	M55[a]	M56[b]	M57[ab]	M58[b]	M59[ab]	M60[b]	M51[ab]
NaCl [g/l] (M)	0 (0)	40.91 (0.7)	87.66 (1.5)	134.41 (2.3)	157.79 (2.7)	181.16 (3.1)	204.54 (3.5)	227.92 (3.9)	251.29 (4.3)	247.67 (4.7)
$MgSO_4.7H_2O$ [g/l] (M)	9.86 (0.04)	9.86 (0.04)	9.86 (0.04)	19.718 (0.08)	19.718 (0.08)	19.718 (0.08)	19.718 (0.08)	19.718 (0.08)	19.718 (0.08)	19.718 (0.08)
KCl [g/l]	2	2	2	2	2	2	2	2	2	2
Na_3Citrate [g/l]	3	3	3	3	3	3	3	3	3	3
Yeast extract (Difco) [g/l]	10	10	10	10	10	10	10	10	10	10
Casamino acids (Difco) [g/l]	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
$FeCl_2.4H_2O$ [mg/l]	36	36	36	36	36	36	36	36	36	36
$MnCl_2.4H_2O$ [mg/l]	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36
Water activity	0.987	0.966	0.921	0.888	0.868	0.854	0.836	0.814	0.79	0.748
pH	7.1	6.9	6.9	6.9	7	7	7	7	7	7
Refractive index (RI)	1.339	1.346	1.353	1.362	1.365	1.368	1.371	1.374	1.378	1.383

Unfortunately, the lockdown of the laboratories (23/03/2020) affected the experiment, therefore the cultures were stored in glycerol at -70°C until the labs opened again (15/07/2020). The strains were revived on Petri dishes and in liquid media and besides visual assessment of clean cultures, taxonomic marker genes were sequenced in order to assess the purity of the strains. Some variation in colony morphology raised suspicions, which proved to be unfounded. Due to the fact that the whole 16S rRNA gene is 100% identical in *Hbt. salinarum* strains NRC-1 and in 91-R6, another gene, coding the RNA polymerase subunit B (‘*rpoB’*) was used as well as a taxonomic marker gene to distinguish these two strains from each other (Minegishi *et al.*, 2010). After the strains were verified to be pure cultures, the experiment continued until 20 months after the start of the experiment. Genomic DNA was extracted from all cultures at the start, after six months, after 11 months (before COVID-19 lockdown of labs), and after 20 months of subculturing.

3.2.5 Genome analysis

3.2.5.1 Genome sequencing

Genomic DNA samples of *Hbt. salinarum* 91-R6 and *H. elongata* 1H9 extracted at the beginning and at the end (19 months) of the experiment were chosen to be taken forward to whole genome sequencing. DNA extracts were visualised using agarose gel electrophoresis (1.0% w/v) to verify the integrity of the samples. DNA extracts were quantified in triplicates via Quant-IT^{TM} PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) using a Nanodrop 3300 Fluorospectrometer (Thermo Fisher Scientific). Genome sequencing and assembly was provided by MicrobesNG (https://microbesng.com), Birmingham (UK), using the Illumina HiSeq sequencing platform. The methods performed by MicrobesNG can be accessed at https://microbesng.com/microbesng-faq/ and are described here briefly. DNA libraries were prepared with the Nextera XT Library Prep Kit (Illumina, San Diego, USA), with certain modifications on the manufacturer's protocol (2x input DNA and 45 s PCR elongation time). DNA quantification and library preparation was conducted on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Pooled libraries were quantified with the Kapa Biosystems Library Quantification Kit (Illumina). The libraries were sequenced with a HiSeq/NovaSeq sequencer using a 250 bp paired end protocol. Adapter sequences were trimmed from reads using Trimmomatic 0.30 with a sliding quality cutoff of Q15 (Bolger, Lohse and Usadel, 2014). SPAdes version 3.7 was used for de novo assembly (Bankevich *et al.*, 2012), while Prokka version 1.11 was used to annotate contigs (Seemann, 2014).

3.2.5.2 Genome annotation and comparison

Forward and reverse reads and contig files were retrieved from MicrobesNG. Contigs smaller than 250 bp were removed from the assembly using BioEdit version 7.2.5 (Hall, 1999). Both

trimmed reads and trimmed contigs were submitted to PathoSystems Resource Integration Center (PATRIC) (Davis *et al.*, 2020). Read files of all samples were subjected to Comprehensive Genome Analysis which includes assembly (Unicycler pipeline, 2 Racon and 2 Pilon iterations, minimum contig length: 300 bp, minimum contig coverage: 5), annotation, identification of nearest neighbours, subsystem summary, phylogenetic tree, and the features distinguishing each genome from its nearest neighbours (Brettin *et al.*, 2015). The phylogenetic placement of the genomes was determined via the NCBI database of reference genomes using the PATRIC global protein families (PGFams). The reference genomes were *Halomonas elongata* strain 1H9 (DSM 2581) and *Halobacterium salinarum* strain 91-R6 (DSM 3754). The protein sequences from the PGFams were aligned with MUSCLE (Davis *et al.*, 2016), and the nucleotides were mapped onto the protein alignment. RaxML was used to analyse the data matric of the amino acid and nucleotide alignments (Stamatakis, 2014). Mutated genes were identified using the Variation analysis in PATRIC, and were verified using Protein Blast of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), then further examined in UniProt (https://www.uniprot.org), along with collecting information from the literature.

3.3 Results

3.3.1 Water activity values of tested media

Initially, an attempt was made to prepare media with a water activity (a_w) close to the currently known water activity limit of life (a_w = 0.585) (Stevenson *et al.*, 2016) that would have been then used to stretch the upper limit for *Halobacterium salinarum*, and could be diluted to the required water activity and salinity for each medium. Out of the 52 media, none of them reached a water activity close to the limits of life without precipitation. The three media with the lowest water activity without precipitation were the following: M39: a_w = 0.690 (2 M NaCl,

1.5 M $MgCl_2$, 1 M Sorbitol; M38: a_w = 0.671 (1.5 M NaCl, 2.5 M $MgCl_2.6H_2O$); M11: a_w = 0.650 (1 M NaCl, 3 M $MgCl_2.6H_2O$). However, in most cases, the media required molar concentrations of $MgCl_2$, which would have led to potential chaotropic stress. Nevertheless, this extensive testing of media has the potential to be used in other applications and experiments. A different strategy was used instead: three media of different salinity were selected for each strain: one that is close to the lower limit of growth, one that is optimal for growth and one that is close to the upper limit of growth. These were then amended by adding the rest of the components of Payne medium (Payne, 1960). Precipitation occurred in the highest salinity medium 50 (M50) (4.8 M NaCl) after cooling, therefore M51 (4.7 M NaCl) was used as the highest salinity medium. The chosen media were the following: M45 (0.4 M NaCl); M46 (2 M NaCl); M48 (4 M NaCl) for *H. elongata* 1H9 and M47b (3 M NaCl); M48 (4 M NaCl); M51 (4.7 M NaCl) for *H. salinarum* 91-R6 (Table 2). All measured water activities are shown in Table 3.1and in Table 3.2.

3.3.2 Growth analysis

3.3.2.1 Growth curve of the pre-cultures of *Hbt. salinarum* 91-R6, *Hbt. salinarum* NRC-1 and *H. elongata* 1H9

For continuous transferring of the microbial cultures, the late exponential phase had to be determined for each strain at each salinity. Pre-cultures were made in triplicate and the OD_{600} was measured for *Hbt. salinarum* 91-R6 and NRC-1, and *H. elongata* 1H9 at three relevant salt concentrations until cultures reached late stationary/death phase. *Hbt. salinarum* 91-R6 and NRC-1 grew similarly in all three media ((M47b (3 M NaCl), M48 (4 M NaCl), M51 (4.7 M NaCl)), although *Hbt. salinarum* 91-R6 grew slower. When subjected to 3 M NaCl media, *Hbt. salinarum* 91-R6 appeared to have a lag phase, however, it quickly reached exponential and then stationary phase, along with the other cultures. Day 7 was determined as the mid-

exponential phase for both haloarchaea (Figure 3.3). *H. elongata* 1H9 grew faster and had higher OD values in the two media with lower salinity (M45 (0.4 M NaCl), M46 (2 M NaCl)), while it grew relatively slowly in the highest salinity medium (M48 (4 M NaCl)), with a visible lag phase. Mid-exponential phase was determined between day 2 and 3 (~ 60 hours) (Figure 3.3). These were the times when each culture was transferred into a new batch of medium with the prediction that in one-year time, cultures will reach between 90-180 transfers, potentially allowing enough time for the cultures to evolve.

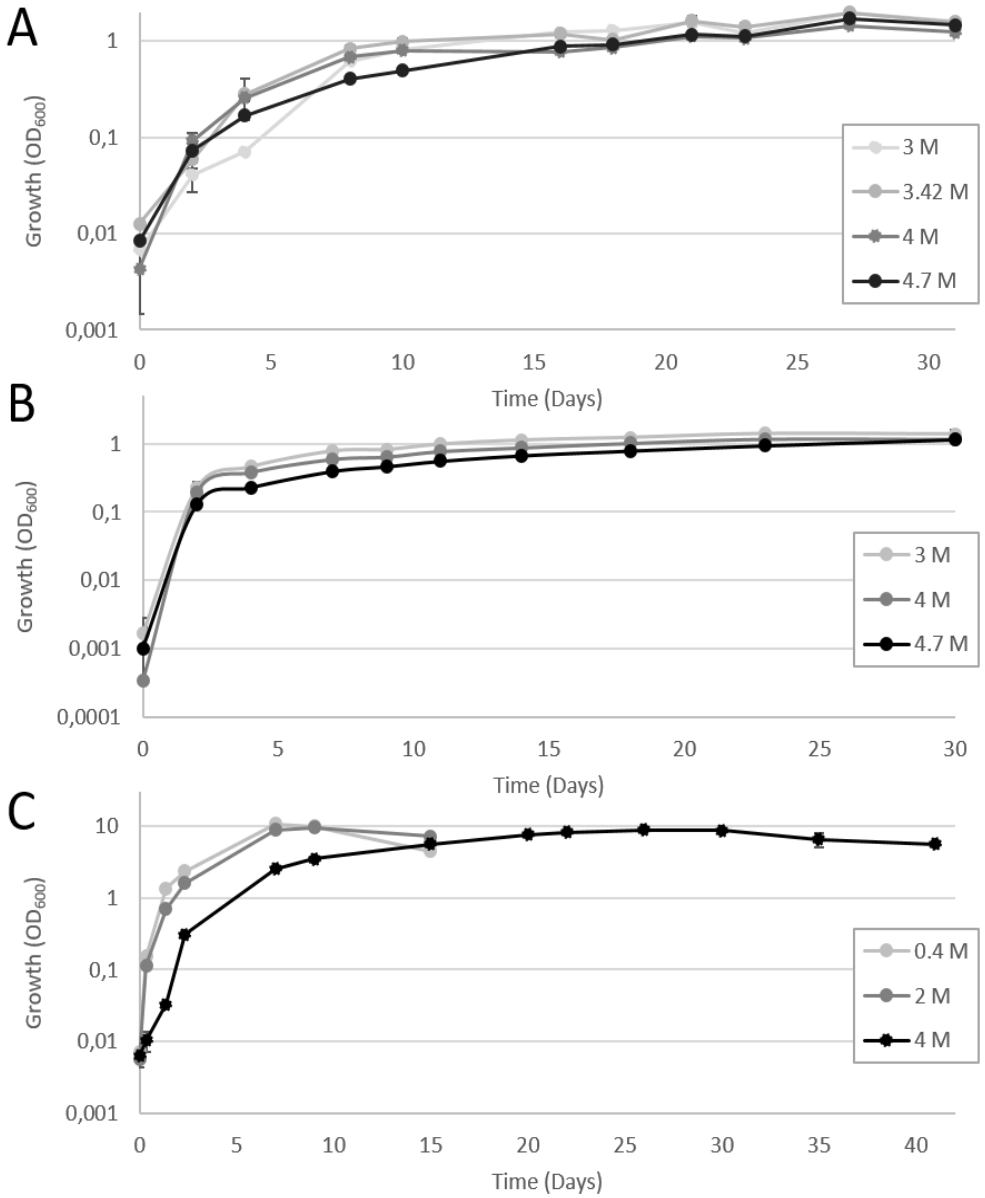

Figure 3.3 Initial growth curves of *Hbt. salinarum* 91-R6 (A), *Hbt. salinarum* NRC-1 (B) and *H. elongata* 1H9 (C) at OD_{600}. Both strains of *Hbt. salinarum* reached early stationary phase by day 2 while *H. elongata* 1H9 reached early stationary phase by day 1. Only *H. elongata* 1H9 appeared to reach death phase. Based on these data, early stationary phase was determined for each strain as the time interval to transfer cultures throughout the experiment (transfer *Hbt. salinarum* strains once a week and *H. elongata* every 3 – 4 days). The additional salinitiy of 3.42 M is shown as that is the salinity of the standard halophile medium used for culturing haloarchaea. Error bars represent standard error. N = 3

3.3.2.2 Growth of the different lab-evolution cultures of *Hbt. salinarum* 91-R6 and NRC-1, and *H. elongata* 1H9 after four months of subculturing

After four months of subculturing, growth over a salinity gradient was measured, after the following number of transfers: 18 in *Hbt. salinarum* 91-R6, 19 in *Hbt. salinarum* NRC-1, 41 in *H. elongata* 1H9 0.4 M and 2 M NaCl media lab-evolution cultures and 34 in the 4 M NaCl lab-evolution culture. All three *Hbt. salinarum* 91-R6 and NRC-1 lab-evolution cultures grew similarly in all salinities, not showing any signs of preference towards lower or higher salts (Figure 3.4, Figure 3.5). All lab-evolution cultures reached stationary phase after three days, except at the highest salinity (4.7 M NaCl), where the cultures grew at a slower rate. All three salinity lab-evolution cultures of *H. elongata* 1H9 grew well across all salinities between 0 and 3.9 M NaCl in the growth-test media (Figure 3.6). The lab-evolution cultures grew better at lower salinities, while they reached exponential phase later in the 3.9 M NaCl growth-test medium compared to other media. The lab-evolution cultures of *H. elongata* 1H9 reached late exponential and stationary phase after the following days when subjected to the highest salinity growth-test medium (4.7 M NaCl): 0.4 M lab-evolution culture – 8 and 16 days, 2 M lab-evolution culture – 7 and 14 days, and 4 M lab-evolution culture – 2 and 7 days, respectively. The salinity of the lab-evolution cultures greatly affected the capacity of *Halomonas elongata* 1H9 to grow in the highest salinity growth-test media of 4.7 M NaCl (Figure 3.6D). As the T_0 OD measurements highly varied between each salinity medium and due to the lack of data points in the exponential phase, the growth rates calculated were not reliable and could not be used, therefore, only the growth curves are presented of each strain in the different salinities (Figure 3.4,Figure 3.5 and Figure 3.6).

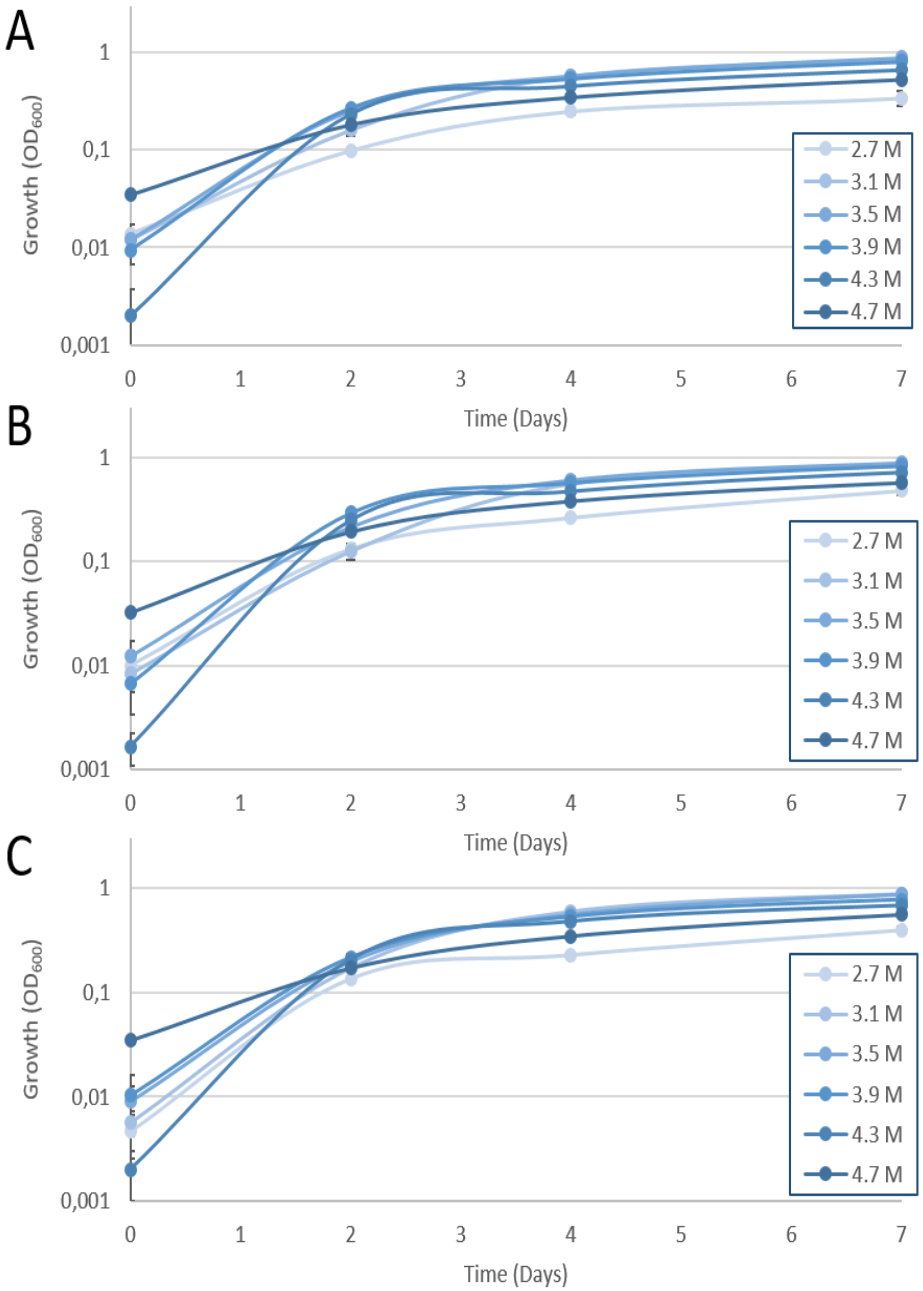

Figure 3.4 Growth curves of the three lab-evolution cultures of *Hbt. salinarum* 91-R6 (3 M NaCl (A), 4 M NaCl (B), and 4.7 M NaCl (C)) when subjected to different growth-test media with salinities from 2.7 to 4.7 M NaCl. Error bars represent standard deviation, however, variation was so small that they are often smaller than the symbol size. N = 3

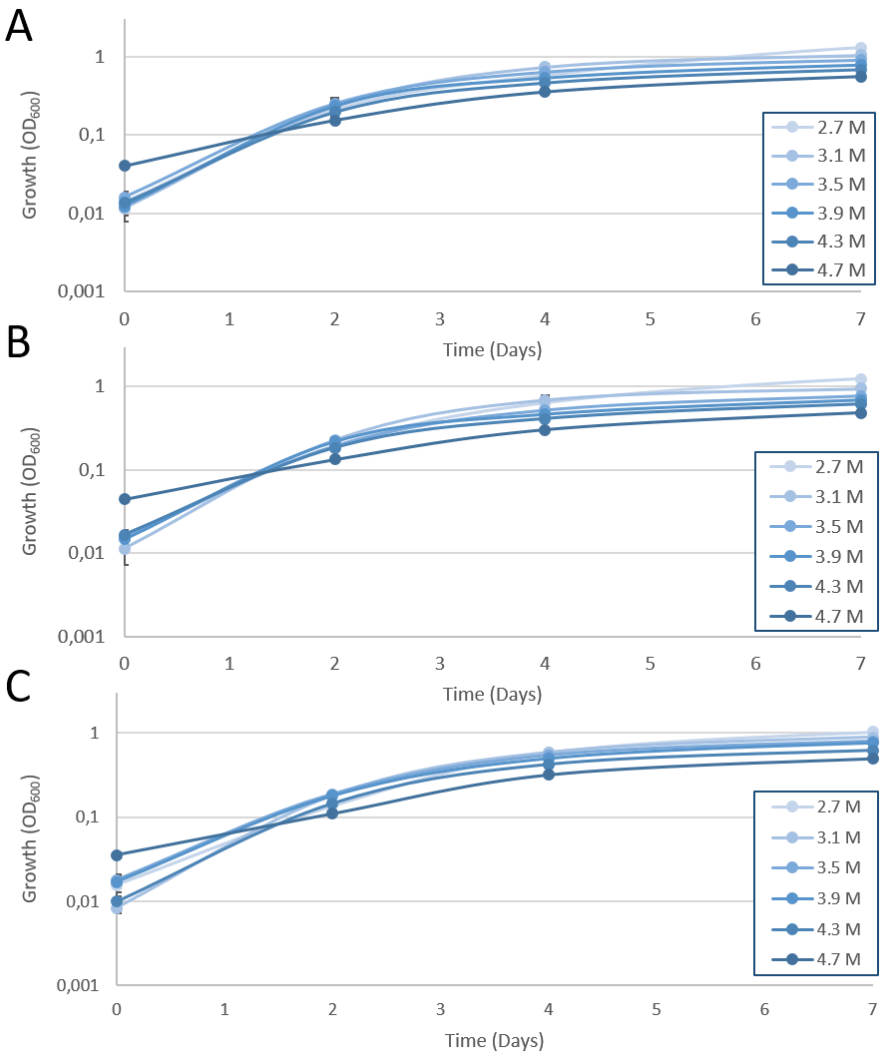

Figure 3.5 Growth curves of the three lab-evolution cultures of *Hbt. salinarum* NRC-1 (3 M NaCl (A), 4 M NaCl (B), and 4.7 M NaCl (C)) when subjected to different salinities from 2.7 to 4.7 M NaCl. Error bars represent standard deviation, however, variation was so small that they are often smaller than the symbol size. N = 3

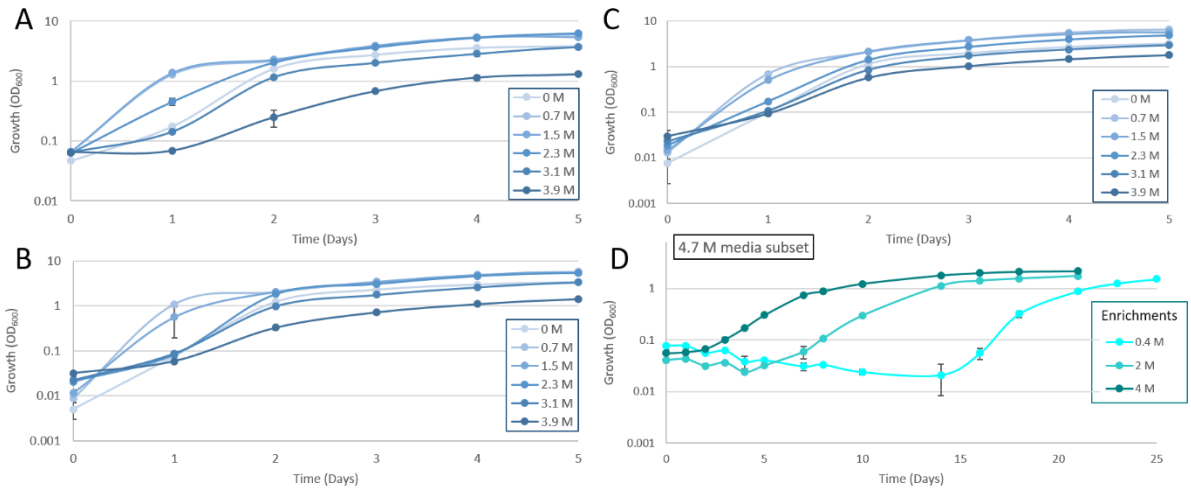

Figure 3.6 Growth curves of the three lab-evolution cultures of *H. elongata* 1H9 (0.4 M NaCl (A), 2 M NaCl (B), and 4 M NaCl (C)) when subjected to different salinities from 0 to 4.7 M NaCl. Panel D represents a subset of the data: the enrichments subjected to 4.7 M NaCl media, where all cultures started to grow much slower than the cultures at lower salinities. There are clear differences in growth between the three enrichments at high salinities. Error bars represent standard deviation, but are not always visible due to the small variation in OD. N = 3, except in the case of the low-salinity-evolved culture in 4.7 M NaCl medium subset where N = 2, as one out of three replicates grew faster than the other two (or became contaminated, or accumulated a beneficial mutation that enabled faster growth), hence it was treated as an outlier and was excluded.

