The effect of chaotropic magnesium chloride on the growth of microbes

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Abstract

Chaotropic agents denature biological macromolecules whereas kosmotropes stabilise macromolecular structures. Magnesium chloride ($MgCl_2$) is one of the most widespread chaotropic solutes that is not expected to support growth above 2.3 M, but few studies have focused on the effects of $MgCl_2$ on microbial growth. This study investigated the effects of $MgCl_2$ in comparison to kosmotropic sodium chloride (NaCl), on microbial growth and community composition, with the focus on $MgCl_2$. Solid (1.5% agar) and liquid media were supplemented with 1% yeast extract and different concentrations of $MgCl_2$ and NaCl, using samples from a salt marsh and agricultural soil (Colchester, UK). Viable counts decreased for both solutes as concentrations increased but $MgCl_2$ had no viable counts at a concentration of 1.5 M and above. PCR amplification showed that salt marsh fungi dominated in $MgCl_2$ enrichments and DGGE analysis of enrichments revealed high community diversity for Bacteria and Archaea but low community diversity for fungi. Sequencing of selected DGGE bands showed the presence of an *Acremonium*-related species in $MgCl_2$ at 1.5 M and *Baeospora myosura* at 1.75 M. Several isolated fungal strains tested in $MgCl_2$ concentrations up to 2.2 M proved to be chaotolerant. A strain from salt marsh potentially grew in 2.2 M $MgCl_2$ but further testing is needed to confirm this as the small sizes of suspected flocks render visual confirmation difficult. Identification and improved knowledge on chaophiles and chaotolerant microbes would have implications for astrobiology and in the search for extra-terrestrial life or the potential of life in chaotropic (or similar) extra-terrestrial environments.

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1 Introduction

1.1 Extremophiles

Earth, the only planet so far known to host life, is extremely diverse in terms of habitats, conditions and perhaps most importantly, in life-forms. Microorganisms especially are highly abundant and diverse with the number of prokaryotic cells estimated to be between 9.2 $x10^{29}$ and $31.7x10^{29}$ on the planet (Kallmeyer *et al*., 2012).

Within the last few decades novel organisms have been isolated from environments previously thought to be sterile or too harsh to support the growth of life forms. These organisms are known as extremophiles – organisms that grow optimally or only grow in extreme conditions. Hyperthermophiles are a type of extremophiles that optimally grow between 80 and 106°C, with some species such as *Pyrolobus fumarii* able to grow up to 113°C and even remarkably able to survive autoclaving at 121°C for an hour (Blöchl *et al*., 1997; see Stetter, 1992 as cited in Stetter, 2006; Stetter, 2006). Many other types of extremophiles have been identified with requirement for extremes of salinity, pH or pressure. Most environments on Earth are also not limited to one such stressor, and extremophiles growing there are able to require or withstand several stresses, and are often called polyextromophiles. For example, *Pyrolobus fumarii*, which in addition to requiring a high temperature, is tolerant to high pressure (Blöchl *et al*., 1997). Alcaide *et al.* (2015) provide evidence in linking pressure adaptation to high temperature adaptation in enzymes from microbes from Lake *Medee*, a deep-sea hypersaline lake under the Mediterranean Sea. Another example of polyextremophiles include the unique group of halophilic alkalithermophiles, which have optimal growth at Na^+ concentrations above 2 M (11.7% w/v), pH above 8.5 and temperatures above 50°C, such as the bacterium *Halonatronum saccharophilum* (see Mesbah and Wiegel, 2012).

Such organisms have generated interest due to their remarkable abilities to grow in extreme conditions and their potential biotechnological applications, such as the use of enzymes

(Elleuche *et al*., 2015), for which the most famous application is Taq Polymerase (from the thermophile *Thermus aquaticus*), which makes possible the invaluable Polymerase Chain Reaction (Brock and Freeze, 1969; Chien *et al*., 1976). Another example of a biotechnological application is the widespread use of *β*-carotene, produced by halophile algae *Dunaliella salina*. *β*-carotene is used in food colouring, cosmetics and health food (reviewed in Oren, 2005).

1.2 Chaotropicity

Despite this popular interest in extremophiles, not all types of extremophiles have been extensively studied or well understood. This even applies to some stressors, such as those that are chaotropic.

Hamaguchi and Geiduschek (1962) made a significant contribution in defining the denaturing effect of chaotropes on nucleic acids. Prior to this, chaotropes were defined on the basis of their capacity to disorder water structure (see Gurney, 1953 as cited in Ball and Hallsworth, 2015). Hamaguchi and Geiduschek (1962) also ranked chaotropic ions based on their abilities to destabilise macromolecular structures. However, as Ball and Hallsworth (2015) note, Hamaguchi and Geiduschek themselves were not clear on the details of how chaotropic mechanisms altered the structure of water. Cray *et al*. (2013a), similarly, quantified and ranked 97 solutes in an order from most chaotropic to most kosmotropic, which stabilises macromolecular structures, using agar, a polysaccharide, as a model macromolecule (examples are shown in Table 1). Specifically, they recorded the concentration of the solute at which the agar gel-point temperature increased or decreased by 1°C. Activity values were expressed in kJ kg^{-1} per mole added compound, as they were calculated using known heat capacity values of 1.5% w/v agar gel and water. The activity values allow clear comparison between solutes and can be applied to environments to determine its net chaotropicity or kosmotropicity value.

Table 1. Activity values determined by Cray *et al.* (2013a) of various chao- or kosmotropic solutes.