3.3.2.3 Growth of the different lab-evolution cultures of *Hbt. salinarum* 91-R6 and NRC-1, and *H. elongata* 1H9 after 20 months of subculturing

As in the previous section after four months, the same growth measurements were performed at the end of the evolution experiment of the three different lab-evolution cultures of the three strains. At first, the OD of the cultures was measured once every day, which did not allow the precise capturing of the exponential phase, therefore the growth measurements were repeated once again. The measurements were conducted after the following amount of transfers in each strain: 60 in *Hbt. salinarum* 91-R6 and NRC-1 3 M and 4 M NaCl lab-evolution cultures, 56 in both 4.7 M NaCl lab-evolution culture, 137 in *H. elongata* 1H9 0.4 M and 2 M NaCl lab-evolution cultures and 127 in the 4 M NaCl lab-evolution culture. In the beginning of the lag phase (0 – 12 h), there was a high variation in OD measurements in the two haloarchaea (*Hbt. salinarum* 91-R6 and NRC-1), making this time period unreliable for calculating growth rate. The frequent measurements allowed the true exponential phase to be captured, which led to accurate growth rate calculations. All evolved cultures of the two

Hbt. salinarum strains grew similarly in the growth-test media across the salinity gradient, with *Hbt salinarum* strain 91-R6 cultures reaching early stationary phase after 48 hours and strain NRC-1 cultures reaching it after 36 hours. The low and the high salinity growth-test cultures of *Hbt. salinarum* strain 91-R6 had a visibly lower growth in the highest (4.7 M NaCl, OD = 0.02 *vs* average value of OD = 0.05 in other growth-test media at hour 36) and in the lowest (2.7 M NaCl, OD = 0.08 *vs* average value of OD = 0.17 in other growth-test media at hours 48) salinity medium, respectively (Figure 3.7). *H. elongata* 1H9 cultures started growing in the first four hours, except for growth-test cultures subjected to the two highest salinities (3.9 and 4.7 M NaCl), which grew much slower, starting to grow between hour 12 and 168, respectively. The low-salinity-evolved culture of *H. elongata* 1H9 did not grow in 4.7 M NaCl medium at all, after measuring it for 504 hours. Compared to the beginning of the experiment, where the two haloarchaeal strains reached stationary phase by day four, both of the measurements after four and 20 months indicated that they reached it by day two (Figure 3.4, Figure 3.5, Figure 3.7, Figure 3.8). At the start of the experiment, *H. elongata* 1H9 cultures reached stationary phase by day two in 0.4 and in 2 M NaCl growth-test media, while in 4 M NaCl media it took three days to reach it (Figure 3.3). As seen in the previous section, the growth of the 0.4 M and 2 M salinity lab-evolution culture decelerated remarkably in the highest salinity media (4.7 M NaCl) after four months of subculturing (Figure 3.6). This decrease in growth was even more prominent after 20 months, as even the lab-evolution cultures in the 3.9 M NaCl medium grew much slower than after four months (Figure 3.9). The cultures reached exponential and stationary phase after the following days in the 3.9 M NaCl media: 0.4 M lab-evolution culture – 3 and 5 days, 2 and 4 M lab-evolution culture – 1 and 3 days, respectively. The cultures reached exponential and stationary phase after the following days in the 4.7 M NaCl media: 0.4 M lab-evolution culture – did not grow, 2 and 4 M lab-evolution culture – 7 and 18 days, respectively.

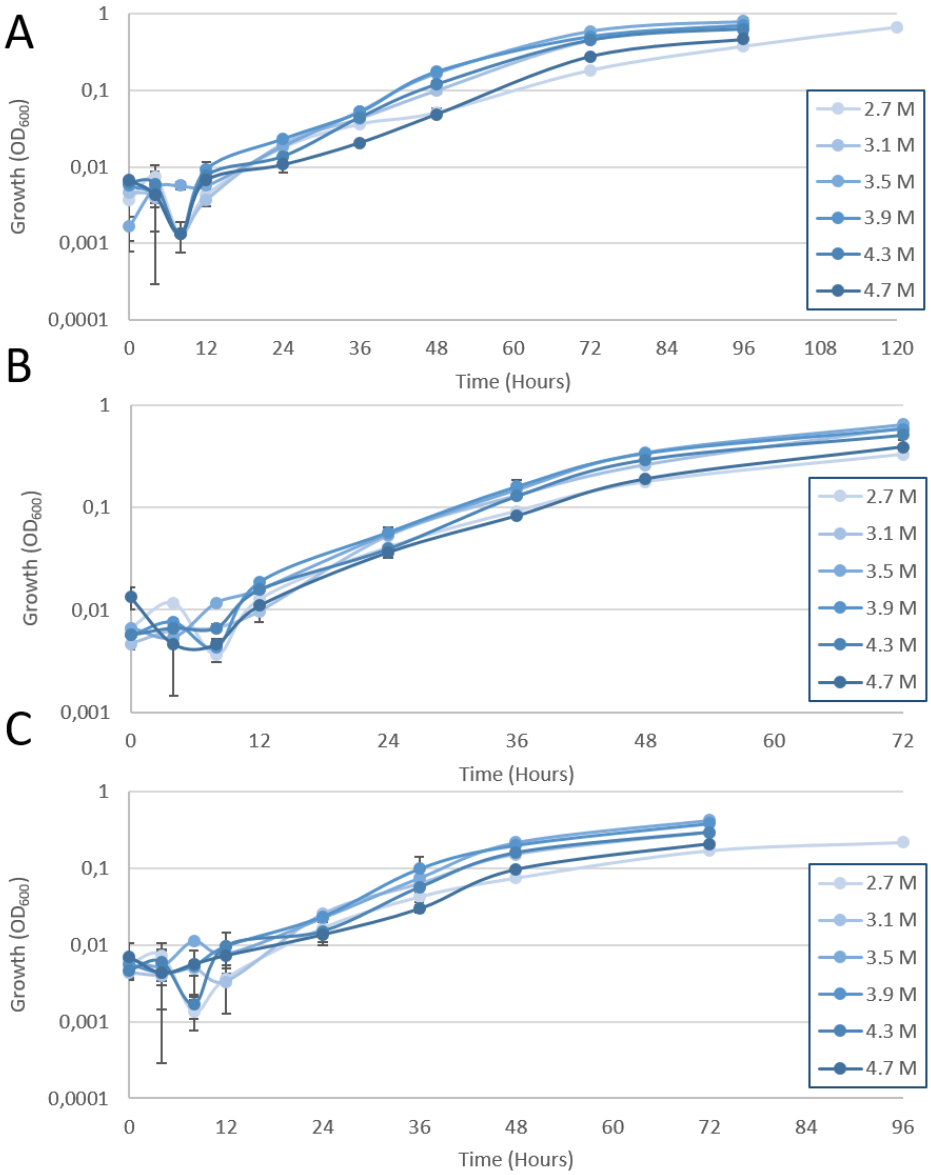

Figure 3.7 Growth curves of the three lab-evolution culture of *Hbt. salinarum* 91-R6 (3 M NaCl (A), 4 M NaCl (B), and 4.7 M NaCl (C)) when subjected to different salinities from 2.7 to 4.7 M NaCl, after 20 months of subculturing. Error bars represent standard deviation, however, variation was so small that they are often smaller than the symbol size. N = 3

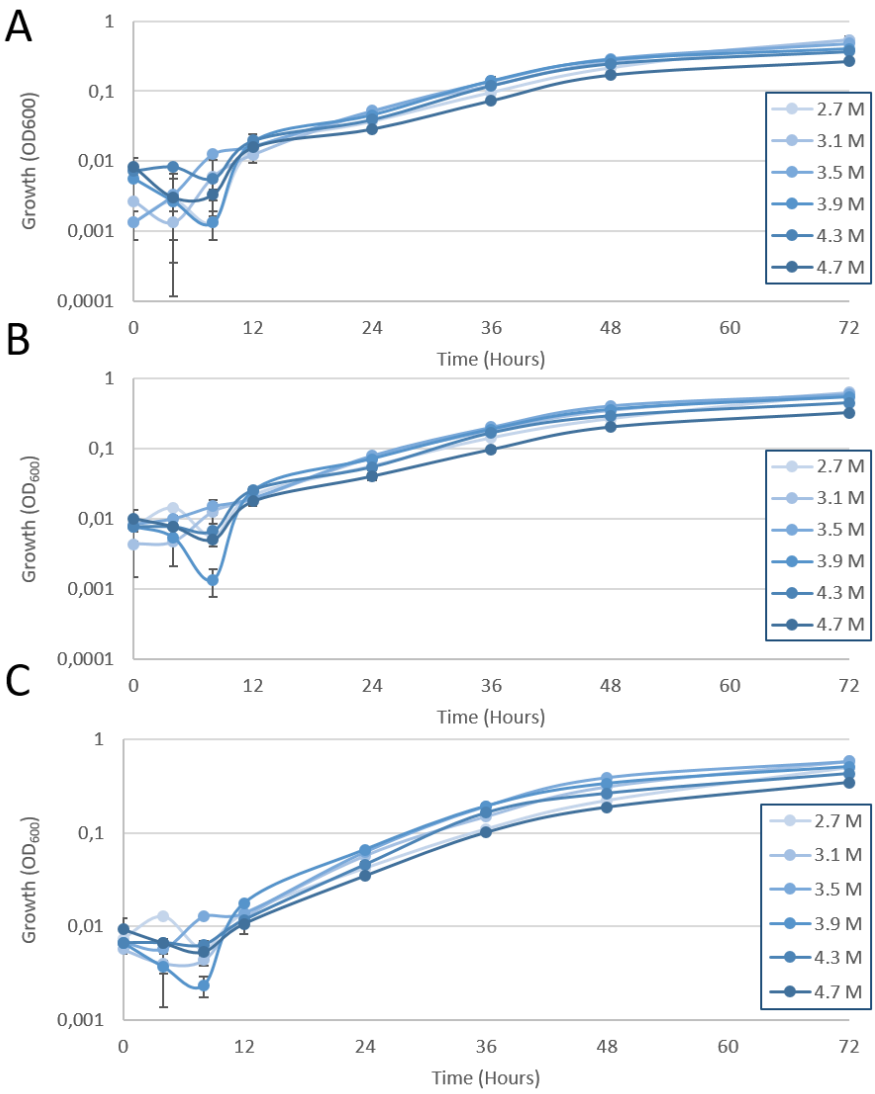

Figure 3.8 Growth curves of the three lab-evolution culture of *Hbt. salinarum* NRC-1 (3 M NaCl (A), 4 M NaCl (B), and 4.7 M NaCl (C)) when subjected to different salinities from 2.7 to 4.7 M NaCl, after 20 months of subculturing. Error bars represent standard deviation, however, variation was so small that they are often smaller than the symbol size. N = 3

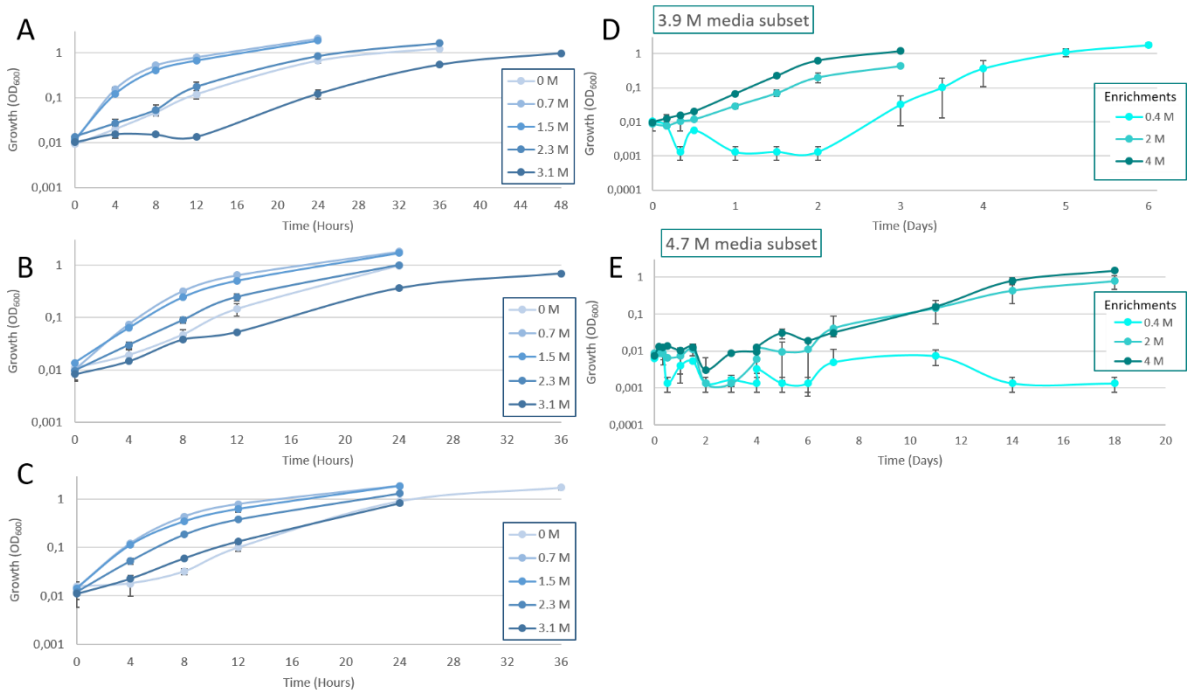

Figure 3.9 Growth curves of the three lab-evolution culture of *H. elongata* 1H9 (0.4 M NaCl (A), 2 M NaCl (B), and 4 M NaCl (C)) when subjected to different salinities from 0 to 4.7 M NaCl, after 20 months of subculturing.. Panel D and E represent a subset of the data: the enrichments subjected to 3.9 and 4.7 M NaCl media, where all cultures started to grow much slower than the cultures at lower salinities. There are visible differences in growth between the three enrichments at high salinities. Error bars represent standard deviation, but are not always visible due to the small variation in OD. N = 3

In order to summarise the 20-month data, specific growth rates were calculated from the final growth measurements. These were plotted for each strain subcultured in the three salinity media against the salinity of the growth-test media ranging from 0 M to 4.7 M NaCl in *H. elongata* 1H9 and from 2.7 M to 4.7 M NaCl in *Hbt. salinarum* strains 91-R6 and NRC-1 (Figure 3.10). A two-way ANOVA was carried out for each strain, with post-hoc Tukey's HSD tests if needed. The growth rate of *Hbt. salinarum* 91-R6 lab-evolution cultures changed over 20 months. Tukey's HSD post-hoc test revealed that there was a significant difference in mean growth rate between the low- and high- (p <0.001), and between the mid- and high-salinity cultures (p <0.001). The 4.7 M NaCl lab-evolution culture had a higher growth rate than the optimal and the low salinity lab-evolution culture at the high salinity growth-test media (3.9, 4.3 and 4.7 M NaCl), with the highest growth rate at 3.9 M NaCl (Figure 3.10). The low salinity (3 M) lab-evolution culture had the highest growth rate at the same salinity (3.9 M)

and its growth rate decreased towards both low and high salinities, making its growth range narrower: between 3.5 and 4.3 M NaCl. The optimal lab-evolution culture (4 M) had its highest growth rate at 4.3 M NaCl, however, it did not grow as well in other salinities. All three lab-evolution cultures had their highest growth rate close to the species' optimum (4 M) (Zeng *et al.*, 2006; Coker *et al.*, 2007; Oren, Arahal and Ventosa, 2009). *Hbt. salinarum* 91-R6 did not grow well (growth rate of ~ 0.05 – 0.06 h^{-1}) towards lower salinities compared to strain NRC-1 (growth rate of ~ 0.08 h^{-1}) (Figure 3.10).

The growth rate of *Hbt. salinarum* NRC-1 was very similar to the strain 91-R6, with the exception of the 4.7 M NaCl media lab-evolution culture, as its highest growth rate was towards 4.3 M NaCl not 3.9 M NaCl (Figure 3.10). Tukey's HSD post-hoc test revealed that there was a significant difference in mean growth rate between the low- and high- (p <0.001), and between the mid- and high-salinity cultures (p <0.001). The low- and mid-salinity cultures were not significantly different from each other. The growth rate of *Hbt. salinarum* NRC-1 cultures was more similar to each other across the different salinities of the growth-test media (range of growth rate: 0.07 – 0.1 h^{-1}) than the cultures of *Hbt. salinarum* 91-R6 (range of growth rate: 0.05 – 0.12 h^{-1}).

Before the evolution experiment started, the original cultures of *Hbt. salinarum* strains 91-R6 and NRC-1 were measured at salinities near their growth limits (March – May 2019). No change in salinity requirement was observed after four months between *Hbt. salinarum* strain 91-R6 repeatedly subcultured at low, optimal and high salinity. However, after 20 months, the lowest-salinity lab-evolution culture, surprisingle, grew more slowly in the lowest-salinity lab-evolution culture reaching exponential phase later when exposed to 2.7 M NaCl than the mid- and high-salinity lab-evolution cultures (Figure 3.7). In addition, all low-salinity lab-evolution cultures took more time to reach late exponential phase (~72 hours, OD_{600} of 0.18 – 0.5) than the other two lab-evolution cultures (~48 hours, OD_{600} of 0.17 – 0.29 of mid-, and OD_{600} of 0.08 – 0.21 for high-salinity lab-evolution cultures).

In general, the two strains of *Hbt. salinarum* had similar growth patterns. The mean growth rate of *Hbt. salinarum* NRC-1 was lower than of *Hbt. salinarum* 91-R6 in all cultures (Figure 3.10). Also, *Hbt. salinarum* NRC-1 appeared to be less affected by long-term exposure to low and high salinities compared to *Hbt. salinarum* 91-R6, as the growth range of the strain stayed more constant at both low (2.7 M) and at high (4.7 M) salt concentrations in the growth-test media (Figure 3.8). None of the three salinity cultures of *Hbt. salinarum* NRC-1 had a growth rate below 0.07 h^{-1} in the lowest salinity growth-test media compared to *Hbt. salinarum* 91-R6 cultures whose growth rate was between 0.05 and 0.06 h^{-1}.

Before the experiment, as for the *Hbt. salinarum* strains, the original culture of *H. elongata* 1H9 was subjected to different salinities near its upper and lower growth limits. Exposing *H. elongata* 1H9 pre-culture to 4 M NaCl medium slowed down its growth (Figure 3.3). Compared to the low- and optimum salinity lab-evolved cultures, which went straight into exponential phase, the high-salinity exposed culture only reached early exponential phase after ~24 hours.

The highest growth rate of the three salinity cultures of *H. elongata* 1H9 was between 0.7 and 1.5 M NaCl (Figure 3.10). The low salinity culture grew much better than the mid- and high-salinity cultures in 0.7 M NaCl medium (p <0.01). The mid-salinity culture had a significantly lower growth rate than the mid- and high-salinity cultures in the 1.5 M NaCl growth-test medium (p <0.001), while the latter ones did not have a significant difference between each other. The low-salinity culture had a wide growth range and a high growth rate in the lower salinity growth-test media (0.7 and at 1.5 M NaCl), which decreased fast with the increase of salinity. Tukey's HSD post-hoc test revealed that there was a significant difference in mean growth rate between the low- and mid- (p <0.001), and between the mid- and high-salinity cultures (p <0.001). The high salinity culture had a higher growth rate towards higher salinities (2.3 – 3.9 M NaCl) compared to the other cultures. Neither of the lab-evolution cultures grew well at the two highest salinities (3.9 and 4.7 M), moreover, the

low-salinity culture did not grow in the 4.7 M NaCl medium at all throughout the optical density measurements of 25 days (Figure 3.9, Panel E).

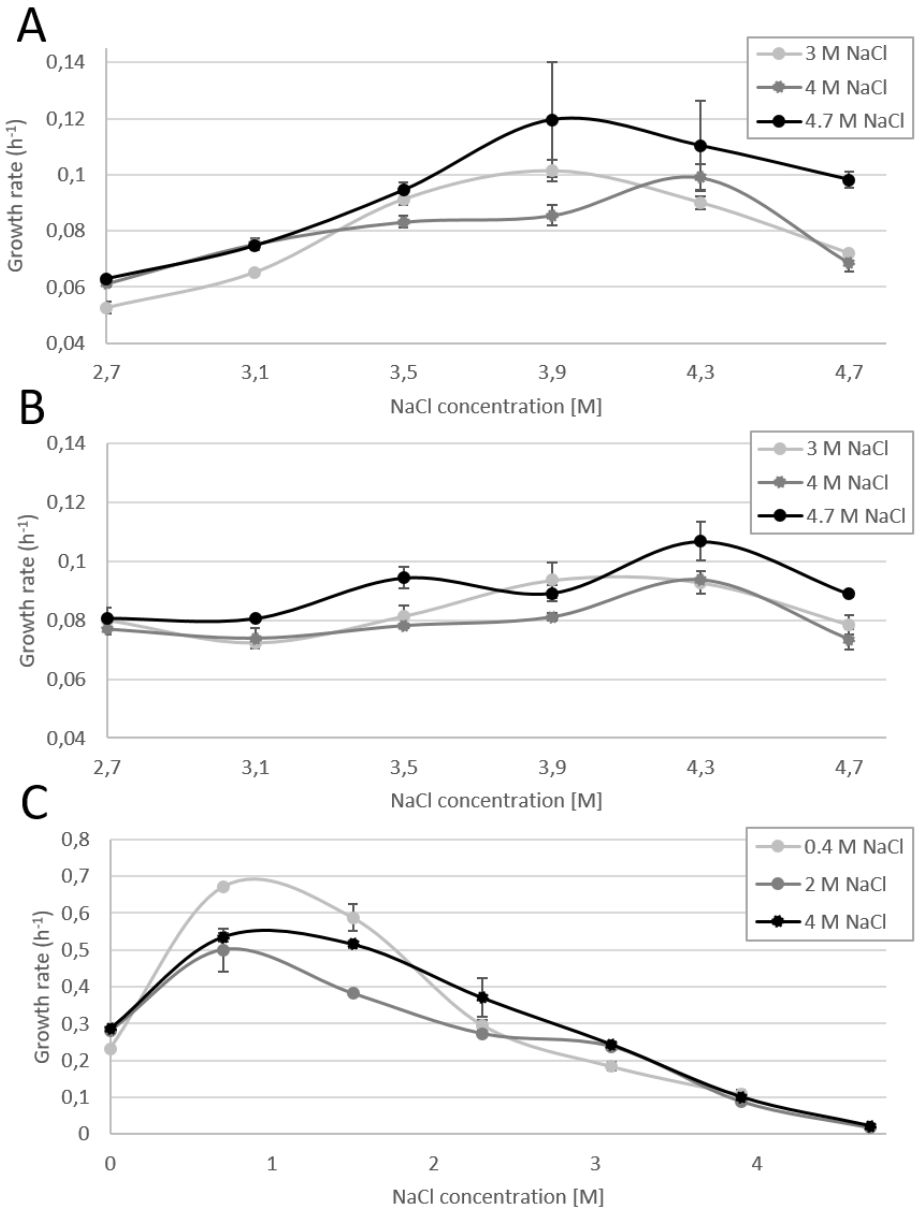

Figure 3.10 Growth rate of *Hbt. salinarum* 91-R6 (A), *Hbt. salinarum* NRC-1 (B) and *H. elongata* 1H9 (C) on a salinity gradient, after 20 months of subculturing in media with different salinities. The different lab-evolution cultures (low, optimal, high) were subjected to a salinity gradient, and OD_{600} was measured until the cultures reached stationary phase. Growth rate was calculated based on the exponential phase. All the lab-evolution cultures of the *Hbt. salinarum* strains had very similar growth rates, growing better at higher salinities. The lab-evolution cultures of *H. elongata* 1H9 had a more distinct growth rate. The lowest salinity lab-evolution culture (0.4 M NaCl) did not grow in 4.7 M NaCl media, while the highest salinity lab-evolution culture (4 M NaCl) grew better than the other two lab-evolution cultures at higher salinites (2.3 M NaCl). Error bars represent standard error, but are not visible due to the lack of variation in OD. N = 3

3.3.3 Cell morphology of the different enrichments of *Hbt. salinarum* 91-R6 and NRC-1, and *H. elongata* 1H9 after 20 months of subculturing

At the end of the evolution experiment, along with the growth curve measurements, all three lab-evolution cultures of all three strains were observed under the microscope, in order to observe any potential differences in cell morphology. All lab-evolution cultures were inoculated into a subset of the salinity media: *Hbt. salinarum* strains into 2.7, 4 and 4.7 M NaCl media and *H. elongata* enrichments into 0, 1.5 and 3.9 M NaCl media. The microscopy images were taken in the exponential phase of each lab-evolution culture in each salinity, calculated based on the previous growth measurements.

All *Hbt. salinarum* 91-R6 lab-evolution cultures had high abundance of rod-shaped cells in 4 and 4.7 M NaCl media, while more spherical cells appeared in the 2.7 M NaCl medium (Figure 3.11). The highest salinity lab-evolution culture only had bigger, round cells in the 2.7 M NaCl medium, with some of them forming clusters. There were more spherical cells found in the 4.7 M medium in the low and high salinity lab-evolution cultures, while the optimum salinity lab-evolution culture consisted of mostly rods (Figure 3.11). The *Hbt. salinarum* NRC-1 lab-evolution cultures had rod-shaped cells in all media across all salinities in high abundance (Figure 3.12). There were some small spherical cells in the low salinity enrichment subjected to 2.7 M NaCl, in clusters of two. However, this was not found in any other *Hbt. salinarum* 91-R6 enrichment.

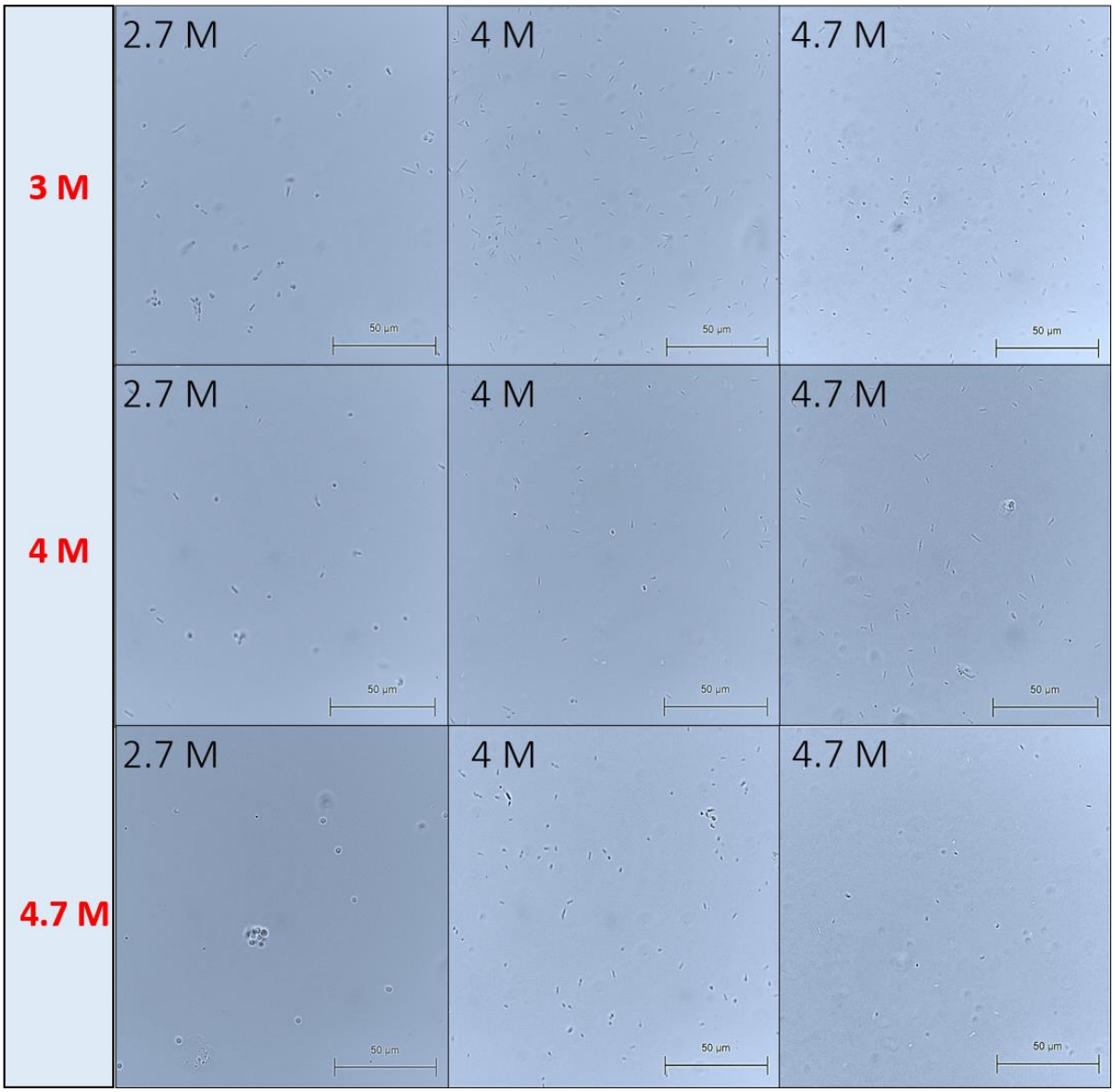

Figure 3.11 Cell morphology of *Hbt. salinarum* 91-R6 lab-evolution cultures when subjected to 2.7, 4, and 4.7 M NaCl media. Lab-evolution cultures (3, 4m 4.7 M) are labelled red, media labelled black.

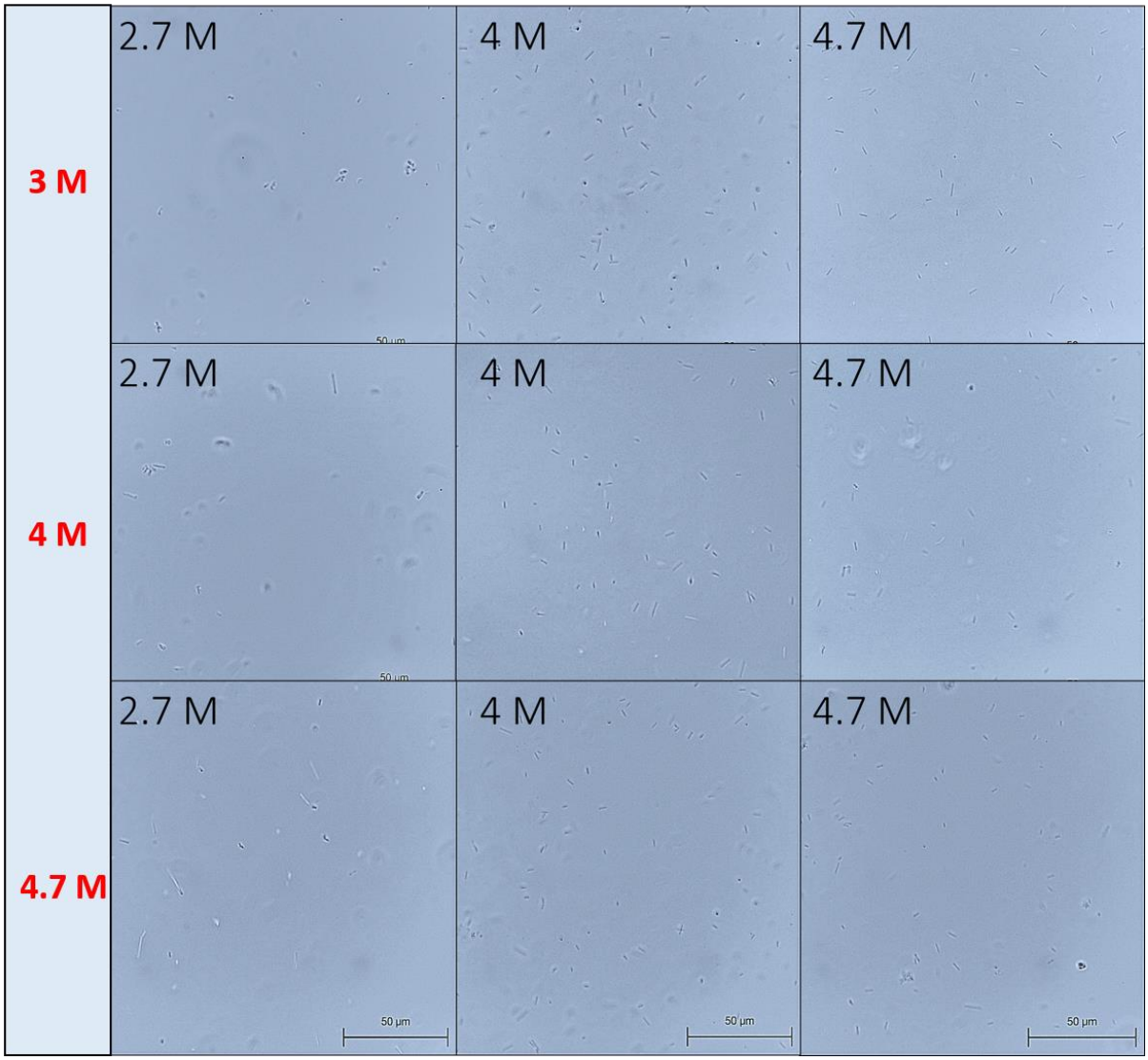

Figure 3.12 Cell morphology of *Hbt. salinarum* NRC-1 lab-evolution cultures when subjected to 2.7, 4, and 4.7 M NaCl media. Lab-evolution cultures (3, 4m 4.7 M) are labelled red, media labelled black.

Most of the lab-evolution cultures of *H. elongata* 1H9 had very elongated cells of up to ~120 µm, sometimes with visible septa (Figure 3.13). The long chains of cells was especially visible in the 0.4 M lab-evolution culture subjected to 1.5 M NaCl medium and in the 2 M lab-evolution culture subjected to 3.9 M NaCl medium (Figure 3.13). The low salinity lab-evolution culture contained fewer cells in the 0 M and 3.9 M NaCl media compared to any other culture.

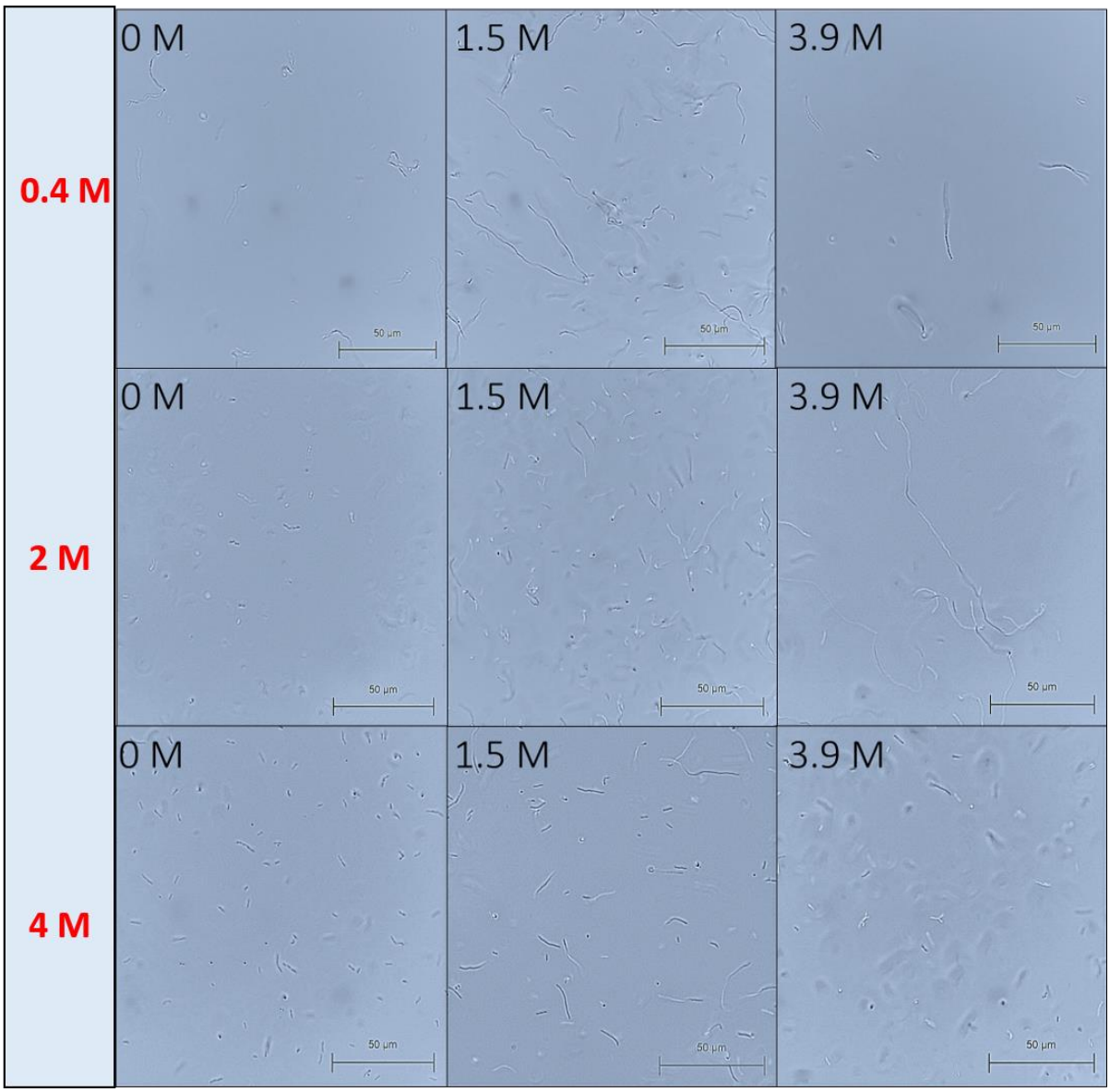

Figure 3.13 Cell morphology of *H. elongata* 1H9 lab-evolution cultures when subjected to 0, 1.5, and 3.9 M NaCl media. Enrichments (0.4, 2, 4 M) are labelled red, media labelled black.

3.3.4 Genome analysis of *Hbt. salinarum* 91-R6 and *H. elongata* 1H9

The genomes of the non-enriched original *Hbt. salinarum* 91-R6 and *H. elongata* 1H9 were annotated using PATRIC, and were used as reference genomes for comparison with the genomes of the three salinity lab-evolution cultures at the end of the experiment (Table 3.4).

Table 3.4 Genome information of the annotated genomes of *Hbt. salinarum* 91-R6 and *H. elongata* 1H9.

	H. elongata 1H9	*Hbt. salinarum* 91-R6
Genome information		
Genome size (bp)	4046999	2396174
G+C content (mol %)	63.63	66.42
Completeness (%)	99.8	100
Contamination (%)	0.4	0
Contigs	61	99
Contig L50	4	2
Contig N50	314096	552916
CDS (coding region)	3874	2666
tRNA	65	48
rRNA	6	3
Repeat regions	8	0
Partial CDS	0	0
Miscellaneous RNA	0	0
Protein features		
Hypothetical proteins	809	1335
Proteins with functional assignments	3065	1331
Proteins with EC number assignments	1043	525
Proteins with GO assignments	890	432
Proteins with Pathway assignments	796	404
Proteins with PATRIC genus-specific family (PLfam) assignments	3717	2283
Proteins with PATRIC cross-genus family (PGfam) assignments	3739	2390
Subsystem (1st no.: subsystems, 2nd no.: genes)		
Metabolism	93, 720	55, 340
Protein processing	42, 225	33, 137
Stress response, defense, virulence	32, 147	14, 30
Energy	31, 270	21, 121
Membrane transport	23, 187	8, 38
DNA processing	19, 94	9, 27
RNA processing	15, 68	15, 71
Cellular processes	15, 127	3, 12
Cell envelope	6, 30	1, 1
Miscellaneous	6, 44	
Regulation and cell signaling	5, 22	

Variation analysis in PATRIC showed that the three salinity lab-evolution cultures of *Hbt. salinarum* 91-R6 had only a few mutations in their genomes, which are listed in Table 3.5. The low salinity lab-evolution culture had two genes knocked out (*flgB1* and *flgB3*) which code FlaB1 and FlaB3 flagellin proteins, which are the main constituents of the archaeal flagella: archaellum (Tarasov *et al.*, 2000; Beznosov, Pyatibratov and Fedorov, 2007). The optimum salinity lab-evolution culture suffered a frameshift mutation in a single-copy translation elongation factor 2 (EF-2), while the high salinity lab-evolution culture had two genes knocked out that code cobalamin synthase and a cell surface glycoprotein that possibly forms the S-layer in the cell wall (Lechner and Wieland, 1989).

Table 3.5 Mutations identified in the genomes of *Hbt. salinarum* 91-R6 enrichments that had moderate or high impact output. Moderate impact: A non-disruptive variant that might change protein effectiveness; high impact: The variant is assumed to have high (disruptive) impact in the protein, probably causing protein truncation or loss of function. Mutations are compared to the genome of the original culture of *Hbt. salinarum* 91-R6.

Mutation type	Lab-evolution culture	PATRIC feature (peg) id	Protein name (PATRIC)
deletion	3 M	fig|2597657.7.peg.1526	Efflux ABC transporter, permease/ATP-binding protein, ~6 copies
nonsynonymous missense	3 M	fig|2597657.12.peg.1955	Flagellin FlaB1, single copy
nonsynonymous missense	3 M	fig|2597657.7.peg.2139	Flagellin FlaB3, single copy
nonsynonymous missense	3 M	fig|2597657.7.peg.511	L-aspartate oxidase (EC 1.4.3.16), single copy
frameshift	4 M	fig|2597657.8.peg.1469	Translation elongation factor 2, single copy
nonsynonymous missense	4.7 M	fig|2597657.6.peg.586	Cobalamin synthase (EC 2.7.8.26), single copy
nonsynonymous missense	4.7 M	fig|2597657.6.peg.1072	Cell surface glycoprotein, single copy

Variation analysis in PATRIC showed that the three lab-evolution cultures of *H. elongata* 1H9 had much more mutations in their genomes compared to *Hbt. salinarum* 91-R6, and are shown in Table 3.6. Although many genes were knocked out in all three genomes, the focus will be on genes mutated in the genomes of the low- and high-salt lab-evolution cultures. All three cultures had mutations in the Fts proteins that play a role in cell division. The FtsZ ("Filamenting temperature-sensitive mutant Z") protein mutated in the 0.4 M culture, along with one copy of the *N*-acetylmuramoyl-L-alanine amidase that is part of the peptidoglycan catabolic process in *Halomonas* spp. and in *Escherichia coli* (Margolin, 2005; Tao, Lv and Chen, 2017). The FtsX ABC transporter-like signalling protein mutated in the 2 M culture, which forms a complex with FtsE and carries out peptidoglycan hydrolysis during cell division in *E. coli* and in *Streptococcus pneumoniae* (Reddy, 2007; Bajaj *et al.*, 2016; Cook *et al.*, 2020). The 4 M culture lost a probable peptidoglycan glycosyltransferase FtsW protein that is responsible for stabilizing the FtsZ cytokinetic ring and facilitating septal peptidoglycan synthesis during cell division in *E. coli* (Mercer and Weiss, 2002; Taguchi *et al.*, 2019; Park *et al.*, 2021). However, this gene has possible three more copies that were not mutated. The dihydroxyacetone kinase (EC 2.7.1.29) protein also mutated in this culture, but it has a

putative extra copy in its genome. Compared to the original genome sequenced before the experiment, all lab-evolution cultures lost two genes thats both have one additional copy in the genome: dihydropicolinate synthase (EC 4.3.3.7) and FliI, a flagellum-specific ATP synthase (EC 7.1.2.2).