Solute	**Chao- or kosmotrope**	**Activity value (kJ kg^{-1} $mole^{-1}$)**
Phenol	Chaotrope	+143
$CaCl_2$	Chaotrope	+92.2
$MgCl_2$	Chaotrope	**+54.0**
Glycerol (6.5–10 M)	Chaotrope	+6.34
Mannitol	Kosmotrope	-6.69
NaCl	Kosmotrope	**-11**
Polyethylene glycol- (PEG-)1000	Kosmotrope	-126

Chaotropicity is also related to the Hofmeister series, which ranks numerous ions' abilities to salt-in or salt-out proteins (Hofmeister, 1888), due to chaotropes exerting salting-in effects. Furthermore, the ranking of the effects of chaotropic anions have been shown to follow the Hofmeister series (Sawyer and Puckridge, 1973; Lo Nostro *et al.*, 2005).

A diverse range of chaotropic ions exist and the particular mechanisms by which they achieve chaotropic effects vary and are complex. Thus, chaotropicity is not well understood (Hua *et al*., 2008; Bhaganna *et al*., 2010) and various mechanisms have been suggested. Washabaugh and Collins (1986) defined chaotropes as "water structure breakers" that have destabilising effects on macromolecular structures and cause a salting-in effect in macromolecules from having weaker interactions with adjacent water molecules compared to bulk water. Collins (1997) later concluded that macromolecular destabilisation occurred at high chaotropic concentrations and argued that chaotropicity should not be assumed to exert the same effects as kosmotropes, "water structure makers" which stabilised macromolecules at high concentrations and caused proteins to salt-out due to kosmotropes having stronger interactions with adjacent water molecules compared to bulk water.

An alternate theory suggests that chaotropes interacted directly with the macromolecules to exert their effects, and not by rearranging the structure of the bulk solvent (Ball and Hallsworth, 2015; Omta *et al*., 2003). Some chaotropes, such as ethanol, have also been found to cause oxidative stress, the cause theorised as released free radicals from disrupted electron transport processes (Russo *et al*., 2001; Albano, 2006; Domínguez-Cuevas *et al*., 2006; Bhaganna *et al*., 2016). Ethanol also readily enters the cell, whereas $MgCl_2$ may not and so cause osmotic stress (de Lima Alves *et al*., 2015; Cray *et al*., 2015).

Whilst numerous studies, such as those listed above, have focused on the interactions of chaotropic ions or alcohols in aqueous solutions and on macromolecules (Cray *et al*., 2015), only a handful of studies have looked at the chaotropic effects on whole cells of living organisms (Hallsworth *et al*., 2003; Duda *et al*., 2004; Lo Nostro *et al*., 2005; Salvi *et al*., 2005; Bhaganna *et al*., 2016). These studies have shown how cellular components, such as membranes and ribosomes, are degraded, and cell growth decreases, as chaotropic concentrations increase.

1.2.1 Limits of life defined by a_w and salinity

Chaotropicity is not as simple to define as a parameter in comparison to salinity and water activity, for example, due to a wide range of chaotropes and their particular effects as discussed earlier.

Water activity is expressed as the mole fraction of water, and is the amount of available water to a cell, impacting the functionality and stability of enzymes, membranes and cells. It is expressed as a_w (Hallsworth *et al*., 2003; Hallsworth *et al*., 2007). To put into context, pure water has the highest water activity value at 1 a_w and the lowest water activity that life has been found to function at is 0.585 a_w from the polyextremophile *Aspergillus penicillioides* (Stevenson *et al*., 2017). Before this study, Stevenson *et al*. (2015b) argued that a common water activity limit exists between the three domains of life at 0.61 a_w. Organisms that can grow down to these levels are of special interest to study as most microbes are only active within ranges between 1

- 0.900 a_w, and therefore may have use in research focusing on the potential of life in environments with low water activity (Stevenson *et al.*, 2015a; Stevenson *et al.*, 2015b).

Salinity is another stress parameter, where organisms that live in high salinity and low water activity (halophiles) are able to do so through adaptations such as producing compatible solutes (moderate halophiles) or importing salt into the cell, for osmotic balance (extreme halophiles). Cells of organisms unable to achieve osmotic balance in high salinity will have water leaving the cells, causing macromolecules to lose structure and function (McGenity and Oren, 2012). The limits of life defined by water activity, salinity in terms of moderately kosmotropic NaCl and chaotropic $MgCl_2$ are shown in Figure 1.