Table 3.6 Mutations identified in the genomes of *H. elongata* 1H9 enrichments that had moderate or high impact output.

Mutation type	Enrichment	PATRIC feature (peg) id	Protein name (PATRIC)
nonsynonymous missense	0.4 M	fig|768066.36.peg.3608	3-oxoadipate CoA-transferase subunit B (EC 2.8.3.6), single copy
nonsynonymous missense	0.4 M	fig|768066.36.peg.1085	alpha-L-arabinofuranosidase (EC 3.2.1.55), 2 copies
nonsynonymous missense	0.4 M	fig|768066.36.peg.2020	Cell division protein FtsZ, single copy
deletion	0.4 M	fig|768066.36.peg.486	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28), 2 copies (1 putative)
nonsynonymous missense	2 M	fig|768066.37.peg.2989	Putative two-component sensor, 2-7 copies
nonsynonymous missense	2 M	fig|768066.37.peg.3037	response regulator receiver domain protein (CheY-like), single copy
deletion	2 M	fig|768066.37.peg.3484	Zinc ABC transporter, substrate-binding protein ZnuA, 1 copy
insertion	2 M	fig|768066.37.peg.984	Cell-division-associated, ABC-transporter-like signaling protein FtsX, single copy
nonsynonymous missense	2 M	fig|768066.37.peg.1639	Dipeptide ABC transporter, ATP-binding protein DppD (TC 3.A.1.5.2), single copy
nonsynonymous missense	2 M	fig|768066.37.peg.2397	Precorrin-8X methylmutase (EC 5.4.99.61), single copy
nonsynonymous missense	2 M	fig|768066.37.peg.176	Uncharacterized MFS-type transporter, 17 copies
nonsynonymous missense	2 M	fig|768066.37.peg.1621	TolA protein, single copy
STOP kodon	2 M	fig|768066.37.peg.3022	Phosphoenolpyruvate carboxykinase [ATP] (EC 4.1.1.49), single copy
insertion	4 M	fig|768066.38.peg.2157	Glycosyl transferase, group 1, 3 copies
nonsynonymous missense	4 M	fig|768066.38.peg.1048	Dihydroxyacetone kinase, ATP-dependent (EC 2.7.1.29), single copy
nonsynonymous missense	0.4 M, 2 M, 4 M	fig|768066.41.peg.3843	Dihydrodipicolinate synthase family PA0223, single copy
nonsynonymous missense	0.4 M, 2 M, 4 M	fig|768066.41.peg.3845	Transcriptional regulator, unknown
nonsynonymous missense	0.4 M, 2 M (insertion), 4 M	fig|768066.41.peg.1832	Glycosyltransferase, 3 copies (2 putative)
nonsynonymous missense	0.4 M, 2 M, 4 M	fig|768066.41.peg.2208	Flagellum-specific ATP synthase FliI, single copy
nonsynonymous missense	0.4 M, 2 M	fig|768066.41.peg.3793	VgrG protein, 7 copies
nonsynonymous missense	0.4 M, 4 M	fig|768066.41.peg.1698	transcriptional regulator MvaT, P16 subunit, single copy

3.4 Discussion

3.4.1 Growth, morphology and evolution in *Hbt. salinarum* 91-R6

Besides its slow growth rate, the low-salinity lab-evolution culture of *Hbt. salinarum* 91-R6 also suffered nonsynonymous missense point mutations in two single-copy genes (*'flgB1'* and *'flgB3'*, and potentially '*flgB2'* on the same operon, now called *'arlB1-B3'* (Figure S3.1). These encode proteins required to form the FlaB (now called ArlB) archaellum (functionally similar to the flagellum in bacteria), a rotating swimming organelle (Lechner and Wieland, 1989; Beznosov, Pyatibratov and Fedorov, 2007; Albers and Jarrell, 2015; Pfeiffer *et al.*, 2020). As this experiment provided these cultures a microbial 'heaven' (complex medium, no competition, cultures refreshed regularly on a shaker), the low-salinity lab-evolution culture evolved not to create a type FlaB (ArlB) archaellum at every single cell cycle. As a decrease in salinity leads to a decrease in viscosity of the medium, it also leads to less resistance to movement (Kwak *et al.*, 2005). Therefore, the need for an archaellum, the biosynthesis of which consumes energy, could have become less (Albers and Jarrell, 2015; Beeby *et al.*, 2020). It has been noted that certain Bacteria produce more FlaB (ArlB) flagellin proteins to cope with low temperature that causes higher viscosity. As the mid- and high-salinity lab-evolution cultures had to cope with increased viscosity too, they were probably required to keep the FlaB (ArlB) archaellum (Gregson et al., 2020). This suggests that evolution was selecting for archaellum-defective random mutants to increase the fitness and survival of the culture and to cope better with low salinity and low viscosity. The more stable FlaA (ArlA) archaellum genes remained intact in the genome, therefore motility was probably still possible to some extent (Tarasov *et al.*, 2000; Albers and Jarrell, 2015). The growth rate was also slower of the low-salinity lab-evolution cultures at the lowest (2.7 and 3.1 M NaCl) and highest (4.3 and 4.7 M NaCl) salinities of the growth-test media compared to the mid- and high-salinity lab-evolution cultures (Figure 3.10). This suggests that H2 hypothesis must be

rejected, as culturing at low salinity did not lead this culture to grow better at low salinities and worse at high salinities but it impaired its general growth capacity.

In contrast, when the high-salinity lab-evolution culture, after 20 months of culturing (53 transfers, ~ 2105 generations), was exposed to the 2.7 M NaCl growth-test medium, it did not have visible growth the first time it was exposed to this salinity (Figure S3.2). As the growth-test measurements had to be repeated in order to capture the exponential phase, the high-salinity lab-evolution culture did grow the second time (Figure 3.7). It is possible that the culture experienced hyposaline stress the first time it was exposed to low salinity. Microscopy showed that the cells of the high-salinity lab-evolution cultures exposed to 2.7 M NaCl growth-test medium formed big, spherical cells, unlike the usual rod-shaped cell of *Hbt. salinarum* (Figure 3.11). These spherical cells were observed previously in *Hbt. salinarum*, and it has been found that cells stop growing when exposed to low salt, but can be revived after exposing them to high salt again (Abram and Gibbons, 1960; Mohr and Larsen, 1963; Stoeckenius and Rowen, 1967; Vauclare *et al.*, 2020). Further research is needed to determine whether *Hbt. salinarum* 91-R6 would be able to recover from hyposaline shock if it was grown at low salinitiy (3 M NaCl) for an extended time period.

3.4.2 Growth, morphology and evolution in *Hbt. salinarum* NRC-1

The consistent growth rate across all lab-evolved cultures of *Hbt. salinarum* NRC-1 suggests that this strain is less affected by salinity below its growth limits (2.7 M NaCl) compared to *Hbt. salinarum* 91-R6. For *Hbt. salinarum* NRC-1, microscopy showed mostly rod-shaped cells in all lab-evolution cultures across all salinities of the growth-test media, with no spherical cells in low salinities (Figure 3.12). As this strain of *Hbt. salinarum* did not show any obvious sign of evolution, its genome was not sequenced at the end of the experiment. Based on these findings, hypotheses H1 and H2 can be rejected. While *Hbt. salinarum* NRC-1 and

R1 are laboratory variants derived from a single isolate, *Hbt. salinarum* 91-R6, the type strain, is an entirely independent isolate (Pfeiffer *et al.*, 2020). The genomes of all three strains are now sequenced, and differences were found in parts of their genome when comparing *Hbt. salinarum* strain NRC-1 and R1 to strain 91-R6. Although the genomes of *Hbt. salinarum* strain NRC-1 and 91-R6 both consist of a chromosome and two large plasmids, while the genome of strain R1 consists of a chromosome and four large plasmids, their chromosomes share an average nucleotide identity of 98% and an *in silico* DNA-DNA hybridization score of 95% (Pfeiffer *et al.*, 2020). The two large plasmids of *Hbt. salinarum* NRC-1 and the four large plasmids of strain R1 share 350 kb of near-identical sequences (Pfeiffer *et al.*, 2008). While *Hbt. salinarum* strains NRC-1 and R1 are auxotroph for amino acids leucine, isoleucine and valine, strain 91-R6 codes genes that biosynthesise all three (Pfeiffer *et al.*, 2020). While the archaellum proteins FlagB1-3 (ArlB1-3) are identical in all three strains, *Hbt. salinarum* 91-R6 only has a single copy of the other archaellum protein FlaA (ArlA) (Pfeiffer *et al.*, 2020). Thus, it is possible that *Hbt. salinarum* 91-R6 is more prone to mutations and knockouts in its archaellum genes. *Hbt. salinarum* strains 91-R6 forms biofilms, while the other two strains do not have this ability (Pfeiffer *et al.*, 2020). As shown above, there are several differences between *Hbt. salinarum* strains NRC-1 and R1 compared to strain 91-R6, which could explain why strain NRC-1 and 91-R6 behaved differently when exposed to different salinities and when examined under the microscope.

3.4.3 Growth, morphology and evolution in *Halomonas elongata* 1H9

Although previous studies reported *H. elongata* growing in up to 5.48 M NaCl in complex medium (Vreeland and Martin, 1980; Vreeland *et al.*, 1980), this experiment used 4 M as the upper salinity limit. The reason for this was to be able to have similar number of transfers in the three salinity cultures and to increase the likelihood of potential mutations arising by increased amount of generations throughout 20 months. Whilst salt precipitation in media

above 4.7 M NaCl did not enable *Hbt. salinarum* cultures to evolve at the very limit of their salinity range, again, this facilitated an increased number of generation times (Robinson *et al.*, 2005). Dividing around twice as fast as *Hbt. salinarum* spp., *H. elongata* 1H9 cultures reached 127 – 137 transfers (~ 12633 generations) by the end of the experiment, while both *Hbt. salinarum* strains reached only 56-60 transfers (~ 2105 generations), which probably is the main factor explaining why more mutations and effects on growth were observed for *H. elongata* 1H9 compared with *Hbt. salinarum* strains.

Interestingly, after four months of subculturing, some of the *H. elongata* 1H9 lab-evolution cultures showed signs of adapting to the salinity of the medium in which they were cultured. The clearest demonstration of this difference was when grown in the 4.7 M NaCl growth-test medium, where lag phase increased as the lab-evolution medium salinity decreased (Figure 3.6D). This lag period was increased even more after 20 months of subculturing, with the low-salinity lab-evolution culture showing no growth by 25 days in the highest salinity growth-test medium (Figure 3.9D). Also, the lag phase of the low-salinity lab-evolution culture was longer when grown in the 3.1 and 3.9 M NaCl growth-test medium (Figure 3.9). Thus, the data show that the growth capacity of the low-salinity lab-evolution cultures to grow at high salinity was greatly impaired, confirming hypothesis H2. Interestingly, while the lab-evolution salinity greatly affected lag phase, it only had a minimal effect on specific growth rate at higher salinities (Figure 3.10). On the other hand, the high-salinity lab-evolution culture showed signs of slower growth in the 0 M NaCl growth-test medium, which suggests that further subculturing would probably have reduced or prevented growth at 0 M NaCl.

All *H. elongata* cultures grew better at low salinities, with the exception of 0 M NaCl, than high salinities (Figure 3.10). Since *H. elongata* 1H9 is a generalist halotolerant bacterium, growing at lower salinities allowed it not to produce or accumulate compatible solutes, which requires energy (Kindzierski *et al.*, 2017; Hobmeier *et al.*, 2020). Microscopy showed several highly elongated cells in all lab-evolution cultures, mainly at mid- and high salinities (Figure 3.13).

The original publication of Vreeland and colleagues (1980) reported that older cultures of *H. elongata* produced elongated cells, which cannot be relevant for this study, as cells were collected at the exponential phase. Recent advances in biotechnology reported the formation of elongated cells, but for a different reason: to further improve cell growth in order to produce polyhydroxyalkanoates (PHA) more efficiently (Tao, Lv and Chen, 2017; Chen *et al.*, 2020). Along with other genes related to cell division, many genes from the Fts group were overexpressed to achieve cell division with more than two daughter cells in one cycle (Chen *et al.*, 2020). Variation analysis of the genome in my study showed that all three lab-evolution cultures suffered losses of Fts genes, sometimes with other genes related to cell division. Due to nonsynonymous missense point mutation, the low-salinity lab-evolution culture lost its single-copy FtsZ gene that is responsible for forming the Z-ring during cell division (Tao, Lv and Chen, 2017). The culture also suffered a deletion of N-acetylmuranoyl-L-alanine amidase (two putative copies), that cleaves the bonds in cell-wall glycopeptides, thus partitioning daughter cells after cell division (Heidrich *et al.*, 2001; Vermassen *et al.*, 2019). The mid- and high-salinity lab-evolution cultures suffered point mutations in the single-copy cell-division-associated, ABS transporter-like signalling protein FtsX and in a probable peptidoglycan glycosyltransferase FtsW, which has three more copies in the genome, respectively. FtsX, along with FtsE, carries out peptidoglycan hydrolysis by regulating the activity of the periplasmic peptidoglycan amidases (such as *N*-acetylmuramoyl-L-alanine amidase) (Bajaj *et al.*, 2016; Cook *et al.*, 2020). FtsW has almost the opposite role as *N*-acetylmuramoyl-L-alanine amidase, as besides stabilizing the Z-ring, it also facilitates the production of septal peptidoglycan to form the new cell wall after cell division (Mercer and Weiss, 2002; Taguchi *et al.*, 2019). In summary, all these proteins are responsible for some part of the cell division, and if single-copy genes are mutated, the cell division process becomes disrupted, and cells will not be able to divide entirely. These mutations probably explain the highly elongated cells in all three salinity lab-evolution cultures (Figure 3.13). Even though these genes are not related to osmoadaptation, further research could answer the questions of whether this was

an event caused by random point mutation, or some other unknown factor related to long-term laboratory evolution. Many other genes were subject to mutation, such as dihydroxyacetone kinase, dihydrodipicolinate synthase and a flagellum-specific ATP synthase FliI, however, most of them have multiple copies in the genome, therefore the cultures can function in normal conditions (Table 3.6). No mutations were found in genes of *H. elongata* 1H9 or *Hbt. salinarum* 91-R6 that was obviously connected to osmoadaptation, even though there were clear differences shown in the grow-test measurements. This rejects hypothesis H4. On the other hand, more mutations happened in the genomes of the *H. elongata* 1H9 lab-evolution cultures than in the *Hbt. salinarum* 91-R6 cultures, which confirms the last hypothesis of this study, H5: double the generation time, double the transfers happening over 20 months, hence more mutations.

3.5 References

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3.6 Supplementary material

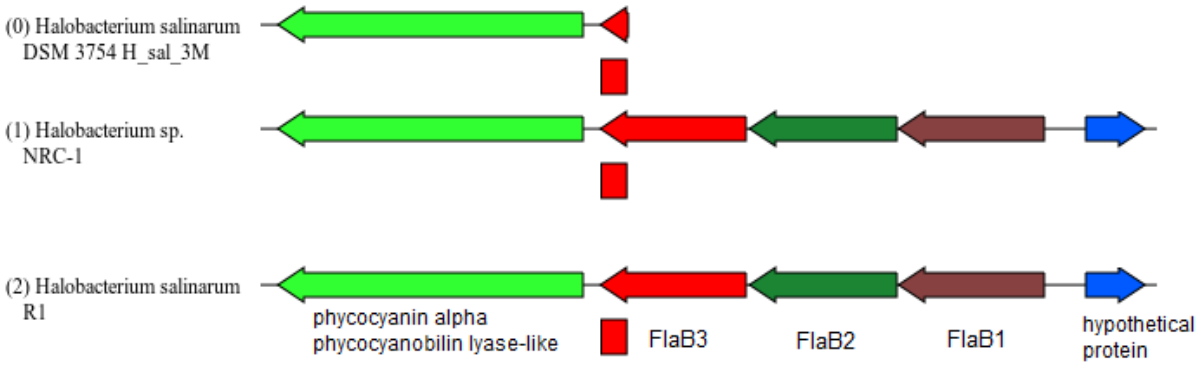

Figure S3.1 Loss of 'flaB' genes that code the FlaB archaellum shown on the operon of *Hbt. salinarum* 91-R6 low-salinity (3 M NaCl) lab-evolution culture after 20 months of culturing. The reference operons shown belong to *Hbt. salinarum* NRC-1 and R1.

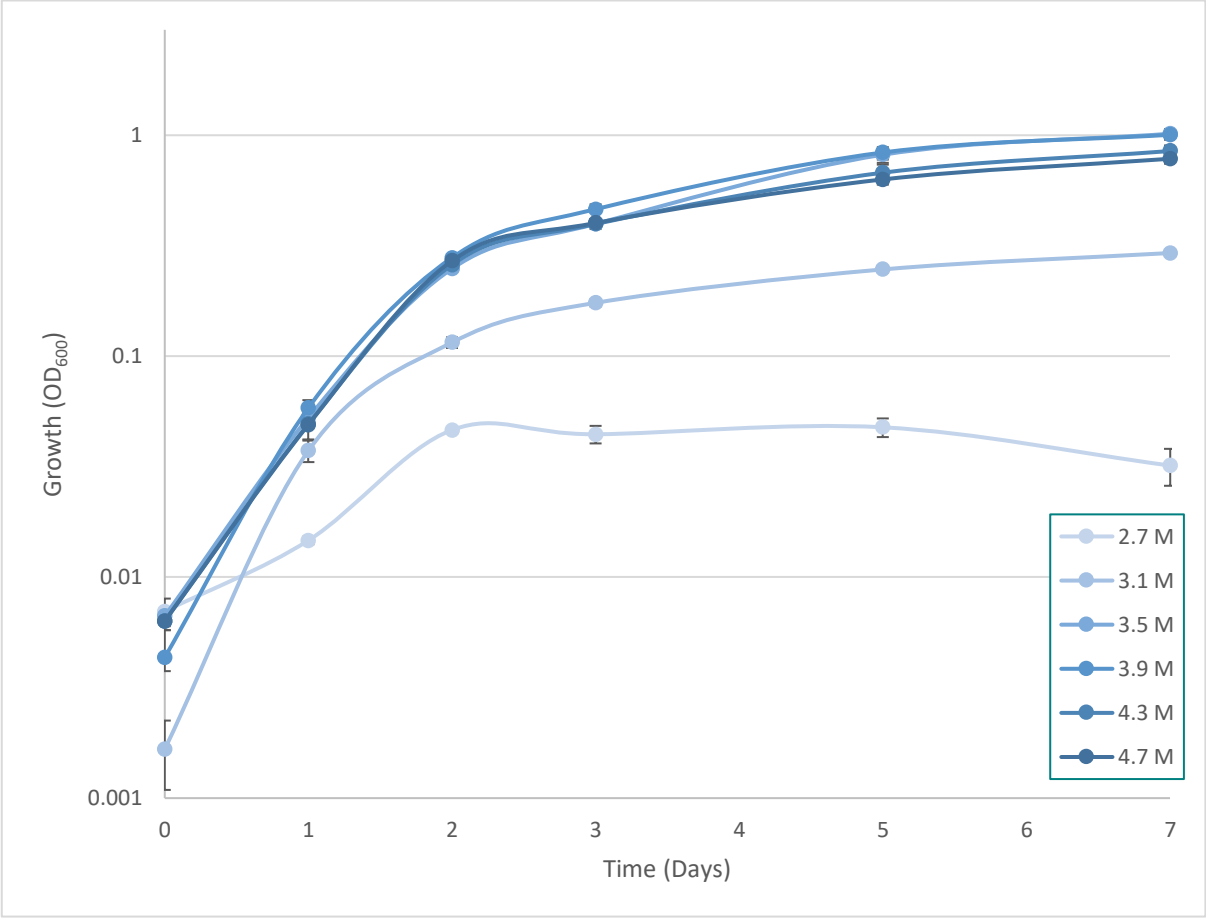

Figure S3.2 Growth curves of the high-salinity lab-evolution cultures of *Hbt. salinarum* 91-R6 (4.7 M NaCl) when exposed to different growth-test media with salinities from 2.7 to 4.7 M NaCl after 20 months of subculturing. Error bars represent standard deviation. N = 3. The culture exposed to the lowest salinity growth-test medium (2.7 M NaCl) did not reach OD = 0.05, and there was no visible growth at all times. Measurements were done after 53 transfers of the culture.

Chapter 4: Evolution of halophilic and halotolerant microbial communities derived from different saline environments

4.1 Introduction

4.1.1 Extremophiles: halophiles and halotolerants

As per the general broad definition from Oren (2008), halophilic microorganisms grow at a minimum 50 g/l (0.85 M) NaCl and are able to tolerate at least 100 g/l salinity (1.7 M NaCl), while extreme halophiles require 2.5-5.2 M NaCl (Oren, 2008b). They are a metabolically diverse group, including oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate-reducers and methanogens (Oren, 2002).

Halophiles and halotolerants have been discovered in all three domains of life, however, many of them only have a few examples in their taxa. In contrast, the three orders of Halobacteria (Halobacteriales, Haloferacales, and Natrialbales) consists entirely of halophiles. Halophilic and halotolerant members of the Archaea, Bacteria and Eukarya use different strategies to cope with high salt, for example modifying their cell envelope, synthesising or accumulating organic osmotic solutes or inorganic ions into the cytoplasm (Gunde-Cimerman *et al.*, 2000; Oren, 2008b).

Adaptations to living at high salt concentrations are often similar to adaptions to low water activity due to the effect of solutes on water activity. The key adaptation is osmoregulation and it includes the utilization of compatible solutes or osmoprotectants (osmolytes) by the cell (Torsvik and Øvreås, 2008). Every halophilic microorganism must cope with both high osmolarity and low water activity by maintaining its intracellular osmotic equilibrium. Two main strategies have evolved for adaptation to high salt: the “salt-in” and the “compatible solute” strategy.

The salt-in strategy involves the accumulation of inorganic ions, mainly K^+ and Cl^- in high concentrations (~ 4.5 M KCl) into the cell and excluding Na^+ ions from the cell to a high degree. As a result, all macromolecules and metabolic processes are adapted to work in this highly saline environment inside the cell. This strategy is energetically effective, although it is highly sensitive to reductions in the external salt concentration (Oren, 2006). A few groups of prokaryotes are using this strategy: all the six families within the haloarchaea (e.g. *Halobacterium salinarum*), the bacterial aerobe genus *Salinibacter* and the bacterial order of the anaerobic fermentative *Halanaerobiales* (McGenity and Oren, 2012).