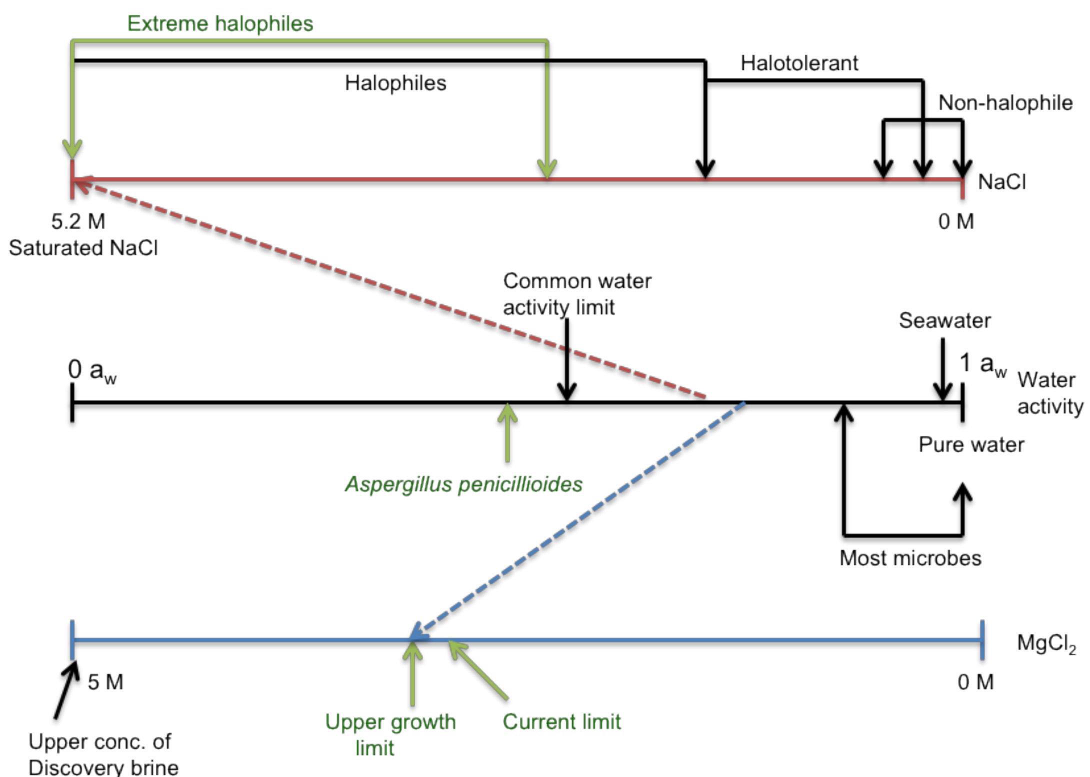


Figure 1. Life limits set by water activity, sodium chloride and magnesium chloride. Optimal NaCl concentrations for growth are shown. Data compiled from Hallsworth (1998); McGenity and Oren (2012); Mesbah and Wiegel (2008); Yakimov *et al.* (2015); Stevenson *et al.* (2015a); Stevenson *et al.* (2015b); Stevenson *et al.* (2017).

1.2.2 Natural chaotropic environments

Since the study by Hallsworth *et al.* (2007), chaotropicity has become a recognised life-limiting stressor and thus can influence the abundance and diversity of life forms (Dartnell, 2011). Since there are many natural environments that are chaotropic, to various degrees, this has huge implications on which microbes can grow where. The original illustration of a natural chaotropic environment that limits life due to its high levels of chaotropicity is the Discovery Basin – a deep hypersaline anoxic lake 3.58 km underneath the surface of the Mediterranean Sea that is characterised by its gradient of $MgCl_2$ concentrations from 0.05 M in seawater to 5.05 M in the brine lake, which makes the Discovery basin unique in comparison to other deep hypersaline anoxic basins (Hallsworth *et al.*, 2007). The $MgCl_2$ originates from bischofite ($MgCl_2 \cdot 6H_2O$), which was formed millions of years ago during the Messinian salt crisis when the Mediterranean Sea evaporated. Cracks appeared in the sediment, caused by tectonic plate movements, leading to bischofite dissolution (MEDRIFF Consortium, 1995; Yakimov *et al.*, 2015).

The Discovery brine was previously thought to be sterile, however van der Wielen *et al.* (2005) in their study of four different DHABs, including Discovery (the other basins being rich in NaCl), showed evidence of active microbial communities in all DHABs, based on processes such as methanogenesis and sulphate reduction. They also showed that the bacterial community in the Discovery brine was different from the communities in the interface and in the overlying seawater. Furthermore, they also compared between the DHABs and found in Discovery higher rates of ectoenzymatic activity but lower rates of methane production and sulphate reduction.

Hallsworth *et al.* (2007) focused on the Discovery basin and based on the findings of van der Wielen *et al.* (2005) on methanogenesis and sulphate reduction, targeted two genes coding for enzymes involved in those processes for qPCR and reverse-transcription qPCR, and analysed for stable DNA, moderately stable 16S rRNA and unstable mRNA sequences. Whilst the 16S rRNA sequences of the genes were found along the chemoline and within the brine, the mRNA

sequences of the functional genes were only found up to two concentrations, the highest being the gene for methanogenesis at 2.3 M. These results suggest that organisms in concentrations above 2.3 M may not be alive (and stable cells and macromolecules are preserved) or not metabolically active, and contradict van der Wielen *et al.*'s findings.

Sass *et al.* (2008) also studied the same four basins as van der Wielen *et al.* (2005): L'Atalante, Bannock, Discovery and Urania, and found 90% of the 89 strains collectively isolated from sediments to be related to spore-forming *Bacillales*. Strains from the Discovery sediments were not able to grow at *in-situ* conditions and therefore Sass *et al.* (2008) suggested these spores could have travelled into the brines or have been further preserved in high salinities, as supported by Hallsworth *et al*. (2007). These studies have shown conflicting results but nevertheless demonstrate how the almost saturation of $MgCl_2$ in the Discovery basin limits growth. Whilst the chaotropic environment of the Discovery basin was explored in detail here, other chaotropic environments of various properties should also be recognised, as shown in Table 2.

Table 2. Characteristics of various chaotropic environments.

Name	Location or examples	Form of chaotrope[a]	Top 3 ions (M)[b]	Natural	Temporary or permanent
Discovery basin[a]	Mediterranean Ridge	Salts	Cl^- 10.15 Mg^{2+} 5.15 SO_4^{2-} 0.11	Yes	Permanent
Lake Kryos[a]	Mediterranean Ridge	Salts	Cl^- 9.04 Mg^{2+} 4.38 SO_4^{2-} 0.32	Yes	Permanent
Don Juan Pond[c]	Antarctica	Salts	Cl^- 12.19 Ca^{2+} 5.83 Na^+ 0.112	Yes	Permanent
Benzene-containing environments[d]	Storage tanks, pipelines, groundwater	Hydrophobic compound		Man-made	Temporary in groundwater
Ethanol-containing environments[e]	Fermentation tank	Alcohol		Man-made	Temporary
Sugar-rich environments[f]	Plant tissues, juices, exudates	Sugar		Yes	Temporary

[a]Compiled and modified from Yakimov *et al.* (2015). Not applicable to all environments listed here.
[b]Cray *et al.* (2013b) used for guidance.
[c]Taken from Marion *et al.* (2003)
[d]Taken from van der Waals *et al.* (2017).
[e]Taken from Roy *et al.* (2015).
[f]Taken from Lievens *et al.* (2015).

1.2.3 Chaophiles

Hallsworth *et al.* (2007) and Williams and Hallsworth (2009) coined the term “chaophiles” – microbes that preferred chaotropicity, with evidence of such microbes from *Xeromyces bisporus* strains that were able to grow at high chaotropic glycerol concentrations, up to 7.60 M, but not in weak chaotropic or kosmotropic conditions. This indicated the strains had a preference for chaotropic effects that destabilised macromolecules since they only grew at high chaotropic concentrations. Zajc *et al*. (2014a) tried to find supporting evidence of chaophiles by testing various fungi on their abilities to grow at high kosmo- and chaotropic concentrations. Whilst they did find fungal growth at high concentrations of $MgCl_2$ and $CaCl_2$ (2.1 M and 2.0 M respectively), they defined these organisms as chaotolerant rather than chaophiles as they were also able to tolerate high kosmotropic concentrations, thus indicating they did not have a preference for chaotropic conditions.

1.2.4 Magnesium chloride and its life limiting concentrations

Magnesium ions and magnesium chloride are abundant in nature and play multiple significant roles for life forms. Magnesium chloride ($MgCl_2$) is not the most chaotropic salt to naturally occur on this planet as indicated in Table 1. (Cray *et al*., 2013a) but Hallsworth *et al*. (2007) argue the chaotropic effects of $MgCl_2$ are greater than most chaotropic solutes as $MgCl_2$ has a high solubility – 5 M $MgCl_2$ would have more than twice the chaotropic effect than saturated phenol. Also, at high concentrations, $MgCl_2$ inhibits cellular systems, thus affecting microbial life in naturally high chaotropic environments, such as the Discovery basin.

Hallsworth *et al*. (2007) in their study on the Discovery basin found mRNA at the upper concentration limit of 2.3 M $MgCl_2$, and thus used this concentration (without compensating kosmotropes) to suggest as the upper limit of life, but they did not exclude the possibility of finding organisms able to tolerate higher concentrations. Zajc *et al*. (2014a) in their search for chaophilic fungi found growth until 2.1 M $MgCl_2$ without compensating kosmotropes (close to Hallsworth *et al*.’s suggested 2.3 M limit). Also working with fungi, Jančič *et al*. (2016) were able

to grow six strains of *Wallemia ichthyophaga* in 2 M $MgCl_2$ without compensating kosmotropes. Nevertheless Zajc *et al*. (2014a) made a significant contribution by redefining the upper life limit dictated by $MgCl_2$, as shown in Figure 2, and this has raised the question if this window can still be pushed further.

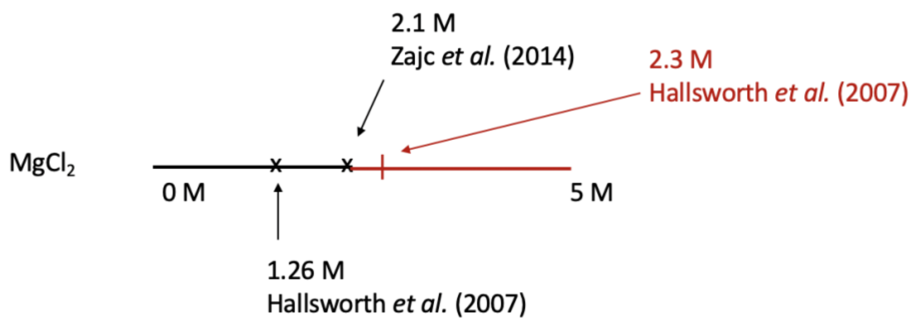


Figure 2. The current life-limiting concentrations of magnesium chloride ($MgCl_2$) as indicated in red, whilst the black line represents where growth is possible.

1.2.5 Chaotropicity versus water activity

Hallsworth *et al*. (2007) demonstrated that it is chaotropicity, rather than water activity, of $MgCl_2$, which is a limiting factor on living organisms. The highest concentration they had growth at in culture was 1.26 M $MgCl_2$ which is 0.916 a_w (sufficient for growth) but a chaotropic value of 26.1 kJ g^{-1}. In comparison, at 3.7 M $MgCl_2$, the water activity level at 0.61 would still allow growth (as shown in Figure 1) but the chaotropic value of 193 kJ g^{-1} is so high that growth has not been found at this level. $MgCl_2$ has been described as “one of the most powerful a_w-reducing solutes known” due to its high solubility in water (Hallsworth *et al*., 2007).

In addition, Williams and Hallsworth (2009) to further support this argument that water activity is not the limiting factor in the presence of chaotropic activity, obtained 157 highly xerophilic fungi from various environments and literature. Their definition of xerophiles were organisms able to grow below 0.85 a_w under two environmental conditions and have optimal growth below 0.95 a_w. Media with a range of low water activities that contained chaotropic fructose or glycerol, or a mixture of other solutes were created, and they found mostly no to slow growth at water activity

of 0.714 at 6.84 M glycerol, but higher growth rates than predicted in media containing a mixture of chaotropic and/or kosmotropic solutes despite the water activities being lower than 0.714 a_w. More recently, Fox-Powell *et al*. (2016) demonstrated that high ionic strength sterilised their simulated martian brines despite the presence of sufficient water activity levels for growth, thus supporting this argument that water activity is not always the limiting factor.