The other, much more widespread strategy to tolerate high salt is the compatible-solute strategy. As its name suggests, it incorporates the use of organic compatible solutes or osmolytes in order maintain metabolic activities in the cytoplasm by keeping intracellular salt concentrations low. Osmolytes can be accumulated and/or biosynthesised. The cell expels excess salt using active transport. It is realised in most halophilic and halotolerant Bacteria (e.g. *Halomonas elongata*), halophilic eukaryotic algae (e.g. *Dunaliella* sp.) and fungi (e.g. *Hortaea werneckii*), and also in the halophilic methanogenic Archaea (*Methanohalophilus portucalensis*) (Gunde-Cimerman *et al.*, 2018). The process of compatible-solute biosynthesis is energetically more demanding than the salt-in strategy, however, it also gives more flexibility against changing conditions, enabling microorganisms to grow across a broad salt concentration range (Oren, 2008a). Most osmolytes are based on amino acids (e.g. proline) and their derivatives, sugars and sugar alcohols (Hoffmann *et al.*, 2012). A few examples include glycerol, glycine betaine, ectoine, sucrose and trehalose (McGenity and Oren, 2012).

There are other salt stress responses found within halophiles. Roberts (2005) identified that many halophilic Bacteria and Archaea use more than one solute to reach osmotic balance unless a solute is readily provided within the medium. These solutes can be a combination of anions and zwitterions or often several solutes with the same net charge (Roberts, 2005).

4.1.2 Experimental evolution in microbial communities

The previous chapter considered evolution of populations in isolation, whereas this chapter focused on how communities change under the same conditions as in Chapter 3. An added question in this chapter was whether communities from a lower and more variable salinity environment, which likely have a greater diversity of microbes, evolved along a different route compared to a community from a stable, high-salt environment. In addition, I wanted to determine how each community responded to three different salinities on a 16-month timescale of continuous culturing. More variable environments are likely to have a more diverse community than those from a stable high-salt environment. In a salt spring environment there will be higher diversity, a larger gene pool, and thus more capacilty to adapt. Given the heterogenous nature of a salt spring that is exposed to open-air migration and fluctuating salinities, an abundance of salinity-flexible microbes are expected to grow: more compatible-solute and less salt-in strategists are expected in this community. More halophilic microbes may be present and dormant until conditions become favourable again (Hubert *et al.*, 2009).

For this study, we chose to carry out an evolution experiment with the serial transfer method, the same way as described in Chapter 3. A fraction of the population will be periodically transferred to fresh media and will be allowed to regrow until it reaches early exponential phase, prior to nutrient depletion, where it will be transferred again, allowing perpetual population growth (Barrick and Lenski, 2013).

The salinity is considered constant in Boulby Mine, whereas it varies in the Cheshire salt springs due to rainfall and evaporation. Previous studies found differences in community composition of saline soils which were due to seasonal changes (Crump *et al.*, 2004) or due to physical and chemical heterogeneity (variable salt concentrations) (Canfora *et al.*, 2014).

Therefore, when exposing these microbial communities to low, medium and high salinities (3.0, 4.0 and 4.7 M NaCl, respectively), we hypothesise that there will be a shift in salt spring microbial community composition after a one to two year continuous culturing period, while the Boulby brine microbial communities will be more similar to each other. Post-experimental microbial communities are expected to have a distinction in the microbial community composition between low- and high-salinity enrichments, especially in samples from the Cheshire salt springs, where microbial diversity is likely higher (Langenheder *et al.*, 2003).

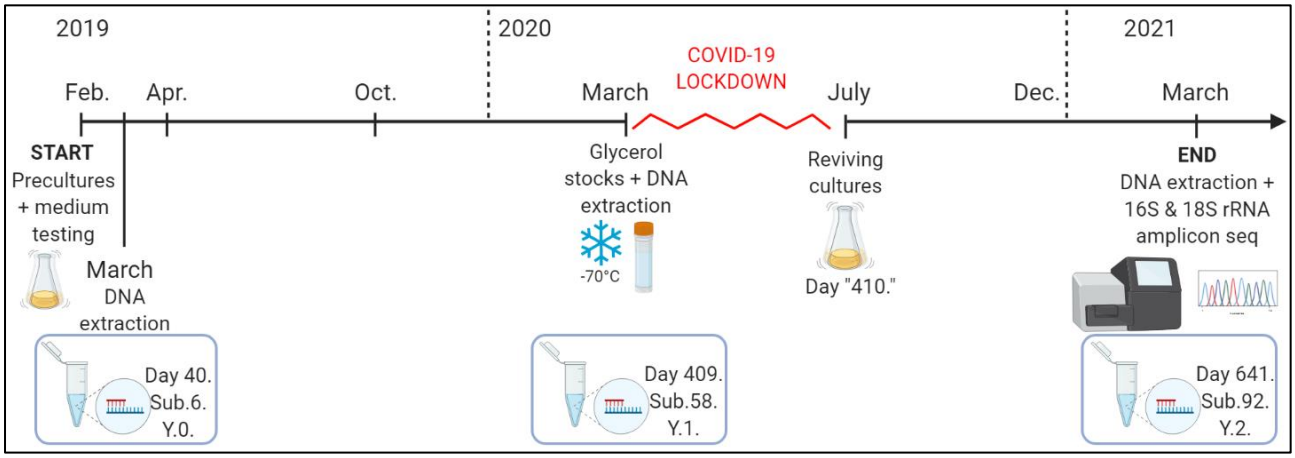

Figure 4.1 Schematic representation of the experiment design. Three microbial communities from two environments with different salinities were subjected to experimental evolution in vitro to investigate how the community composition changes when communities are cultured in media of different salinities.

The experiment focused on identifying extreme halophiles or halotolerants from microbial communities enriched in the highest salinity medium. Extreme haloarchaea such as *Halobacterium* sp. are expected to be found in the salt mine sample 'LH' and halotolerant bacterial (*Halobacillus* sp. and *Halomonas* sp.) or archaeal species (*Haloferax* sp., *Haladaptatus* sp.) are expected to be found in the salt spring samples, which have much lower and also varying salinities compared to a mostly constant high salinity environment such as Boulby Mine.

Boulby mine is a working potash mine near Whitby (UK) sylvite (KCl) is mined, and is home to Boulby International Subsurface Astrobiology Lab (BISAL). The mine is ~1.1 km deep below the surface, and has a constant temperature of ~40-42°C (Norton, McGenity and

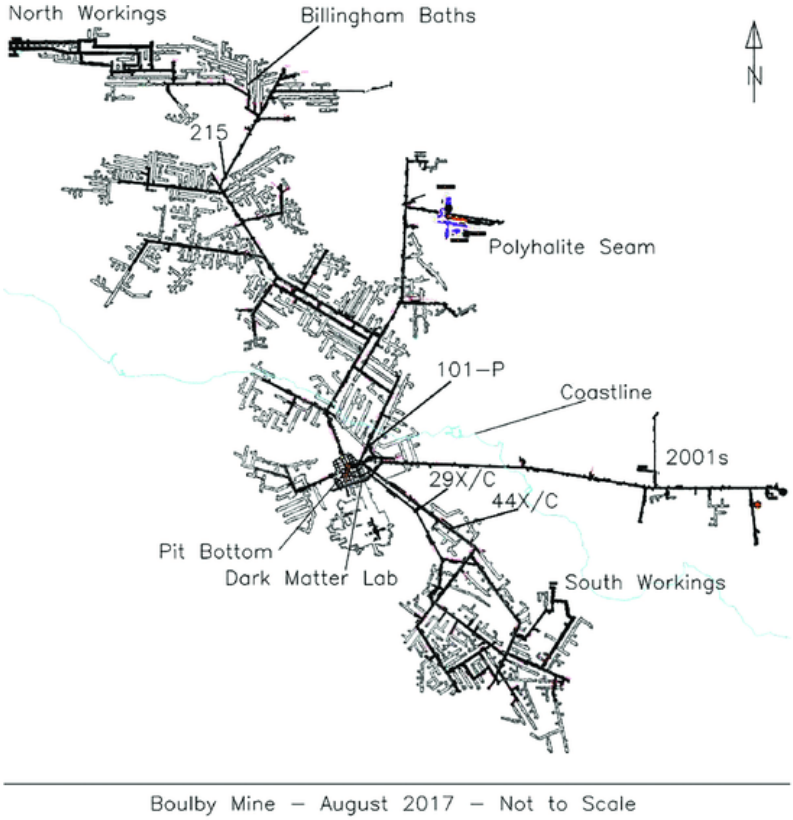

Figure 4.2 A schematic representation of Boulby Mine in 2017. Samples were acquired from 29X/C brine seep, and artificial brines using 29X/C as an inoculating brine. New West brine seep is not represented on the schematic. The coastline is outlined in light blue; the northeast part of the mine is under the sea. Figure was adapted from Payler et al. (2019)

Grant, 1993). The mine allows to direct sampling from a Permian (225 – 270 million years old) Zechstein salt deposit, that existed as an ancient sea across Europe (Norton, McGenity and Grant, 1993; Payler *et al.*, 2019). The thick halite layer is overlaid by sylvite (KCl). Several brines are present within the mine, where some of the brines were isolated from the mining works for over 20 years. Samples were taken from a pressurised brine seep, directly from the evaporite, where a pumping system drains the oldest part of the mine works. They have subsequently flooded with a water inflow from the aquifer that is above the mine that seeped through the Bunter Sandstone (Norton, McGenity and Grant, 1993). The water that seeps out from the aquifer is near saturation, almost entirely of Permian salt and potash material, which means that the process is so slow that the water can accumulate enough salts to become a brine.

Cheshire is home to the UK's oldest working mine, Winsford Salt Mine, where current mining works are extracting Triassic salt (235 – 260 million years ago) via pumping brine at the Halford brine fields (Kelbrick, Abed and Antunes, 2021). It is a much shallower mine compared to Boulby, lying around 150 meters below the surface (Kelbrick, Abed and Antunes, 2021). As mining was established here earlier, in 1844, the mine and the halite became more exposed to human influence. The salt deposits in this mine are connected with the surface-derived groundwater, creating brine that emerges on the surface as salt springs, such as Anderton brine springs (Kelbrick, Abed and Antunes, 2021). Multiple interlocking brine pools fluctuate in salinity (0.2 – 2.2 M NaCl) depending on precipitation, evaporation, and drainage (Kelbrick, Abed and Antunes, 2021).

4.2 Methodology

4.2.1 Field sampling

Multiple over-saturated brine samples were taken from Boulby Mine from brines created by the mixture of surrounding salt and filtered aquifer water on 11th July 2018. Brines named “29X/C L/H BOP” (LH) and “29X/C R/H BOP” (RH) were acquired, with the help of Thomas Edwards, Senior Exploration Geologist). After the brines were carried back to the laboratory at the University of Essex, 5 mL of each brine was enriched in 10 mL of 2.8 M NaCl Payne medium in a 50 mL sterile Falcon tube and was incubated at 30°C (Payne, Sehgal and Gibbons, 1960). Only LH brine showed growth (visual inspection, then plating onto agar plates), therefore this was chosen for future experiments. After the first observation of microbial growth (~ nine days), further subculturing was done in 2.8 – 3.4 M NaCl Payne medium (Payne *et al.*, 1960). Brine and sediment samples were obtained from the Cheshire salt springs (A1, A2 and A3 salt springs) on 15th October 2018 and two were chosen for the experiment from A2 salt spring: A2.1 brine and A2.2 sediment (top 0.5 cm) samples. Samples were stored refrigerated until the start of the experiment. Water activity values (a_w) were measured with a Novasina AW SPRINT TH-500 instrument at 25°C. Refractive index measurements were carried out using a hand-held refractometer (DIGIT-100 ATC, CETI).

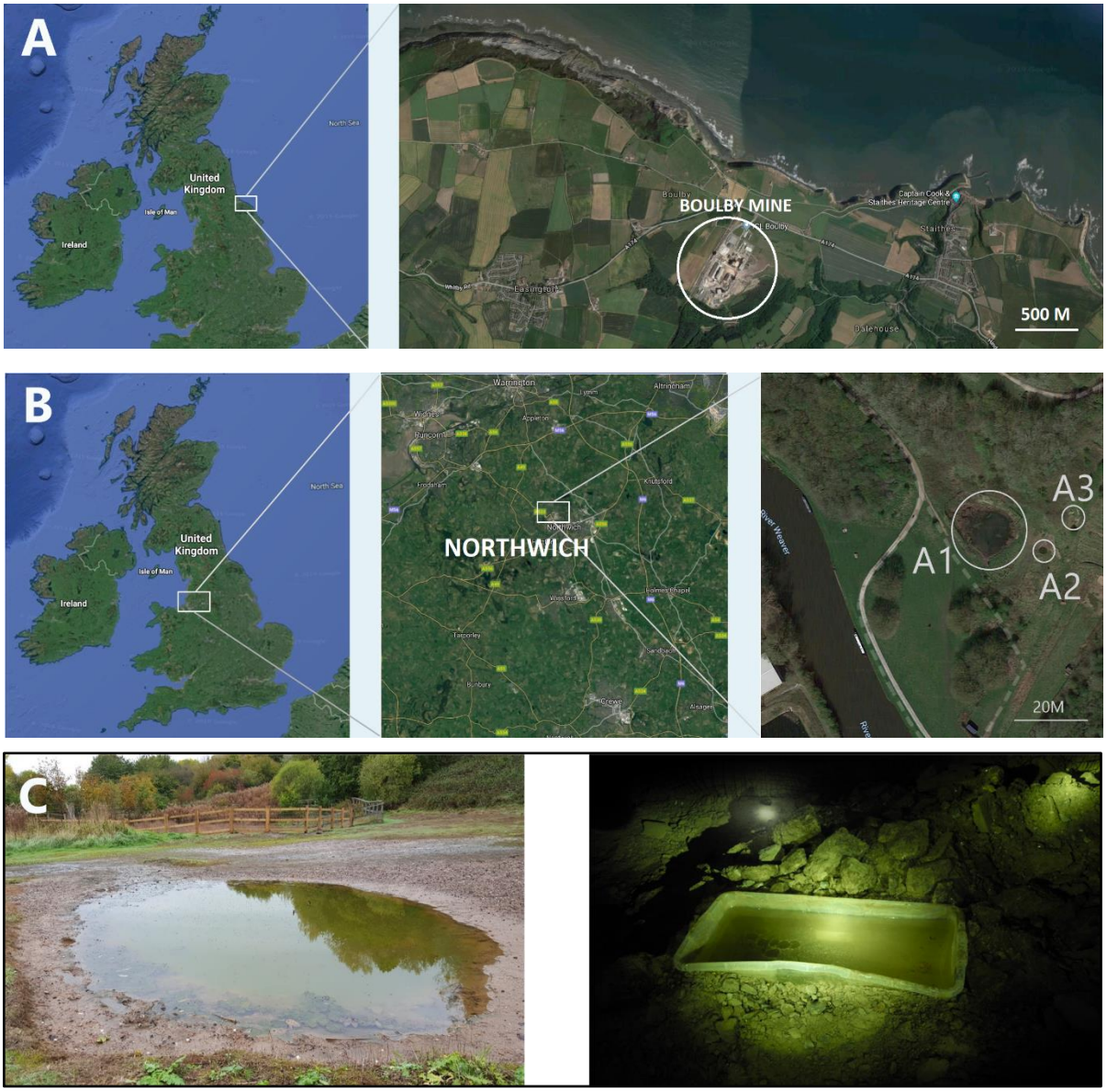

Figure 4.3 Location of the sampling sites for the halophilic microbial communities (Google Maps, 2019). A: Boulby Mine, lat 54.554, lon -0.823, 1.2 km underground; B: Salt springs in the Cheshire Salt District near Northwich, lat 53.272, lon -2.524; C: Left: 'A2' salt spring, Right: 'BRIMY 2', an artificial brine created from the surrounding salts and inoculated with the LH Bop brine which was used in this study. No pictures were made of LH brine.

4.2.2 Establishing NaCl concentrations for low, mid, and high salinity media which yield growth of halophilic/halotolerant microbial communities from Boulby and Cheshire

Based on the measured refractive index (RI) and water activity of the brines and sediment (only water activity of the latter one), we inferred that all samples potentially contain halophiles or halotolerants. Three media were selected, within the growth limits of a general halophilic microorganism, based on *Halobacterium salinarum*. The A2.2 wet sediment samples (1 g) were suspended in 10 mL sterile 2.56 M NaCl (150 g/l) salt solution, vortexed for 15 minutes,

then sonicated for 10 minutes in a water bath. The vortexing and sonicating steps were repeated twice, and the final solution was determined as the zero dilution. Ten-fold serial dilutions were made in four steps (10^{0}-10^{-4}) from the zero dilution with a final volume of 10 ml, using 2.56 M NaCl salt solution. Both brine samples (5 mL each of the original LH sample and A2.1) and 1 mL of the 10^{-4} diluted A2.2 sediment sample were inoculated (T_0, Day 1, 08/02/2019) in 10 mL media with three different salinities: M47b (3 M NaCl), M48 (4 M NaCl) and M51 (4.7 M NaCl) (Table 3.2). After growth was established, 100 µL of each enrichment was transferred into 10 mL of new batches of the same salinity media once every week when OD reached ~ 0.5. After 13 months, the cultures were stored in glycerol at -70°C for four months during the COVID-19 lockdown, then revived, and the experiment continued for four more months. Genomic DNA was extracted at different time points of the experiment for community composition analysis (Table 4.1).

4.2.3 Metagenetic sequencing

4.2.3.1 Amplicon sequencing of the microbial communities

Genomic DNA was extracted (Griffiths *et al.*, 2000) from the original brine and sediment samples, and from the cultures at the start of the experiment, after 13 months of subculturing, and at the end of the experiment (Table 4.1). The extracted DNA was visualized by agarose gel electrophoresis (1% w/v), using 10 mg/ml ethidium bromide staining, and was diluted accordingly in order to normalize DNA concentration before performing PCR. All extracted DNA was stored at -20°C until use. Two primer pairs were used to amplify taxonomic marker genes: prokaryotic primers 515F (Parada, Needham and Fuhrman, 2016) and 806R (Apprill *et al.*, 2015) specific to the 16S rRNA gene of Archaea and Bacteria, and universal primers 515F (Parada, Needham and Fuhrman, 2016) and 926R (Quince *et al.*, 2011) specific to the 16S rRNA prokaryotic and 18S rRNA eukaryotic genes. Protocols of the modified primer pairs and amplicon sequencing can be found here: EMP 16S Illumina Amplicon Protocol

(dx.doi.org/10.17504/protocols.io.nuudeww), Fuhrman Lab 515F-926R 16S and 18S rRNA Sequencing Protocol V.1 (dx.doi.org/10.17504/protocols.io.nkhdct6).

Table 4.1 Main time points of the evolution experiment. DNA was extracted at the start, after 13.6 months, and at the end (21.4 months) of the experiment for microbial community analysis. Cultures were in glycerol stock at -70°C for 115 days, after which the experiment continued. Lockdown days: 525-410 = 115. Year 2 DNA extraction: 756 – 115 = 641 days after start.

	DNA extraction	Date	Days after T_0	Months after T_0	No. of transfers
START	-	08/02/2019	0	0	0
	Year 0	19/03/2019	40	1.3	4
	Year 1	22/03/2020	409	13.6	56
Lockdown	-	23/03/2020	410	13.7	-
	-	16/07/2020	525	17.5	-
END	Year 2	04/03/2021	641	21.4	90

Table 4.2 Primers used for Illumina MiSeq metagenetic sequencing shown with and without MiSeq adapter sequences

Primer	Name	Sequence (5'->3')
Prokaryotic primers - 16S rRNA gene of Archaea and Bacteria		
Forward	515F	GTGYCAGCMGCCGCGGTAA
Reverse	806R	GGACTACNVGGGTWTCTAAT
Forward + MiSeq adapter	515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA
Reverse + MiSeq adapter	806R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT
Universal primers - 16S rRNA gene of Archaea and Bacteria + 18S rRNA gene of Eukaryotes		
Forward	515F	GTGYCAGCMGCCGCGGTAA
Reverse	926R	CCGYCAATTYMTTTRAGTTT
Forward + MiSeq adapter	515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA
Reverse + MiSeq adapter	926R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGYCAATTYMTTTRAGTTT

Library preparation was performed following the modified protocol provided by Illumina (16S Metagenomic Sequencing Library Preparation (15044223 B)). The first PCR was performed in a 96-well microplate with a final volume of 25 µl, consisting of the following: 1 µL DNA extract, 0.5 µL each of forward and reverse primers (10 µM), 12 µL appTAQ RedMix (2x) DNA polymerase (Appleton Woods LTD), and 11 µL PCR-grade water. A negative control

was included in the PCR run, using 1 µL of PCR-grade water instead of DNA extract. The microplate was sealed with Microseal A film (Bio-Rad). After the initial denaturation step at 95°C for two minutes, PCR amplification was performed on both primer sets according to the following, in 30 cycles: denaturation at 95°C for 15 seconds, annealing at 50°C for 15 seconds, and extension at 72°C for 30 seconds. A final extension step followed at 72°C for five minutes. The original brine and sediment samples were amplified in 38 cycles, while two samples (A2.2 from 2021 in medium M48 and LH original brine) did not amplify after several attempts, regardless the number of cycles or changing other PCR conditions. PCR products were visualized and their size was verified as stated above.

In order to remove free primers, primer dimers, and loading dye from the PCR products, a first clean-up step was performed using AMPure XP beads. After a short centrifugation step of the PCR microplate, 20 µL of homogenised room-temperature AMPure XP beads were added and mixed into each sample well and were incubated at room temperature without shaking for five minutes. The supernatant was cleared by placing the microplate on a magnetic stand (Thermo Fisher Scientific) and letting the beads settle. After the supernatant was discarded, the beads were washed with 200 µL of feshly prepared 80% v/v ethanol for 30 seconds. After the supernatant was removed, this wash step was repeated, and the beads were air-dried for 10 minutes. The microplate was removed from the magnetic stand, and 52.5 µL of 10 mM Tris (pH 8.5) was added to each sample well to resuspend the beads, which then were incubated at room temperature for two minutes. The microplate was placed on the magnetic stand again to settle the beads. Finally, 50 µL of the supernatant of each sample well was carefully transferred to a new 90-well microplate and stored at -20°C. Purified amplicons were visualized by agarose gel electrophoresis as described above.

A second PCR was performed in order to attach dual indices and Illumina sequencing adapters to the amplicons, using the Nextera XT Index Kit v2 (Illumina). The PCR with a final volume of 50 µL consisted of the following: 5 µL of purified amplicons, 5 µL of Nextera XT

Index Primer 1 (N7XX), 5 µL of Nextera XT Index Primer 2 (S5XX), 25 µL appTAQ RedMix (2x) DNA polymerase (Appleton Woods LTD), and 10 µL PCR-grade water was added into a new 96-well microplate and was sealed with Microseal A film (Bio-Rad) and centrifuged briefly. After the initial denaturation step at 95°C for three minutes, PCR amplification was performed in 8 cycles: denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 15 seconds. A final extension step followed at 72°C for five minutes. Indexed and non-indexed PCR products were visualized side-by-side by agarose gel electrophoresis as described above to verify that the dual indices and the Illumina sequencing adapters had successfully attached. A second clean-up step was performed on the indexed PCR products following the protocol described above, except that 56 µL of AMPure XP beads was added, and after the second wash step, amplicons were resuspended in 27.5 µL of 10 mM Tris (pH 8.5). Finally, 25 µL of supernatant was added into a new 96-well microplate from each sample well and stored at -20°C. Purified amplicons were visualized by agarose gel electrophoresis as described above, and quantified via a Quant-IT™ PicoGreen assay (Thermo Fisher Scientific) at 520 nm using a NanoDrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific).

Individual gene libraries were prepared by pooling the quantified amplicons in equal concentrations, and were quantified again via a Quant-IT™ PicoGreen assay (Thermo Fisher Scientific) as described above. DNA concentration (nM) in each library was calculated by dividing the average DNA concentration (ng/µl) by the average molecular weight of dsDNA (660 g/mol) multiplied by the average library size, then multiplied by 10^6. Due to the low concentration, the individual gene libraries were concentrated using the AMPure XP beads as described above, except that they were resuspended in 20 µL of 10 mM Tris (pH 8.5). The individual gene libraries were measured again via Quant-IT™ PicoGreen assay (Thermo Fisher Scientific) as described above and via a NEBNext Library Quant Kit (Illumina) using a CFX96 real-time PCR detection system (Bio-Rad). The individual gene library sizes were

verified via a 2100 Expert Software (Agilent) using a 2100 Bioanalyzer Instrument (Agilent). The DNA concentration was calculated again as described above, and the prokaryotic 16S rRNA and universal 16S and 18S rRNA gene libraries were pooled using a ratio of 1:1. The final amplicon library was quantified twice via Quant-IT^{TM} PicoGreen assay (Thermo Fisher Scientific) as described above, and via a NEBNext Library Quant Kit (Illumina) as described above.

The final dilution was prepared by diluting the final library to 4 nM with elution buffer (Qiagen), then measured via a NEBNext Library Quant Kit (Illumina) as described above. To prepare samples for library denaturing and MiSeq sample loading, 5 µL of 4 nM pooled final library was mixed with 5 µL of 0.2 N NaOH, vortexed briefly, then centrifuged at 280 x *g* at 20°C for one minute. After five minutes of incubation at room temperature, 990 µL of pre-chilled Hybridization Buffer (HT1) was added to the denatured DNA to get a 20 pM denatured library in 1 mM NaOH. To achieve a 4 pM library, 120 µL of 20 pM denatured DNA was diluted with 480 µL pre-chilled HT1. The PhiX control was denatured and diluted the same way as described above, then 480 µL of 4 pM denatured DNA library was mixed with 120 µL of 4 pM denatured PhiX control. The tube of the combined library mixture was denatured at 96°C for two minutes, inverted twice, then immediately placed in an ice-water bath for five minutes. The final library mixture was loaded into a MiSeq reagent cartridge and sequenced on the Illumina MiSeq system using the MiSeq Reporter Metagenomics Workflow.

4.2.3.2 MiSeq bioinformatics

All bioinformatics analyses were performed in R statistical software version 4.0.4 (R Core Team, 2021). Individual raw demultiplexed paired-end reads were processed using the package ‘dada2’ (Callahan *et al.*, 2016). Forward and reverse sequence reads amplified by the ‘Prokaryotic’ primers were quality filtered and trimmed based on quality read profiles and quality scores. Instead of averaging quality scores, sequences were filtered based on the

maximum allowed expected errors, which are calculated from the nominal definition of the quality score. Filtered reads were trimmed from sequencing adapters, primers and from reads that match against the phiX genome. All sequencing errors (substitutions and indel errors) were removed from the filtered and trimmed sequencing reads based on the estimated error rates to reveal the members of the sequenced community. Denoised paired-ends reads were merged with a minimum of 20 base overlap between the forward and reverse reads and contig sequences were assembled. An amplicon sequence variant (ASV) table was created, that determined which exact sequences were observed and how many times they were observed in each sample, hence it is a higher resolution version of an operational taxonomic unit (OTU) table. The samples in the ASV table were independently checked, and a consensus decision was made on each sequence variant, removing chimeric sequences. The same protocol was followed for the 'Universal' sequences, except that only the forward reads were used for the analysis because the reverse reads were too short after quality trimming and did not overlap with the forward reads at merging.

Taxonomy was assigned to the sequence variants using reference datasets of the SILVA rRNA database project for 16S rRNA (Quast *et al.*, 2013; Yilmaz *et al.*, 2014; Callahan *et al.*, 2016) for both 'Prokaryotic' and 'Universal' sequences and the Protist Ribosomal Reference database (PR2) for 16S and 18S rRNA (Guillou *et al.*, 2013) for only the 'Universal' sequences. The exact databases used can be found at https://zenodo.org/record/4587955 and at https://github.com/pr2database/pr2database/releases, respectively (Callahan *et al.*, 2016). The ASV table was combined with the taxonomy data and the sample information to create a comprehensive ASV table. Since there were no Eukaryotes present in any of the enriched samples, further analysis was performed only on 16S rRNA sequences. Sequences identified as chloroplasts or mitochondria were removed from the comprehensive ASV table. All the statistical analyses were performed using the package 'vegan' (Oksanen *et al.*, 2020) and all figures were produced using the package 'ggplot2' (Wickham, 2016). After the ASV

table was rarefied, alpha diversity indices were calculated and analysis of variance (ANOVA) was performed examining how time, salinity and sample type affects ASV richness. Beta diversity was visualized by non-metric multidimensional scaling (NMDS) and Permutational Multivariate Analysis of Variance (PERMANOVA) was performed using the package 'pairwiseAdonis' to examine how time, salinity and sample type affects community composition (Martinez Arbizu, 2017). All dominant ASVs were further assessed using the BLASTN programme of the National Center for Biotechnology Information (NCBI) (Available at https://blast.ncbi.nlm.nih.gov/Blast.cgi), and are described in the Discussion section.

4.3 Results

The refractive index (RI), water activity, and pH values were the following: RI = 1.386, a_w = 0.727, pH 7.47 for LH brine, and RI = 1.339-1.340, a_w = 0.931, pH 7.4 for A2 salt spring. The water activity and chemical composition of the three saline media is shown in Table 3.2. Growth appeared in all enrichments after two weeks, and the experiment continued by subculturing each enrichment once every week.

Metagenetic sequencing of the microbial communities revealed how community composition changed over time and over different salinities. Time was described a the year in which DNA was extracted: every March in 2019, 2020, and 2021. Although the 'Universal' primers had a higher coverage of 16S rRNA sequences than the 'Prokaryotic' primers, visualization and statistical analysis showed very similar results. Besides the original samples of A2.1 and A2.2, all the enrichments had low ASV richness (10 – 30) at the start of the experiment, which significantly decreased (p <0.001) by the end of the first year (2 – 10) and stayed low by the end of the second year (2 – 8), regardless of the sample type or salinity. The 16S rRNA ASV richness of the original samples was the following: A2.1 – 555 and 1099, A2.2 – 399 and 1475 sequenced by the Prokaryotic and Universal primers, respectively.

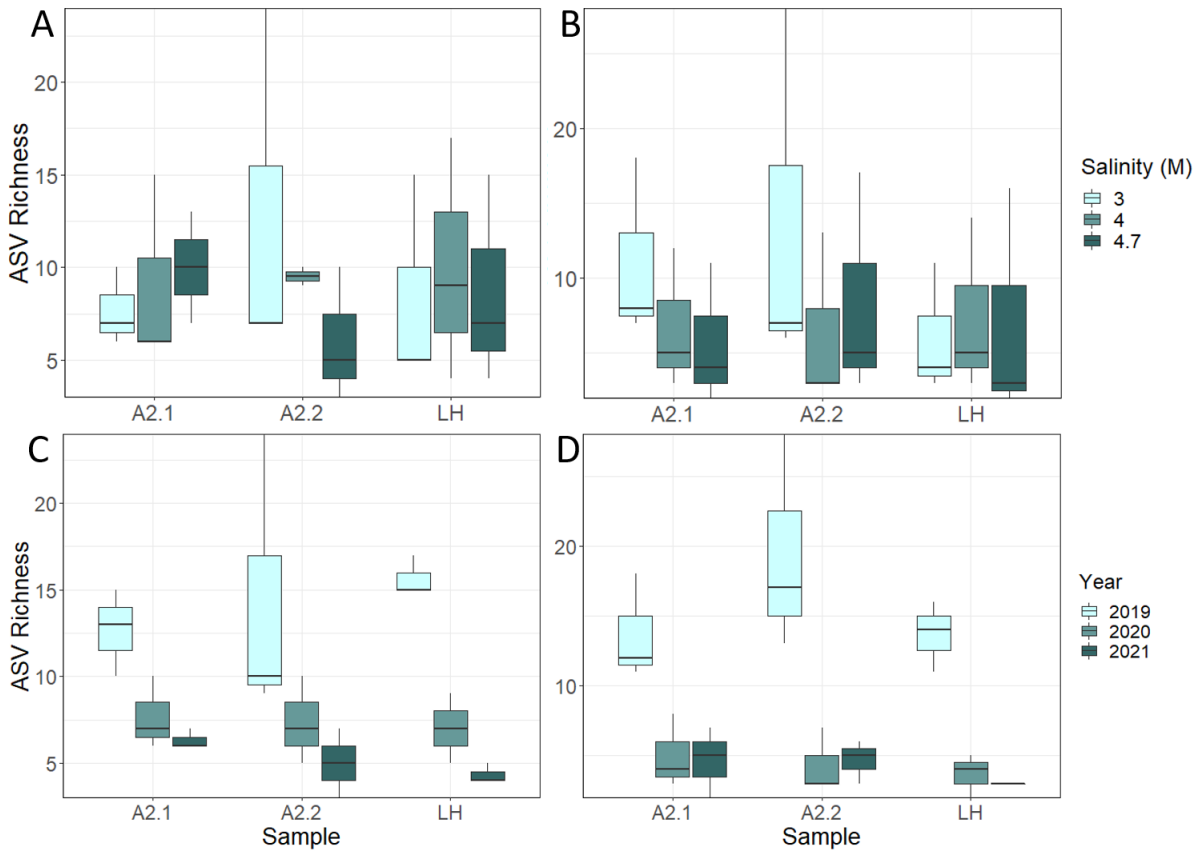

Figure 4.4 ASV richness of three samples across salinity (A-B) and year (C-D). X axis shows the three different enrichment samples, Y axis shows ASV richness. A and C are sequences amplified by the 'Prokaryotic' primers, B and D are sequences amplified by the 'Universal' primers. A and B shows ASV richness based on salinity, while C and D shows ASV richness based on year. ASV richness was significantly (p < 0.001) affected by the time (Year) only, not by salinity.

Permutational Multivariate Analysis of Variance (PERMANOVA) showed that microbial community composition was driven by both sample type (p <0.001), salinity (p <0.001), and time (Prokaryotic primers (Prok): p <0.05, Universal primers (Univ): p <0.01) (Figure 4.5) (Table S4.3). There was a statistically significant interaction between the effects of sample type and salinity (Prok: p <0.001, Univ: p <0.01) and between sample type and year (Prok: p <0.05, Univ: p <0.01) on community composition (Table S4.3). Microbial communities were distinct in the lowest salinity (3 M NaCl) enrichment from the medium (4 M NaCl) and high salinity (4.7 M NaCl) enrichments, while the communities in the two latter enrichments were

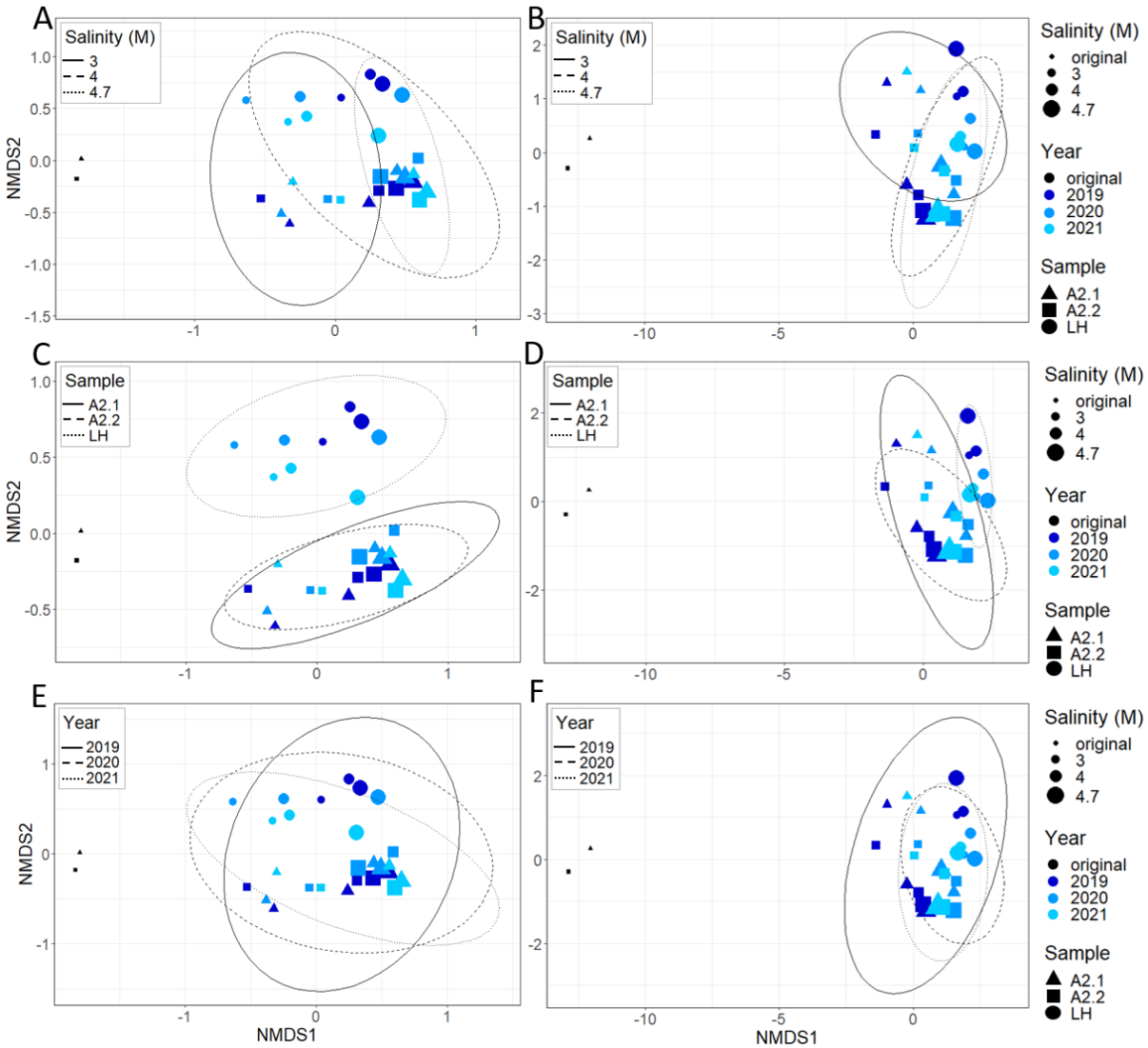

Figure 4.5 Non-metric Multidimensional Scaling (NMDS) of microbial community composition. Figures on the left (A, C, E) represent sequences from communities amplified by the 'Prokaryotic' primers, while figure on the right (B, D, F) represent communities from sequences amplified by the 'Universal' primers. Legends within the plots indicate which factor the confidence ellipses were based on: A & B: salinity, C & D: sample type, E & F: year. Note that there were only two original communities sequenced, A2.1 and A2.2 (black symbols on the left of the plots), as it was not possible to extract DNA from the original LH Bop salt mine sample.

more similar to each other (Figure 4.5). The community composition of the two samples from the same salt spring in Cheshire, A2.1 and A2.2, were similar to each other, while being highly distinct from the sample LH from Boulby salt mine. Interestingly, time alone was not significantly affecting community composition, like in te case of ASV richness, but only when interacting with the other two factors: salinity and sample type (Table S4.3).

There was little to no discrepancy in community composition between 16S rRNA sequences amplified by the 'Prokaryotic' versus 'Universal' primers, therefore they are described here together. The microbial communities from Boulby salt mine and Cheshire salt springs already had a low diversity at the start of the experiment, which declined even more after one year of subculturing and stayed constant until the end of the experiment. There were only a few dominant species in each enrichment (Figure 4.6). Community composition was very similar between the Cheshire samples (A2.1 and A2.2) but was distinct from the Boulby samples, LH, which had the lowest diversity out of all three samples. While *Halopenitus* sp. (*Hpt.*) was present in 80% in the lower salinity (3 M NaCl) enrichment of LH, *Halobacterium* sp. (*Hbt.*) was the most dominant genus in both mid- (4 M NaCl), and high salinities (4.7 M NaCl) at the start of the experiment (~78% and ~92%, respectively). Other less abundant genera included *Halorhabdus* sp. (*Hrd.*), *Halorientalis* sp. (*Hos.*), *Halomonas* sp., *Halomicrobium* sp. (*Hmc.*), *Halapricum* sp. (*Hpr.*), and *Haloferax* sp. (*Hfx.*) (Oren and Ventosa, 2013). However, after a year of subculturing, more than 99% of the lower and higher salinity enrichment consisted of *Halorhabdus* sp. The mid salinity enrichment was more diverse, with *Halorhabdus* sp. (~67%), *Halomicrobium* sp. (~16%), and *Halapricum* sp. (16%) being dominant. At the end of the experiment, *Halorhabdus* sp. dominated (~99%) across all enrichments, while *Halomonas* sp. and *Haloferax* sp. were the only other genera found in all the enrichments.

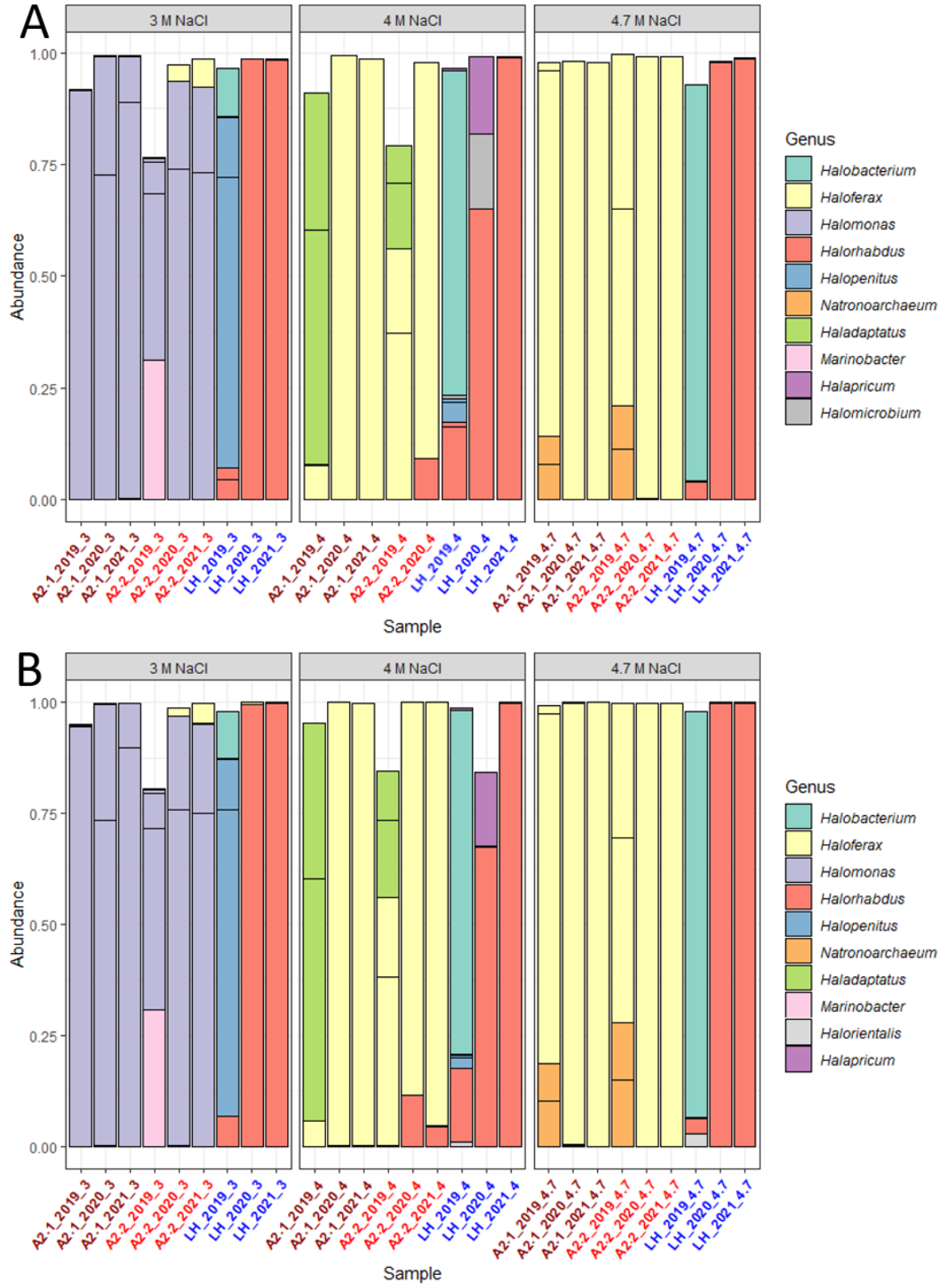

Figure 4.6 Community composition of the most abundant genera in each enrichment across different salinities. Sample names indicate sample type (A2.1 – dark red, A2.2 – red, LH Bop – blue), year the community was sampled, and NaCl concentration (M) of the enrichment medium. Panel A: 18 most abundant ASVs amplified by the 'Prokaryotic' primers, panel B: 17 most abundant ASVs amplified by the 'Universal' primers. More than one ASV can belong to one genus, as indicated by the horizontal lines within bars representing a genus. Halorientalis (panel B) was found using both Prokaryotic and Universal primers, but in very low abundance. Halomicrobium (panel A) was identified when using the 'Prokaryotic' primers to genus level, but it was only amplified to Family level by the 'Universal' primers, hence it is not shown on panel B. Y axis shows relative abundance, 1 = 100%. Hence only the 17-18 most abundant genera were plotted, the relative abundance value does not always reach 100%.