1.3 Kosmotropic effect of Sodium chloride

Sodium chloride (NaCl) is the most common salt in hypersaline environments (Hallsworth *et al*., 2007) and halophiles, microbes that require or thrive in high salinities, have been extensively studied and have been applied to various fields in biotechnology (reviewed in Oren, 2010). In response to osmotic stress caused by salts such as sodium chloride, many halophiles have been observed to produce compatible solutes, such as chaotropic glycerol (Zajc *et al*., 2014b). Interestingly, glycerol has also been produced in order to cope with chaotropic stress (Hallsworth, 1998; Bhaganna *et al*., 2016).

Kosmotropes have also been of interest as they can offset chaotropic effects by lowering the net chaotropicity, or cells have produced kosmotropes in the form of compatible solutes in order to cope with chaotropic stress, such as betaine and sorbitol (de Lima Alves *et al*., 2015). Therefore cells in the presence of kosmotropes in high chaotropic conditions can grow at higher concentrations than those without compensating kosmotropes, despite the lowered overall water activity (Hallsworth *et al*., 2007).

1.4 Extremophiles in non-extreme environments

Extremophiles do not exist only in their well-suited extreme environments. Brock and Freeze (1969) isolated thermophilic *T. aquaticus*, which are usually found in thermal aquatic habitats such as thermal springs, in soil in a greenhouse.

Dispersal could be one potential explanation as to why extremophiles have been found in non-extreme environments or in environments not particularly suited to their needs, such as via water (Hubert *et al*., 2009; Muller *et al*., 2014). However dispersal limitations should also be taken into account as this could affect the organism's likelihood to grow and settle into new environments or habitats (Hanson *et al*., 2012). Therefore dispersed extremophilic organisms must also be able to tolerate or adapt to the conditions, stresses and biotic factors in regards to microbial communities that are associated with the new habitat. Their long-time survival could also be made possible if a wider range of conditions than perceived exists in the new environment (thus not limiting them further) and/or their extremophilic abilities do not negatively affect their growth in non-extreme conditions, which Low-Décarie *et al*. (2016) found to be the case with extremophilic communities from a freshwater lake that could also grow in less extreme conditions. However Low-Décarie *et al*. did acknowledge these organisms were not obligate extremophiles as they grew in a non-extreme environment and thus did not solely require or prefer extreme conditions for growth.

Alternatively, Domínguez-Cuevas *et al*. (2006) suggested microbes from dominant species in their communities are able to withstand multiple stresses, such as *Pseudomonas putida*, a metabolically versatile soil microbe tolerant to many solutes, not just chaotropes (Hallsworth *et al*., 2003). This could imply that dominant non-extremophilic organisms may potentially be able to compete against extremophiles when faced with certain stresses that they can tolerate in their shared environment.

1.5 Agricultural soil and salt marsh environments

Soil is a diverse and rich environment, and its microbes drive or are involved in various key processes in the ecosystem. This study argues since microbes from soil may be exposed to chaotropes or other conditions that may exert similar effects as chaotropicity, some soil microbiota may be able to grow in chaotropic conditions. One example includes soil exposed to chaotropic pollutants, such as urea, which is a popular fertiliser. A more natural example is root

exudates where plant roots release compounds, such as chaotropes as a form of protection against pathogenic organisms in the rhizosphere (reviewed in Baetz and Martinoia, 2014).

Salt marsh is another diverse environment, characterized by its interesting ever-changing environmental conditions of salinity, water and nutrients for example. This would put constant stress on the living cells from a variety of factors and Kearns *et al*. (2016) argue this induces dormancy in some cells until the organism encounters a favourable condition for their growth. From this constant pressure, one could argue there could be microbes from this environment able to withstand chaotropic stresses, as they could be encountering similar stresses such as reduced water activity regularly in their own natural habitat, and perhaps if they are dormant, they may end their dormancy when encountering chaotropic stress if it is favourable for them. In addition, the second most common cation in salt marsh is magnesium, and compared to agricultural soil, salt marshes have a higher abundance of magnesium salts (Adam, 1993). Therefore it could be argued that microbes in salt marsh are regularly exposed to chaotropic conditions and to a much higher concentration than microbes in agricultural soil.

Furthermore, as discussed in the previous section, dispersal could be one explanation as to why extremophilic organisms are found in non-extreme environments. Microbes in environments containing chaotropes could arguably disperse to soil or salt marsh environments. Overall, finding microbes from agricultural soil and salt marsh able to grow at high chaotropic conditions may support or contradict previous research that have looked at extremophilic organisms from non-extreme environments and more importantly, the limits of life defined by $MgCl_2$.

1.6 Implications

Since many pollutants are chaotropic, discovering or further understanding chaophiles that are able to utilise chaotropic compounds may play a crucial role in bioremediation – the use of microbes in facilitating the removal of pollutants from an environment (Hallsworth *et al.*, 2003).

This may include introducing chaophiles to clean polluted water caused by chaotropic fertilisers, or in industrial tanks where chaotropic products affect the microbes needed for biofuel production (Cray *et al.*, 2015). The potential advantages to studying chaotropicity and chaophiles also extend beyond Earth as the search for extra-terrestrial life or potential habitats is ongoing and chaotropic effects may be close to some of the effects experienced in these harsh environments. There is substantial evidence Mars had and may currently have hypersaline brines (Bridges & Schwenzer, 2012; Ojha *et al.*, 2015; Fox-Powell *et al.*, 2016). Europa, Jupiter's moon, is also thought to have a briny ocean and the potential stress parameters on Europa that limit life are reviewed in Marion *et al*. (2003). McKay *et al.* (2014) argue Saturn's moon Enceladus is the best target for habitability as energy sources, carbon, nitrogen and a subsurface water reservoir are present and this environment is the most similar to Earth.

1.7 Aims and Hypotheses

This study's aim is to investigate how chaotropic magnesium chloride concentrations affect growth of microbes and microbial community composition from agricultural soil and salt marsh in comparison to kosmotropic sodium chloride. The first hypothesis is that as the concentration of magnesium chloride increases, microbial growth from both environmental samples will decrease. The second hypothesis is that growth will decrease more in $MgCl_2$ than in NaCl. The third hypothesis is that salt marsh will have more microbes growing at high $MgCl_2$ concentrations compared to microbes from agricultural soil. The objectives are to measure microbial growth from both environments in $MgCl_2$ or NaCl media of different concentrations; analyse communities in different concentrations of $MgCl_2$ and NaCl from liquid enrichments using DGGE; perform genomic analysis on strains grown in high $MgCl_2$ concentrations, and to perform further testing on these strains, such as determining their limits of growth in $MgCl_2$.

2 Methods

2.1 Sampling methods

Soil samples were taken in triplicate from the top 2 cm of soil of an agricultural field (51°52’15N, 0°56’28E) and salt marsh (51°52’24N, 0°56’54E) in Wivenhoe, Colchester, United Kingdom. Samples (0.6 g) were serially diluted in 5.4 ml of autoclaved distilled water to 10^{-4} dilution.

2.2 Testing the parameters of liquid media

The refractive index, water activity and pH were measured for each medium used in the growth enrichments. The instruments used were an Eclipse hand held refractometer, Novasina A_w Sprint TH-500 and Jenway pH meter respectively.

2.3 Growth enrichments

Solid and liquid media were prepared using 1% yeast extract (BD Difco), and either sodium chloride (Fisher Scientific) or magnesium chloride (Sigma-Aldrich) at the desired concentrations, and 1.5% agar (BD Difco) for solid media. Liquid enrichments containing NaCl were prepared until 3.5 M and $MgCl_2$ enrichments prepared until 2.5 M, as shown in Table 3. All media were inoculated with 100 µl of 10^{-1} to 10^{-4} dilutions of the samples. Enrichments and plates were incubated at 21°C for two months and checked regularly. Colonies on plates were counted and turbidity in liquid enrichments was inspected. Colony-forming units were calculated from plates using the number of colonies and the dilution of the sample. Isolated colonies were subcultured onto new agar plates with medium at the same salt and concentration as the medium from which they were originally isolated.

Table 3. The prepared concentrations of sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) in solid and liquid media for growth enrichments as indicated by "X". No-solute is the control. Both media also had 1% yeast extract, and solid media in addition had 1.5% agar.

Solute	Concentration	Type of medium	
		Solid	Liquid
None	0	X	X
NaCl	0.5	X	X
	1	X	X
	1.5	X	X
	2	X	X
	2.5	X	X
	3	X	X
	3.5	X	X
$MgCl_2$	0.5	X	X
	1	X	X
	1.25	X	X
	1.5	X	X
	1.75	X	X
	2		X
	2.25		X
	2.5		X

2.4 DNA extractions and PCR amplification of liquid enrichments

After 27 days of incubation, all liquid enrichments showing signs of growth had 1.2 ml transferred to sterile new microcentrifuge tubes and centrifuged at 11,337 x *g* for 10 minutes. The supernatant was discarded and the pellet frozen at -20°C. DNA was extracted from the thawed pellets using the method of Griffiths *et al.* (2000). Agarose gels (1%) in 1% Tris-acetate-EDTA (TAE) were run at 105 V for 50 minutes to visualise DNA. Gels were stained in 0.4 μg/ml ethidium bromide and viewed under UV light. Extracted DNA served as the template for PCR amplification in 25 μl reactions using Appleton AppTaq RedMix (2X) and primers and conditions as shown in Tables 4 and 5. Agarose gel electrophoresis as above was used to confirm amplification.

Table 4. Primers and conditions for PCR of extracted DNA from liquid enrichments from agricultural soil and salt marsh, for DGGE community analysis.

<table>
<tr><th rowspan="2">Target</th><th rowspan="2">Forward primer</th><th rowspan="2">Reverse primer</th><th colspan="5">PCR conditions</th></tr>
<tr><th>Initial denaturation</th><th>Denaturation</th><th>Annealing (55°C)</th><th>Extension (72°C)[1]</th><th>No. of cycles</th></tr>
<tr><td rowspan="2">Archaea</td><td rowspan="2">968F-GC Clamp</td><td rowspan="2">1401R</td><td colspan="2">94°C</td><td rowspan="2">20 s</td><td rowspan="2">45 s</td><td rowspan="2">30</td></tr>
<tr><td>5 min</td><td>15 s</td></tr>
<tr><td rowspan="2">Bacteria</td><td rowspan="2">F341-GC Clamp</td><td rowspan="2">534R</td><td colspan="2">95°C</td><td rowspan="2">15 s</td><td rowspan="2">15 s</td><td rowspan="2">30</td></tr>
<tr><td>1 min</td><td>15 s</td></tr>
<tr><td rowspan="2">Fungi</td><td rowspan="2">ITS3-GC clamp</td><td rowspan="2">ITS4</td><td colspan="2">94°C</td><td rowspan="2">45 s</td><td rowspan="2">1 min</td><td rowspan="2">35</td></tr>
<tr><td>5 min</td><td>45 s</td></tr>
</table>

[1]The final extension time for all cycles was 7 minutes, reactions were held at 4°C.

Table 5. Primer pairs for PCR of extracted DNA from liquid enrichments from agricultural soil and salt marsh, for DGGE community analysis.