Cheshire samples A2.1 and A2.2 consisted of the same genera but in different abundance in the first enrichments, in addition, all initial A2.2 enrichments were more diverse, having 15 additional genera but in very low abundance (Figure 4.6). *Halomonas* sp. was the most abundant genus in both A2.1 and A2.2 samples in the low salt enrichments (~94% and ~54%, respectively), followed by *Halobacillus* sp. (~6%) in A2.1, and *Marinobacter* (~30%) and *Anaerophaga* sp. (~5%) in A2.2. The mid salt enrichments were dominated by the same three genera in both A2.1 and A2.2 samples, but in different abundances: *Haladaptatus* sp. (~87% and ~20%, respectively), *Haloferax* sp. (~6% and ~55%, respectively), and *Halobacillus* sp. (~6% and ~9%, respectively). *Haloferax* sp. dominated the high salt enrichments of both A2.1 and A2.2 (~80% and ~75%, respectively), followed by *Natronoarchaeum* sp. (~16% and 24%, respectively). Other less abundant genera included *Salimicrobium* sp., *Halobacterium* sp., *Halorhabdus* sp., *Halorubrum* sp. (*Hrr.*), *Halovibrio* sp., *Halopenitus* sp., *Haloterrigena* sp. (*Htg.*), *Halomicroarcula* sp. (*Hma.*), *Alkalibacterium* sp., and *Paraliobacillus* sp. The community composition became much less diverse after one year of subculturing, with only a few genera found in each enrichment. *Halomonas* spp. still dominated both A2.1 and A2.2 samples at low salinities (~99% and 95%, respectively), while the mid- and high salinities were solely dominated by *Haloferax* sp. (~99% in A2.1 at both salinities and in A2.2 high salinities). The mid salt enrichment of A2.2 consisted ~90% of *Haloferax* sp. and ~10% *Halorhabdus* sp. Other less abundant genera included *Alkalibacterium* sp., *Halobacterium* sp., *Paraliobacillus* sp., and *Halopenitus* sp. By the end of the experiment, after two years of subculturing, the enrichments were reduced to a few genera only. *Halomonas* sp. stayed the dominant genus of the low salt enrichments with ~99% abundance in the A2.1 sample, and ~94% in the A2.2 sample, followed by ~5% of *Haloferax* sp. The mid salt enrichment was ~99% dominated by *Haloferax* sp. in the A2.1 sample, while A2.2 consisted of ~95% *Haloferax* sp. and ~4.5% *Halorhabdus* sp. The high salinity enrichments of both A2.1 and A2.2 were almost entirely dominated by *Haloferax* sp. only (~99.96 - 100%). The other ~1% of genera in the mid-, and high salt enrichments included *Paraliobacillus* sp., *Halomonas* sp.,

Halobacterium sp., *Massilia* sp., *Gillisia* sp., *Mycobacterium* sp., and an uncultured genus ‘KCM-B-112’ from the family Acidithiobacillaceae.

4.4 Discussion

4.4.1 Enrichments from Boulby salt mine

There was an expected high ratio of Archaea compared to Bacteria (99.8:0.2, amplified by the Prokaryotic primers, respectively) in all the enrichments from Boulby Mine, a relatively closed and ancient salt mine system (Norton, McGenity and Grant, 1993; Payler *et al.*, 2019). Although various archaeal species were found in the initial enrichments, this number decreased significantly after one year of subculturing, and only three (*Halomonas, Haloferax,* and *Halorhadus*) genera remained after two years, with only *Halorhadus* being dominant across all salinities by the end of the experiment. This confirmed the hypothesis that microbial communities in Boulby mine are more similar to each other compared to Cheshire samples. Archaea dominated 99.8% or more of most enrichments, and the only bacterium found was *Halomonas taeanensis* at less than 0.2% in any enrichment. *H. taeanensis* being an extreme halotolerant, this bacterial genus survived until the end of the second year of subculturing in all enrichments (Lee *et al.*, 2005).

One of the genera in the initial low salt enrichment of the LH sample included two abundant ASVs making up to 80% of the enrichment. One of the ASVs was 100% identical to many *Halopenitus* (*Hpt.*) strains, including *Halopenitus persicus* strain DC30, while the other ASV was 100% identical to *Hpt.* sp. strain BbpA.3, which was previously isolated from Boulby Mine (McGenity *et al.*, 2000; Amoozegar *et al.*, 2012). Interestingly, the three currently characterised and named *Halopenitus* species were not isolated from deep subsurface salt deposits but from an inland salt lake and from a brine sample resulting from the process of making salted brown alga using crude sea salt (Amoozegar *et al.*, 2012, 2013; Han, Cui and Li, 2014). On the other hand, Thompson and colleagues (2021) recently isolated halophiles from a stalactite formed of NaCl, where *Halopenitus persicus* was only found inside the stalactite and in a brine pool within the same salt mine but not in a nearby non-saline soil

sample. Both halite samples from the two mines are from the Triassic, and could be from the same halite basin, the Larne Basin Deposit (Thompson *et al.*, 2021).

The other abundant ASV (73 – 90% abundance) in the initial mid- and high salt enrichment from Boulby mine was 100% identical to several strains of *Halobacterium* (*Halobacterium*), including *Halobacterium noricense* and *Halobacterium hubeiense* strains. This coincides with an isolate that I obtained from Boulby Mine earlier in 2018 (unpublished), and MinION whole genome sequencing (Oxford Nanopore Technologies) verified that it is 100% identical to *Halobacterium hubeiense*. In contrast to *Halopenitus.*, *Halobacterium noricense* strain A1 was isolated from alpine Permian (225 – 280 million year old) salt deposit, while *Halobacterium hubeiense* JI20-1 was isolated from early Cretaceous (~123 million year old) drilled core of halite from ~2026 meters depth. These species are also known to be obligate extreme halophiles, hence they were in low abundance in the low-salt enrichment. This could indicate that not only this abundant ASV, but most other abundant genera (including *Halopenitus* ASVs) from this experiment may have originated from the ancient halite itself. It is important to mention that this ASV was only 96.05% similar to *Halobacterium salinarum* strains 91-R6 and NRC-1, which can tolerate NaCl levels up to saturation but was not found in the enrichments. The high abundance of *Halopenitus* ASVs in the low salt enrichment can be explained by the lower salt tolerance they have (no growth below 1.7 M NaCl) compared to the *Halobacterium* ASV at mid- and high salinities with a higher salt tolerance (no growth below 2.5 M NaCl) (Aharon Oren, 2002; Amoozegar *et al.*, 2012).

Two ASVs dominating the latter enrichments are closely related to *Halorhabdus* sp. One of the ASVs was in high abundance only in the high salinity enrichments after one and two years of subculturing. This ASV was 100% identical to an uncultured archaeon clone MIG-A2 from a hypersaline oil reservoir (GenBank: JQ690686.1), 99.6% similar to *Halorhabdus* strain T2.1 from a solar saltern (GenBank: KY827051.1), and 99.21% similar to *Halorhabdus rudnickae* WSM-64^{T} from a salt mine borehole (Albuquerque *et al.*, 2016; Gales *et al.*, 2016).

Interestingly, some of the *Halorhabdus* ASVs found were 100% identical to *Halorhabdus* strains that were repeatedly found in ancient halite. The other ASV was abundant in the low- and mid-salt enrichments after one and two years of subculturing, and was 100% identical to several *Halorhabdus* strains, including *Halorhabdus rudnickae* WSM-64^T. Even though they were isolated from various environments, all three *Halorhabdus* species have a wide range of growth in salt, especially *Halorhabdus rudnickae*, growing between 0.8 and 5.1 M NaCl (Albuquerque *et al.*, 2016). The other two species were isolated from a deep hypersaline anoxic basin and from the Great Salt Lake, while *Halorhabdus rudnickae* was isolated from a salt mine (Wainø, Tindall and Ingvorsen, 2000; Antunes *et al.*, 2008; Albuquerque *et al.*, 2016). The high level of adaptability to different salinities could explain why these two ASVs were the most abundant across all salinities in the last two years of the experiment.

While *Halorhabdus* dominated the 2020 and 2021 LH enrichments, another ASV was in relatively high abundance (~17%) only the mid-salinity enrichment and only after one year of enrichment (2020), disappearing in 2021. The first ASV was 100% identical to *Halapricum* (*Hpr.*) *desulfuricans* strain HSR12-1, which was isolated from a hypersaline lake in Russia (Sorokin *et al.*, 2021). The second ASV was 99.21% identical to an uncultured clone DSFBPENV12arc_5D from a hypersaline pool of the Salton Sea, possibly in the family Haloarculaceae (KC465613.1). The optimum salinity for growth of *Halapricum desulfuricans* is 4 M, therefore we can assume that the two ASVs function at their best and able to outcompete other genera (e.g. *Halobacterium noricense* and *Halobacterium hubeiense*) only at this salinity. It is probable that it is more specialised to 4 M than to 3 M or 4.7 M NaCl. Both of these ASVs were outcompeted by *Halorhabdus* by the end of the experiment. This could indicate that the *Halorhabdus* ASVs possess certain genes that allow them to perform better in any salinity compared to other microbes. Two ASVs 100% identical to *Halomonas taeanensis* strain BH539^T (solar saltern) and *Haloferax* (*Haloferax*) species (*Haloferax massiliensis, Haloferax chudinovii* (Permian salt deposit)*, Haloferax sulfurifontis, Haloferax*

denitrificans) were the only two other ASVs found besides *Halorhabdus* at the end of the experiment in less than 0.2%, repressed by *Halorhabdus*.

4.4.2 Enrichments from the Cheshire salt springs

The initial enrichments of both Cheshire samples had a larger variety across salinities compared to the enrichments from Boulby Mine. The dominant genus was different for all three salinities in the A2.1 enrichments. An ASV 100% identical to *Halomonas taeanensis* strain BH539^T dominated the low salt A2.1 enrichments from the start to the end of the experiment. However, the same *Halomonas* ASV was outcompeted by the same *Halorhabdus* ASV in the Boulby enrichments; therefore, we can assume that certain conditions allowed the *Halomonas* ASV to persist better in the A2.1 enrichments. For example, it could have stayed dominant because coexisting with other microbes because of different environmental origin (open salt spring *vs* deep salt mine), etc. Having matching *Halomonas* ASVs in the Cheshire and in the Boulby enrichments does not necessarily mean that the organism is exactly the same regarding other genes. As the 16S rRNA taxonomic marker gene is highly conserved, other, more variable genes would be preferred to compare, such as the '*rpoB'* gene as a starting point. Then, it would require to sequence the whole genome, e.g. via cultuting or metagenome-assembled genomes from enrichments of both organisms. In contrast to the Boulby samples, the dominant ASV at low salinities belongs to Bacteria, not to Archaea. As *Halomonas taeanensis* is an extreme halotolerant and is capable of adapting to salinities from 0.17 to 5.1 M NaCl, it was most probably able to outcompete other, less adaptable haloarchaea such as *Haloferax, Halobacterium, Haladaptatus* and *Natronoarchaeum.* However, as soon as salinity was increased from 3 M to 4 M NaCl or more, haloarchaea outcompeted *Halomonas*. The second most abundant genus with many ASVs in the initial low- and mid-salt enrichments was 100% identical to several *Halobacillus* species, out of which some were isolated from hypersaline environments such as solar

salterns and the Great Salt Lake (Spring *et al.*, 1996; Yoon *et al.*, 2005). However, *Halomonas* ASVs must have had an advantage over not only haloarchaea but other Bacteria as well, as they were not found in any of the enrichments after a year of subculturing.

The mid-salinity enrichments were dominated by three ASVs which were similar to several *Haladaptatus* (*Haladaptatus*) species, including *Haladaptatus paucihalophilus* (99.21% similar) and *Haladaptatus* sp. strain W1, 30AH, and HA15 (100% identical). The latter strains were isolated at salt marshes on the coast of East of England (Purdy *et al.*, 2004). The two dominant *Haladaptatus* ASVs matched two different copies of 16S rRNA gene of *Haladaptatus* strain W1, and they were both 99.21% similar to the type strain, *Haladaptatus paucihalophilus* DX253^{T}. Although the optimum salinity for growth of *Haladaptatus* species is between 2.5 and 3.4 M NaCl, *Haladaptatus* strain W1 was previously found to be able to grow slowly at seawater salinity with an optimum salinity of 1.7 M NaCl (Purdy *et al.*, 2004; Savage *et al.*, 2007; Cui *et al.*, 2010; Roh, Lee and Bae, 2010; B.-B. Liu *et al.*, 2014). This might indicate why the *Haladaptatus* ASV was able to dominate the mid-salt enrichment over other genera. It was present in the low- and high-salt enrichments as well, but in very low abundance (0.01%). The reason for this might be because the *Halomonas* ASVs in the low-salt enrichment and the dominant *Haloferax* ASVs in the high-salt enrichment outcompeted it due to their preference of salinity. Therefore, the only salinity it was able to grow in high abundance was the 4 M NaCl enrichment. Both *Halomonas* and *Haloferax* species grow well in general halobacteria medium (Payne medium), depending on salinity, while *Haladaptatus* strain W1 was observed (unpublished) to grow better at a modified halophile (“Nils”) medium with added salts and trace elements (Stevenson *et al.*, 2014; Bartha, 2017). This could have contributed to its initial fast growth and dominance at 4 M NaCl, then being outcompeted by other microbes, which grow better in general halobacteria medium. This was one of the limitations of the study, as only one halophile medium was used with varying salinity instead of multiple different halophile-selective media.

The same *Haloferax* (*Haloferax*) ASV that was outcompeted by *Halorhabdus* (*Halorhabdus*) ASVs in the Boulby enrichments. it dominated all mid- and high-salt enrichments of both A2.1 and A2.2 samples in a very high abundance. Interestingly, most *Haloferax* species have an optimum salinity for growth of ~2.5 – 3 M NaCl, but are able to grow near saturation level (Tomlinson, Jahnke and Hochstein, 1986; Torreblanca *et al.*, 1986; Elshahed *et al.*, 2004; Saralov, Baslerov and Kuznetsov, 2013). This might have given the *Haloferax* ASV the advantage in the 4 M and 4.7 M NaCl enrichments compared to other halophilic salt spring microbes, which might not be able to cope so well with higher salinity. The Cheshire salt spring system is also connected to a Triassic salt deposit, therefore *Haloferax* species found in Kilroot salt mine in Northern Ireland could be related to the one that was dominant in the Cheshire samples (Megaw *et al.*, 2019; Thompson *et al.*, 2021). For example, *Haloferax chudinovii* was isolated from a Permian salt deposit in Russia, but was able to grow at salinities between 1.19 M and 4.6 M NaCl (Saralov, Baslerov and Kuznetsov, 2013). Although the *Haloferax* ASV was only 99.6% similar to *Haloferax mediterranei*, we should not exclude the possibility that it can act similarly when interacting with other microbes. *Haloferax mediterranei* was explored as a potential 'microbial weed': 'a species that is able to dominate communities in open (microbial) habitats' (Cray *et al.*, 2013; Oren and Hallsworth, 2014). It has traits that makes it able to outcompete other halophiles when coexisting with them: it has the fastest growth rate out of all species of haloarchaea; has a wide tolerance to salt (like many *Haloferax* species); is metabolically diverse (utilizes many carbons sources and inorganic and organic substrates); produces gas vesicles; and excretes halocins that kill other Archaea (Oren and Hallsworth, 2014). Because most other *Haloferax* species were not studied this extensively, we can only assume that the *Haloferax* ASV acted as a 'microbial weed', being more than 90% abundant at mid- and high salinities in both Cheshire enrichments after one year of subculturing, and being more than 99% abundant by the end of the experiment (>95% abundant in A2.2 mid-salt enrichment). The only other ASV which was able to coexist with the *Haloferax* ASV was in the mid-salt enrichment of A2.2 after one

(~10% abundant) and two years (~5% abundant) of subculturing. It was 100% identical to the same *Halorhabdus* that dominated the LH high-salt enrichments in 99% abundance. Although this ASV was able to coexist with the *Haloferax* ASV, based on its abundance, it would probably not have been able to survive for longer term. This either suggests that the same taxa behave differently when sampled from different environments, or that the 16S rRNA taxonomic marker gene is not sufficient and isolates would require whole genome sequencing to thoroughly assess the traits of dominant microbes.

Microorganisms exist in a balance that is highly subjected to seasonal changes when assessing a salt spring. It is likely that many of the halotolerants and halophiles found in the enrichment are somehow also playing an active part of the usual, slightly halotolerant microbial community in the original environment, even if they exist in low abundance. This is especially true for samples from the Cheshire salt springs, where the community composition and salinity varies based on season, temperature, light, and precipitation. Opposed to Boulby Mine samples, where Archaea dominated all enrichments, the low salinity enrichments were mainly occupied by Bacteria in the Cheshire samples. This could be due to the generally lower salinities in the Cheshire 'A2' salt spring at the time of sampling (around seawater salinity, ~0.4 M NaCl). Generally, the optimum salinity for many halotolerant Bacteria is lower (0.5 – 2.05 M NaCl) than that of haloarchaea (2.1 – 4.6 M NaCl), allowing them to outcompete extreme haloarchaea at low salinities (Spring *et al.*, 1996; Wainø, Tindall and Ingvorsen, 2000; Gruber *et al.*, 2004; Yoon *et al.*, 2005; Lee *et al.*, 2005; Antunes *et al.*, 2008; Amoozegar *et al.*, 2012; Saralov, Baslerov and Kuznetsov, 2013; Song *et al.*, 2014; Albuquerque *et al.*, 2016; Jaakkola *et al.*, 2016; Sorokin *et al.*, 2021). As Boulby Mine is a relatively secluded environment and there is little variability in physicochemical conditions (no seasons, no temperature changes, constant salinity, no light), we cannot expect a large variation in microbial communities, especially not Bacteria (Payler *et al.*, 2019). The high initial abundance of Bacteria in the Cheshire salt springs could be explained by temperature:

it highly varies, but the air was not warmer than ~15°C at the sampling time, which does not facilitate the growth of haloarchaea but Bacteria. As Cray and colleagues described it (2013), it is not stationary-phase or closed communities (like Boulby Mine) which select for ‘microbial weeds’, but open habitats that facilitate the emergence ‘weed phenotypes’ such as *Haloferax* in the Cheshire samples. *Halorhabdus* in the Boulby samples and *Halomonas* in the Cheshire samples could not be considered as microbial weeds as they do not have such traits that allow them to entirely outcompete other microbes.

Based on microbial community composition, the qualities of the two different environments, we can conclude that the environmental conditions at Boulby salt mine selects for extreme halophiles, especially *Halorhabdus* sp., while the Cheshire salt brines are selective for *Halomonas* sp. and *Haloferax* sp., when continuously subcultured in a general halophile medium over two years. This confirmed the hypothesis that the microbial community composition shifted towards low and high salinities in the Cheshire communities compared to the Boulby mine cultures where one genus was dominant across all enrichments. For future experiments, it would be beneficial to test microbial growth in many different enrichment media with long-term subculturing across more saline environments to observe any differences in community composition and re-assess how ubiquitous and adaptable ancient extreme halophilic microbial life is.

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4.6 Supplementary material

Table S4.1 Analysis of Variance (ANOVA) showing factors that affect ASV richness. Left: 16S rRNA ASVs amplified by the 'Prokaryotic' primers, Right: 16S rRNA ASVs amplified by the 'Universal' primers. DF: Degrees of Freedom. Only Year (time) had a significant effect on ASV richness. The interactions of Sample and Salinity was excluded from the post-hoc test as it was only significant for one primer set.

	Prokaryotic					Universal				
Effect	DF	Sums of Squares	Mean Squares	F value	*p* value	DF	Sums of Squares	Mean Squares	F value	*p* value
Sample	2	3.22	1.61	0.29	0.755	2	14.89	7.44	0.83	0.468
Salinity	1	8.46	8.46	1.53	0.251	1	51.05	51.05	5.67	0.041
Year	2	359.78	179.89	32.55	<0.001	2	748.22	374.11	41.57	<0.001
Sample x Salinity	2	74.56	37.28	6.75	<0.05	2	50.98	25.49	2.83	0.111
Sample x Year	4	22.68	5.67	1.03	0.450	4	22.22	5.56	0.62	0.661
Salinity x Year	2	20.80	10.40	1.88	0.214	2	2.53	1.27	0.14	0.871
Sample x Salinity x Year	4	40.92	10.23	1.85	0.213	4	65.10	16.28	1.81	0.211
Residuals	8	44.22	5.53	-	-	9	81.00	9.00	-	-

Table S4.2 Tukey HSD post-hoc test comparing ASV richness across years. ASV richness significantly decreased after one year of subculturing, but it did not decrease significantly further between the first and second year of enrichment.

		Prokaryotic			Universal		
Factor	Interaction	Difference between means	95% Confidence Intervals	*p* value	Difference between means	95% Confidence Intervals	*p* value
Year	2020 x 2019	-6.44	[-9.97],[-2.92]	<0.01	-11.11	[-15.6],[-7.16]	<0.001
Year	2021 x 2019	-8.74	[-12.37,[-5.11]	<0.001	-11.22	[-15.17],[-7.27]	<0.001
Year	2021 x 2020	-2.29	[-5.92],[1.34]	0.228	-0.11	[-4.06],[3.84]	0.997

Table S4.3 Permutational Analysis of Variance (PERMANOVA) showing how community composition was affected by sample type, salinity, and time. All factors had a significant main effect on community composition, and there was a significant interaction between Sample and Salinity, and between Sample and Year.

	Prokaryotic					Universal				
	DF	Sums of Squares	R^2	F value	*p* value	DF	Sums of Squares	R^2	F value	*p* value
Sample	2	2.32	0.23	6.19	<0.01	2	3.02	0.30	10.06	<0.01
Salinity	1	1.84	0.18	9.84	<0.01	1	1.67	0.17	11.13	<0.01
Year	1	0.51	0.05	2.71	<0.05	1	0.60	0.06	4.01	<0.01
Sample x Salinity	2	1.42	0.14	3.79	<0.01	2	1.03	0.10	3.44	<0.01

Sample x Year	2	0.83	0.08	2.20	<0.05	2	0.93	0.09	3.10	<0.01
Residual	17	3.18	0.32	-	-	18	2.70	0.27	-	-
Total	25	10.10	1.00	-	-	26	9.95	1.00	-	-

Chapter 5: General Discussion

5.1 Extremotolerants in non-extreme environments

Understanding the interplay between connectivity and dispersal and the environmental filtering of microbial communities remains an important challenge in the field of microbial biogeography. Extremophilic and extremotolerant microbes are good models to better understand microbial biogeography, as although they are mostly found in extreme habitats, there is increasing evidence that they can be as ubiquitously distributed as any other microbe. The second chapter of this thesis demonstrated that it is possible to enrich microbial communities from non-extreme aquatic environments that can grow in different extreme conditions. Stressor-enriched communities mainly consisted of extremotolerant and/or extremophilic genera, where the type of stressor determined the bacterial community composition, while the same fungal genera were abundant across most enrichments. To the best of my knowledge, this thesis, combined with the findings of Low-Decarie and colleagues (2016), is the first to report microbial communities from riverine and marine environments actively growing both in saline, chaotropic, hyperosmotic, acidic, and heavy-metal-rich conditions, along with no-stressor control conditions.