Community	Primers	Sequence (5′ – 3′)	Reference
Archaeal	968F*	AACGCGGAAGAACCTTAC	Nübel *et al.* (1996)
	1401R	CGGTGTGTACAAGACCC	
Bacterial	341F*	CCTACGGGAGGCAGCAG	Muyzer *et al.* (1993)
	534R	ATTACCGCGGCTGCTGG	
Fungal	ITS3*	GCATCGATGAAGAACGCAGC	White *et al.* (1990)
	ITS4	TCCTCCGCTTATTGATATGC	

* With the addition of the GC Clamp, CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG (Muyzer *et al.*, 1993)

2.5 Denaturing Gradient gel electrophoresis (DGGE)

DGGE was performed on all amplified DNA using the Bio-Rad DCode™ Universal Mutation Detection System. The denaturant gradient ranges were 34.4%-65.5% for bacterial and archaeal samples and 30-60% for fungal samples, where 100% denaturant consisted of 20% v/v acrylamide, 40% v/v formamide, 2% 50x TAE buffer and 42 g urea. The gels were run at 60°C in 1x TAE buffer for a minimum of 16 hours. Fixing solution of 100 ml ethanol, 5 ml acetic acid and 895 ml water was added to the gels for 30 minutes, stained with 0.001% w/v of silver nitrate for 30 minutes, developing solution with 0.015% w/v sodium hydroxide and 0.008% v/v formaldehyde added for 15 minutes and fixing solution added again for 10 minutes. Gels were then scanned and presence/absence matrixes were created using the eye. These then were inputted into Microsoft Excel and formatted to be opened by R, where distance matrixes were created using the "jaccard" method. Dendrograms were produced with the "raup" method (R Core Team, 2013).

2.6 Sequencing of selected DGGE bands

Selected bands from fungal DGGE gels were cut using a sterile, new scalpel for each band and placed in 100 µl of sterile MilliQ water and left in a fridge overnight before amplification with the same primers (without the GC-clamp) and conditions used for fungi (Table 3). The amplification products were purified using the Sigma GenElute™ PCR Clean-Up Kit. Agarose gel electrophoresis was run to confirm amplification and purification. Purified PCR product was sent to Source Bioscience for Sanger sequencing with primer ITS4 and the results were edited using 4Peaks (Nucleobytes). The edited sequences were searched against the nucleotide database in NCBI BLAST (Altschul *et al.,* 1990) and close relations to 18S rRNA gene sequences were identified. Original sequences, closest sequences from BLAST and related sequences (38 in total) were assembled into MUSCLE (Edgar, 2004) for alignment and trimming. The alignment was exported to MEGA7 (Kumar *et al.,* 2016) to create a phylogenetic tree through Maximum Likelihood method and Tamura 3-parameter model. The bootstrap method was chosen for 500 replicates.

2.7 Illumina sequencing

Eleven samples were amplified with the primers targeting bacterial 16S rRNA genes (Klindworth *et al.*, 2013): F (5′-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNGGCWGCAG-3′) and R (5′-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACHVGGGTATCTAATCC-3′) that had overhang adapter sequences indicated in bold, with cycling conditions of initial denaturation at 95°C for 3 minutes, 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final elongation at 72°C for 5 minutes, in 25 µl reactions. PCR products were viewed on 1% agarose gel.

Tom Huby prepared and sequenced the metagenomic library on the Illumina MiSeq platform at University of Essex, following the Illumina 16S Metagenomic Sequencing Library Preparation guide (please see Bibliography for link). For PCR product purification, PCR products and then 20 µl of SPRI beads (AxyPrep™ Mag PCR Clean-up) were loaded onto a 96-well PCR plate, mixed using a pipette and then incubated at room temperature for 5 minutes to allow the PCR products to bind to the beads. The supernatant was then removed after placing the PCR plate onto a magnetic plate stand (IMAG™ Handheld Magnetic Separation Device) for 2 minutes and the samples were washed twice with 80% ethanol, then air dried for 10 minutes. The plate removed from the stand, the samples were then eluted in 52.5 µl Buffer EB (Qiagen) and incubated at room temperature for 2 minutes. The plate was then placed onto the stand and incubated for a further 2 minutes at room temperature to separate the beads from the sample containing DNA. From each sample, 50 µl is transferred to a new 96-well PCR plate.

This new plate was used for Index PCR to attach dual indices and Illumina sequencing adapters, using Nextera XT Index Kit v2 (Set A). The samples were amplified with the following cycle conditions: initial denaturation at 95°C for 3 minutes, 8 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final elongation at 72°C for 5 minutes, in 50 µl reactions. The index PCR plate was purified again following the previous purification steps,

however 56 µl SPRI beads and 27.5 µl EB buffer were used instead. Following purification, 25 µl from each sample was transferred to a new plate.

For the quantification of the products, the Quant-iT[TM] PicoGreen[TM] dsDNA Assay Kit (Invitrogen) was used and samples were 50-100-fold diluted, and 10 µl were pipetted in triplicate onto a 384-well microtiter plate. The samples were mixed with 10 µl of PicoGreen dye solution and quantified using FLUOstar Omega Microplate Reader (BMG Labtech). NanoDrop 3300 (Thermo Scientific) was used on samples that needed to be quantified again.

Samples were run on a 1% agarose gel to estimate amplicon library size and molar concentrations of libraries were calculated using the formula in the protocol. Libraries were then quantified and individually pooled and the concentrated final library was diluted with EB buffer to 4 nM. Both the DNA library and PhiX were diluted to 6 pM, combined in an 80:20 ratio and sequenced.