Freshwater- and marine-derived microbes enriched in extreme conditions had a significantly wider tolerance to stressors than microbes from the same samples enriched in control conditions with no stressor. However, the growth rate of nearly all stressor-enriched microbial communities was lower than the growth rate of the control-enriched communities at the lower concentration end. This suggests that being able to grow both in extreme and in non-extreme conditions comes with a trade-off for these extremotolerant or moderate extremophilic microbes. The so-called “true” extremophiles are specialists; they can only grow in extreme conditions and cannot compete against extremotolerants or mesophilic microbes in less extreme conditions. However, extremotolerants having to compete against mesophiles in

non-extreme conditions and against true extremophiles in extreme conditions means that their trade-off results in a slower growth and a lower growth rate compared to mesophiles and extremophiles. This is especially true for polyextremotolerants that have the ability to grow in different extreme conditions. Polyextremotolerant bacterial representatives from the findings of Chapter 2 include *Staphylococcus, Arenimonas, Acetobacter, Arcobacter*, and *Salinicola*, which were found in as least two different extreme conditions. As Gostinčar (2022) highlighted in his recent findings, fungal polyextremotolerants are of very common occurrence in extreme conditions, which was confirmed in this chapter too. The same OTUs of *Penicillium, Candida*, and *Cladosporium* were found in almost all stressor enrichments. A limitation of this study was that there is no information on the bacteria:fungi ratio in each enrichment; only visible fungal growth (mycelia and spores in the $MgCl_2$- and in the sorbitol enrichments) was an indicator of potentially more fungi in these enrichments compared to bacteria. Regardless the species and its mechanisms of adaptation, maintaining growth in one or more extreme conditions is still heavily energy demanding, which often results in slow growth (Gostinčar et al., 2022). In the case of fungi, energy-requiring processes can be synthesizing or accumulating osmolytes, or constantly operating membrane transporters to exclude toxic ions of inorganic salts from the cytoplasm (Gostinčar et al., 2022). Certain bacteria and fungi were shown to cope better with sudden exposure to stress compared to their fast-growing counterparts (Gostinčar et al., 2022). This behaviour could be related to bet-hedging: adaptation to unpredictability (Hellweger et al., 2014; von Hegner, 2022). This was established in plants and insects, and it is a risk-spreading strategy that creates phenotypic plasticity within a population to adapt to unknown future changes by producing dormant/not active non-optimal phenotypes along with actively growing optimal ones (Bradford and Roff, 1993). Some of the extremotolerants emerging from non-extreme environments in this study could possibly be part of the bet-hedging strategy of microbial populations or simply be part of the microbial seed bank, waiting for stressed conditions. Another important factor is the cohabitation with other elements such as higher eukaryotes

and even viruses. Viruses were found to deliver extremotolerance genes to their hosts in a hyperarid desert, contributing to the shaping of microbial evolution, which could possibly occur in other extreme environments too (Yunha et al., 2022). Future studies should include sequencing and analysing other communities as well, including cyanobacteria, plants, viruses, and potentially other eukaryotes, and identifying common extremotolerance genes that could have been shared among taxa. Holland and colleagues (2014) showed that phenotypic heterogeneity in „wild yeast" populations provides a survival advantage in heavy-metal polluted environments. They showed that *Candida sake* was the most abundant yeast near a lead-mine outflow, which could be relevant to this study where OTU2 (99.3% similar to C. sake) was in the top 3 most abundant fungi in the copper-, HCl- and zinc enrichments (Holland et al., 2014). Another study found that acidotolerant fungi isolated from an acidic and heavy-metal-rich environment are not equally adapted to the metal levels characteristic to that environment with regards to their metal-tolerance level, growth patterns, and time needed to germinate, while pH was found not to be a limiting factor for growth (Álvarez-Pérez et al., 2011). It is important to mention that these cultures consisted of a mixture of microorganisms. Therefore, we cannot deduce how each individual taxon grew across the stressor gradient. This also suggests that there could have been a positive or negative interaction between taxa (competition/antibiosis, mutualism, etc.), allowing one to grow better than the other (Moënne-Loccoz et al., 2014). These findings raise the question about the role that extremotolerants could play in ecosystem functioning.

As extremotolerants are present in non-extreme environments, they have the potential to be used in many biotechnological applications, which require microbial functioning in harsh conditions, while they can be isolated from almost any environments compared to extremophiles. Polyextremotolerants are especially useful in biotechnology, tolerating two or more extreme conditions (Harrison et al., 2013). For instance, four alkali-thermo-halotolerant *Bacillus* strains from desert environments were found to be able to produce cellulose that can

be converted into biofuel in a more sustainable way (Souii et al., 2020). Although most of the type strains of the four species (*B. mojavensis, B. vallismortis, B. sonorensis, and B. safiensis*) were isolated from salt-saturated brines or soils with a pH of ~5.6, the cellulose-producing strains do not require exceptionally high salinities for growth like extreme halophiles and are able to grow in pH 7 - 10 (Roberts et al., 1994, 1996; Palmisano et al., 2001; Satomi et al., 2006; Souii et al., 2020). The polyextremotolerant bacterial and fungal taxa found in this study highlights the potential for biotechnological applications based on previous findings (Gostinčar and Turk, 2012; Raddadi et al., 2015).

5.2 Experimental evolution of halophilic and halotolerant microorganisms at different salinities

Experimental evolution studies are a popular in vitro tool to quantify phenotypic and genotypic changes such as adaptation and acclimation in real time. However, to date, only a handful of studies have conducted experimental evolution in halophiles and halotolerants, examining the high salt adaptation of the black fungus *Hortaea werneckii* and the acid tolerance of *Halobacterium salinarum* strain NRC-1 (Kunka et al., 2020; Gostinčar et al., 2021). The third chapter of this thesis demonstrated that the haloarchaeon *Halobacterium salinarum* strain 91-R6 is able to develop a preference to high salinities (4 – 4.7 M NaCl) when grown in 4.7 M NaCl for an extended period and evolve not to be able to grow at 3 M NaCl anymore. This was the case in another haloarchaeon, *Natrinema pallidum*, which was able to grow in 0.7 M NaCl originally but being kept at high salinities, its minimum salinity requirement changed to 1.8 M NaCl (McGenity et al., 1998). Moreover, subculturing *Halobacterium salinarum* strain 91-R6 at 3 M NaCl for 16 months impaired its general growth capacity at all salinities. On the other hand, *Halobacterium salinarum* strain NRC-1 did not show any changes in its growth patterns, highlighting the difference between the two strains (Pfeiffer et al., 2020). While *Hbt.*

salinarum strain R1 and NRC-1 are simply laboratory variants derived from the same isolate, *Hbt. salinarum* strain 91-R6 is an entirely independent isolate. While their growth patterns and genome composition is very similar, they have subtle differences in their chromosomes and plasmids. For example, the arginine fermentation cluster (arcDBCAR) is encoded on plasmids of all three strains, but strain 91-R6 is missing a 31 kb section which encodes a gas vesicle cluster and the kdpFABCQ cluster coding a potassium uptake system (Pfeiffer et al., 2020). It is possible that the mutations occurred in the arginine fermentation cluster observed in the acid-evolved *Hbt. salinarum* strain NRC-1 study conducted by Kunka and colleagues are due to these genomic differences between *Hbt. salinarum* strain NRC- and 91-R6. However, their acid evolution experiment resulted in 500 generations, while the research in this chapter only reached at a maximum of 60 generations. It is likely that even *Hbt. salinarum* strain NRC-1 would have evolved to be adapted to higher salinities than its original optimum with the increase in generations. On the other hand, the arginine fermentation cluster located on a plasmid could be more susceptible to changes in pH rather than salinity. The halotolerant bacterium *Halomonas elongata* strain 1H9 served as a comparison, where subculturing at low salinity (0.4 M NaCl) resulted in its imparied ability to grow at higher salinities and not to grow at all at 4.7 M NaCl. Some of the strains that evolved to have different growth capacities also showed changes in cell morphology and suffered mutations in their genomes. To the best of my knowledge, this thesis is the first to report physiological and genetic changes in two strains of *Halobacterium salinarum* in direct comparison with *Halomonas elongata* strain 1H9 after continuously subculturing them at their salinity limits for 16 months in an experimental evolution setting.

Halomonas elongata strain 1H9 cultures accumulated approximately an average three times more mutations in their genome in total compared to *Halobacterium salinarum* stain 91-R6 cultures at different salinities compared to the original, non-evolved cultures. This is noteworthy because while most bacteria, like *H. elongata* strain 1H9, have an average

mutation rate of 10^{-7} to 10^{-10} base substitutions per nucleotide per generation (Westra et al., 2017), haloarchaea, and some thermophiles and acidophiles were found to have an average genomic mutation rate of 10^{-3} to 10^{-4} spontaneous mutations per genome per replication (Busch and DiRuggiero, 2010). We also measured an average almost six times faster growth rate for *H. elongata* than *Hbt. salinarum* strains at their optimum salinities. In addition, *H. elongata* strain 1H9 could have less DNA mismatch repair mechanisms compared to *Hbt. salinarum* strains that are polyploid with approximately 20 copies of its chromosome per cell (Busch and DiRuggiero, 2010; Schwibbert et al., 2011; Zerulla and Soppa, 2014). This means that *H. elongata* strain 1H9 cultures had a higher probability of accumulating mutations throughout 20 months, especially with a three to four day subculturing period compared to *Hbt. salinarum* strains which were subcultured once a week.

Future experiments could be improved by adding transcriptomics and/or proteomics analysis to the study. Several previous studies confirmed the potential of *Hbt. salinarum* to be easily manipulated genetically in the laboratory, using both knockout-, microarray-, or protein overexpression systems (Ng et al., 2000; Wang et al., 2004; Jaakola et al., 2005; Dassarma et al., 2006; Twellmeyer et al., 2007; Schwaiger et al., 2010; Kahaki et al., 2014). As the exploration of the archaellum systems of *Hbt. salinarum* were successfully employed (Tarasov et al., 2000; Beznosov et al., 2007), and this study was essentially focusing on long-term osmoadaptation, a knockout system could be designed for genes that have a more direct role in osmoadaptation. Targets could include ionic transport and compatible solute transport and biosynthesis genes (found in Hbt. salinarum NRC-1 and R1): K^+/H^+ symporter and ABC transporter (K^+ in), Ca^{2+}/Na^+ exchanger and Na^+/H^+ antiporter (Na^+ out), glycine betaine transporter, choline uptake and glycine betaine synthesis, and proline uptake (compatible solute transport and biosynthesis), Cl^- transporter, PO_4^{2-} transporter, and compatible solute chemotaxis genes (Becker et al., 2014). Once the target genes are selected and deletion mutants are created, another long-term exposure to different salinities

would give more information on the extent to what each mutant strain is able to adapt to hyper- and hyposaline conditions compared to the wild type strain, and which genes have the most crucial role in reacting to osmotic stress long term. The osmoadaptation mechanisms of *H. elongata* to different salinities has been extensively studied, including well-working gene knockout systems (Schwibbert et al., 2011; Kindzierski et al., 2017; Hobmeier et al., 2020, 2022; Vandrich et al., 2020). Besides its well-studied ectoine metabolism, Hobmeier and colleagues proposed a new idea of a mixed strategy for osmoadaptation as a result of transcriptome profiling of *H. elongata* at different salinities (Hobmeier et al., 2022). It was suggested that the high cytoplasmic sodium levels could be explained by the accumulation of potassium and sodium into the cell, which provides a basic resistance to (and dependence from) salt, while ectoine accumulation is only triggered at especially high salinities (Hobmeier et al., 2022). Based on the extensive research on *H. elongata* and its prominent differences from *Hbt. salinarum*, future experimental evolution studies should probably focus on comparing *Hbt. salinarum* strain NRC-1, R1 and 91-R6, with carefully designed deletion mutants and potentially protein overexpression.

Fendrihan and colleagues (2012) observed *Halobacterium salinarum* strains among other haloarchaea to form 3 – 4 small spherical cells (0.4 µm) from one rod-shaped cell when exposed to low water activity (4 M NaCl or 4 M LiCl buffer, plus 10 mM Tris-HCl, pH 7.4), which then were able to regrow to normal rods in nutrient medium. The light microscopy images of *Hbt. salinarum* strains NRC-1 and 91-R6 produced in this thesis did show a few small spherical cells at 4 M and at 4.7 M NaCl medium, but around ~95% of cells were rods, which could be explained by using nutrient medium instead of buffer. Regardless, future experiments should include the use of electron microscopy to observe how cell shape changes over time at different salinities (including the potential loss of archaellum ArlB) and whether results correlate with previous findings about spherical cells at high salinity and about the defective archaellum (Tarasov et al., 2000; Fendrihan et al., 2012). However, the

significantly bigger spherical cells clearly visible in the high-salinity-evolved *Hbt. salinarum* 91-R6 culture subsequently grown in 2.7 M NaCl medium verify the previous research, where cells experienced hyposaline stress, but were also able to regrow to normal rods in optimal salinity medium (Abram and Gibbons, 1960; Mohr and Larsen, 1963; Stoeckenius and Rowen, 1967; Vauclare et al., 2020). The cultures of *H. elongata* strain 1H9 already started to adjust to their relevant salinity conditions only after four months, which resulted in even more distinct growth patterns between the low-, medium-, and high salinity-evolved culture after 20 months of subculturing. Once again, *H. elongata* strain 1H9 proved its fast adaptability to changing salinity conditions. The polyploidy and efficient DNA mismatch repair mechanisms of haloarchaea could help to better understand long-term survival and may facilitate fast adaptations to environmental stress.

Both *H. elongata* strain 1H9 and *Hbt. salinarum* strain 91-R6 accumulated mutations in their genomes when kept at their optimum salinities. Clearly, salinity selection pressure was not the determinant factor of the evolution of these strains. Mutations in *Hbt. salinarum* strain 91-R6 could be explained by the high variability of the genome of *Halobacterium salinarum* strains in general, by accumulating much more insertion sequence mutations (ISHs) than single nucleotide polymorphism (SNPs) (Kunka et al., 2020). ISHs are responsible for most of the genetic variability of *Hbt. salinarum* and other archaea, and it was found that large deletions and loss of function mutations might serve as a trade-off for adapting to environmental stress in *Sulfolobus* (Brügger et al., 2002; Pfeiffer et al., 2008; Kunka et al., 2020). The relatively small number of mutations in *Hbt. salinarum* strain 91-R6 at optimal conditions are consistent with the findings of Kunka and colleagues (2020) where *Hbt. salinarum* strain NRC-1 was exposed to acid stress in an experimental evolution setting. This study showed that experimental evolution is a useful tool to identify important environmental stress-related genes in halophiles that are not necessarily related to osmoadaptation. Many previous studies showed that certain laboratory working cultures have a tendency to develop

genotypic variation over repeated subculturing (Iguchi et al., 2002; Kim et al., 2002; Cross et al., 2011; Safavi, 2011). For pathogens, this most frequently appears as loss of virulence efficiency (Iguchi et al., 2002; Kim et al., 2002; Safavi, 2011). Cross and colleagues showed that many laboratories are using genetically different control strains of *Listeria monocytogenes* and *Staphylococcus aureus* from the reference strains (Cross et al., 2011). Nevertheless, in the case of the slow-growing haloarchaea, growing a fresh stock from a freeze-dried culture can sometimes take weeks or months, therefore, it could be more effective to keep a fresh culture on an agar plate, in liquid medium, or even enclosed in a salt crystal as a method of preserving the original genome.

5.3 Experimental evolution of halophilic and halotolerant microbial communities from different saline environments

The 16S rRNA genes were 100% identical in the two most abundant high-salinity enrichments (*Haloferax* and *Halorhabdus* ASVs) from the two separate environments. However, the *Haloferax* amplicon sequence variants (ASVs) were only abundant in the high salinitiy enrichments of Boulby mine while the *Halorhabdus* ASVs were only abundant in the high salinity enrichments of the Cheshire salt springs. This verifies that the two ASVs from the two environments are distinct from each other as the same taxa would have had to become abundant in both enrichments, which was not the case. Although haloarchaea are well known to possess many heterogenous copies of their 16S rRNA gene, the closest matching species (100% identical 16S rRNA genes) to the *Halorhabdus* and *Haloferax* ASVs both only have a single copy of the 16S rRNA gene, therefore only genome sequencing of the isolates would be sufficient enough to compare these ASVs (Tomlinson et al., 1986; Elshahed et al., 2004; Saralov et al., 2013; Albuquerque et al., 2016; Khelaifia and Raoult, 2016; Louca et al., 2018). The microbial communities are highly subjected to seasonal changes (temperature, light,

precipitation, salinity, etc.) in the Cheshire salt springs. Tucker and colleagues (2014) found that environmental variability highly affects microbial community composition in a nectar community, while it also somewhat counteracts priority effects (the strength of the order and timing of species arrival during community assembly) (Tucker and Fukami, 2014; Debray et al., 2021). This allows better species coexistence compared to constant environmental conditions (Tucker and Fukami, 2014). The results of Chapter 4 verify this, with only a few species left in any culture that was subjected to constant salinity for 16 months, even though the original community consisted of between 399 to 1475 ASVs (depending on brine/soil sample and prokaryotic/universal primer sets). It is important to mention that aerobic heterotrophic halophiles have versatile metabolism, being able to utilize many substrates, which means microorganisms can interact with each other in a community or with any other living creature or inanimate object in the habitat (Oren, 2015). Microbial communities could have beneficial relationships which could help them recover after a perturbation event compared to the response of a single strain after stress (Low-Decarie et al., 2015). There was a shift, then recovery in a halophilic microbial community in the Atacama desert after heavy rainfall (Uritskiy et al., 2019). Microbial communities specialized to an extreme environment are more vulnerable to environmental changes than communites living in a less extreme habitat like the Cheshire salt springs with lower salinity. General seasonal changes would likely not affect these communities as much as subjecting them to harsh salinities, which resulted in the emergence of only the most resilient haloarchaea, *Haloferax* sp. Future studies could establish a microbial community composition profile in different seasons through years to acquire more information on community shifts, including the determinant environmental factors.

The most abundant *Haloferax* ASV outcompeted every other taxa, being 99% abundant at almost all mid- and high salinites (4 and 4.7 M NaCl) from both Cheshire samples. The reasonably low salinity (~0.4 M NaCl) and air temperature (average high 20°C and low 13°C

in summer) at the salt springs in Northwich favours halotolerant bacteria rather than haloarchaea. This explains how were *Haloferax* species in such low abundance in the original samples (0.12% in the brine and 0.03% in the sediment sample), simply being part of the microbial seed bank. Cray and colleagues (2013) established the concept of microbial weed species which can dominate a whole ecosystem if conditions are favorable or at all times, depending whether the dominating taxa are specialists (e.g. *Halomonas* sp. in the NaCl-enriched communities in Chapter 2, Table 2.1) or generalists (e.g. *Penicillium* sp. in all enrichment in Chapter 2, Table 2.2). Extreme halophilic species were identified as potential weed species, including *Dunaliella salina, Salinibacter ruber, Haloquadratum walsbyi*, and *Haloferax mediterranei* (Cray et al., 2013; Oren and Hallsworth, 2014). The latter species can grow faster than any other haloarchaea, has a wide salt tolerance, a wide range of substrates to metabolize, can grow anaerobically, and can produce halocins that kill other archaea. Although the *Haloferax* ASV in this chapter is only 99.6% similar to *Hfx. mediterranei* based on the 16S rRNA gene, it could potentially be identified as a microbial weed, outcompeting every other taxa when conditions became severe. This further supports the findings that variable environmental conditions can counteract priority effects (Tucker and Fukami, 2014).

Both the *Halopenitus* ASVs in the low salinity enrichment and *Halobacterium* ASVs in the high salinity enrichment from Boulby mine were overtaken by the *Halorhabdus* ASVs by year 1. The 16S rRNA genes of the *Halobacterium* ASVs were 100% identical to *Hbt. noricense* and *Hbt. hubeiense*, which coincides with a previous isolate „LH Bop", with its genome sequence being 100% identical to *Hbt. hubeiense*. As these microbes were originally isolated from ancient salt deposits (Gruber et al., 2004; Jaakkola et al., 2016), and their genomes are surprisingly similar to modern haloarchaeal genomes, this could suggest that these species are very similar to the sulfate-reducing *Candidatus* Desulforudis audaxviator (CDA), the bacterium that dominates the whole deep-subsurface ecosystem across most continents (Chivian et al., 2008; Becraft et al., 2021). CDA possessess all the genes required to maintain

a whole ecosystem single-handedly, including several genes inherited from archaea by horizontal gene transfer that may help with living in extreme environments (Chivian et al., 2008). It has been suggested by Jaakkola and colleagues (Jaakkola et al., 2016) that ancient subsurface haloarchaea could easily exchange large genomic material between modern surface haloarchaea by the same frequent lateral gene transfer when the deposited salt becomes exposed due to natural processess and human action. Near identical taxonomic marker genes of *Hbt. noricense* and *Hbt. hubeiense* strains have also been found all over the world in different salt deposits of close geological origin which suggests that these halophiles could be part of a remnant microbial community originally inhabiting paleozoic brines (Gruber et al., 2004; Jaakkola et al., 2016). We could assume that deep subsurface salt mines are similarly isolated ancient environments as deep subsurface fractures, where life must be maintained independently of the photosphere at high temperature and pressure, using chemical or other energy for metabolism and potential slow cell division. Based on the recent discovery of the anaerobic lithoheterotrophic sulfur-respiring haloarchaea in hypersaline inland lakes, solar salterns, lagoons, and deep submarine anoxic brines (Sorokin et al., 2017), science is not far away from discovering the next unusual group of anaerobic haloarchaea in the deep subsurface.

5.4 Conclusion

The presented research demonstrated that although microbes are ubiquitous on Earth, the environment, along with priority effects, defines their distribution and community composition in this experimental evolution study, and that isolated environments are ideal model system to study priority effects and the change in community composition in response to stress. Furthermore, this research also shows that experimental evolution is an effective approach to pinpoint genes for salinity stress response in halophiles. This provides valuable insights in our understanding of microbial evolution and biogeography.

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