After sequencing, Boyd McKew processed raw data by quality trimming paired reads using Sickle (Joshi and Fass*.,* 2011) and used the BayesHammer algorithm (Nikolenko *et al.,* 2013) in SPAdes (Nurk *et al.,* 2013) for error correction. Sequences were pair end aligned using PEAR (Zhang *et al.,* 2014) in PANDASeq (Masella *et al.,* 2012). Duplicate pair end sequences were removed and sorted by abundance before OTU centroids were selected using VSEARCH (Rognes *et al.,* 2016) at 97% similarity. To identify de-novo and reference based chimera sequences, UCHIME (Edgar *et al.,* 2011) was used and the RDP Classifier (Wang *et al.,* 2007) was used to classify taxonomy. Using Microsoft Excel, an OTU table was created and singleton OTUs, OTUs with sequences below 400 base pairs, OTUs with less than 1000 reads and OTUs with de-novo chimeras were deleted from the table and data was normalised. Plots were generated in STAMP (Parks *et al.,* 2014), and in Microsoft Excel analysing genera whose sum of sequences were more than 5%.

2.8 DNA extractions and amplification of strains

Colonies of strains isolated from previous enrichments were transferred to microcentrifuge tubes containing 1.2 ml of $MgCl_2$ at 1 M or 1.5 M as these were the closest or exact concentrations of the plates the strains were subcultured onto in order to minimise stress from lower or higher chaotropic concentrations. The tubes were centrifuged at 11,337 x *g* for 10 minutes. DNA extractions following the method of Griffiths *et al.* (2000) were performed on the pelleted cells. Agarose gels as previously described in Section 2.4 were run to confirm presence of DNA, which were then amplified using primers and conditions as shown in Tables 6 and 7, in 25 µl reactions using Appleton AppTaq RedMix (2X).

Table 6. Primers and conditions for PCR of bacterial and fungal genes of strains isolated from agricultural soil and salt marsh.

Target	Forward Primers	Reverse Primers	PCR conditions				
			Initial Denaturation	**Denaturation**	**Annealing (55°C)**	**Extension (72°C)[1]**	**No. of cycles**
Bacteria	27F	1492R	95°C		15 s	15 s	30
			1 min	15 s			
Fungi	ITS3	ITS4	94°C		45 s	1 min	35
			5 min	45 s			
Fungi	F BT2a	R BT2b	94°C		45 s	1 min	35
			5 min	45 s			

[1]Final extension lengths for all cycles were 7 minutes and held at 4°C.

Table 7. Primer pairs for PCR of target genes of bacterial and fungal strains isolated from agricultural soil and salt marsh.

Gene	Primers	Sequence (5′ – 3′)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	Lane *et al.* (1985)
	1492R	GGTTACCTTGTTACGACTT	
ITS	ITS3	GCATCGATGAAGAACGCAGC	White *et al.* (1990)
	ITS4	TCCTCCGCTTATTGATATGC	
B-tubulin (*benA*)	F BT2a	GGTAACCAAATCGGTGCTGCTTTC	Glass & Donaldson (1995)
	R BT2b	ACCCTCAGTGTAGTGACCCTTGGC	

2.9 Phylogenetic analysis of strains

The same methods as described in Section 2.6 were used.

2.10 Testing the parameters of media used for strain experiments

For media used in the high-$MgCl_2$-concentration and floating-filter experiments, the refractive index, water activity and pH were measured for each medium with the same instruments listed in Section 2.2.

2.11 Testing of strains in high magnesium chloride concentrations

Liquid media were prepared using 1% yeast extract (BD Difco) and Sigma-Aldrich magnesium chloride at concentrations of 1.4, 1.6, 1.8, 2.0 and 2.2 M. A control medium containing no magnesium chloride was also prepared. All media were autoclaved before inoculating with 100 µl of strains suspended in media, with each strain being tested at the triplicate level at each concentration. Enrichments were incubated as previously described and checked regularly.

2.12 Strains on floating filter papers

Liquid media were prepared as described in Section 2.11 for magnesium chloride concentrations of 0.5, 1.0, 1.5 and 2 M. A control medium containing no magnesium chloride was prepared. All media, tweezers and 47 mm hydrophilic Millipore membrane filters of 0.22 µm pore size were autoclaved. Media were poured into petri dishes and filter papers placed on the surface using sterile tweezers. Filter papers were inoculated and incubated as above. Diameters of colonies were measured using a ruler.

2.13 Microscopy of strains

Colonies of strains were streaked onto a microscope slide using a sterile loop, either directly from its liquid enrichment, or from a 1.5 M $MgCl_2$ plate and suspended in a droplet of sterile 1.4 M $MgCl_2$. The strains were viewed under an Olympus BH2 microscope and immersion oil was used for x1000 magnification. Images were taken using a Samsung phone.

3 Results

3.1 Testing of parameters of liquid media used for enrichments

In order to determine the validity of the solute concentrations, the water activity, refractive index and pH of the media used for liquid enrichments were measured (Figure 3), showing that as the concentration of added salt increased, the refractive index increased. At 2.5 M, the highest concentration shared between the two salts for comparison, $MgCl_2$ had a higher refractive index of 1.388 than NaCl with 1.361.

As for water activity, the control had 0.993 a_w, which was expected. At 0.5 M, the water activity was lower for NaCl and $MgCl_2$ than the control, at 0.975 a_w and 0.971 a_w respectively. As concentrations of both salts increased, the water activity further declined; at 2.5 M NaCl had a water activity of 0.889 a_w and $MgCl_2$ had 0.764 a_w. This also showed at the same salt concentrations, $MgCl_2$ had lower water activity than NaCl.

In regard to pH, as the concentrations of the salts increased, the pH increased with $MgCl_2$ but decreased with NaCl. At 0.5 M, the pH for $MgCl_2$ was higher than the control at pH 6.7, which increased until 1.5 M to pH 7.5, and decreased to pH 7 at 2.5 M. With sodium chloride, the pH was lower than the control at 0.5 M with a pH of 6.3, which decreased to pH 6 at 3.5 M. Across all concentrations, $MgCl_2$ had a higher pH than NaCl – there was a difference of 1 pH between the two salts at 2.5 M.

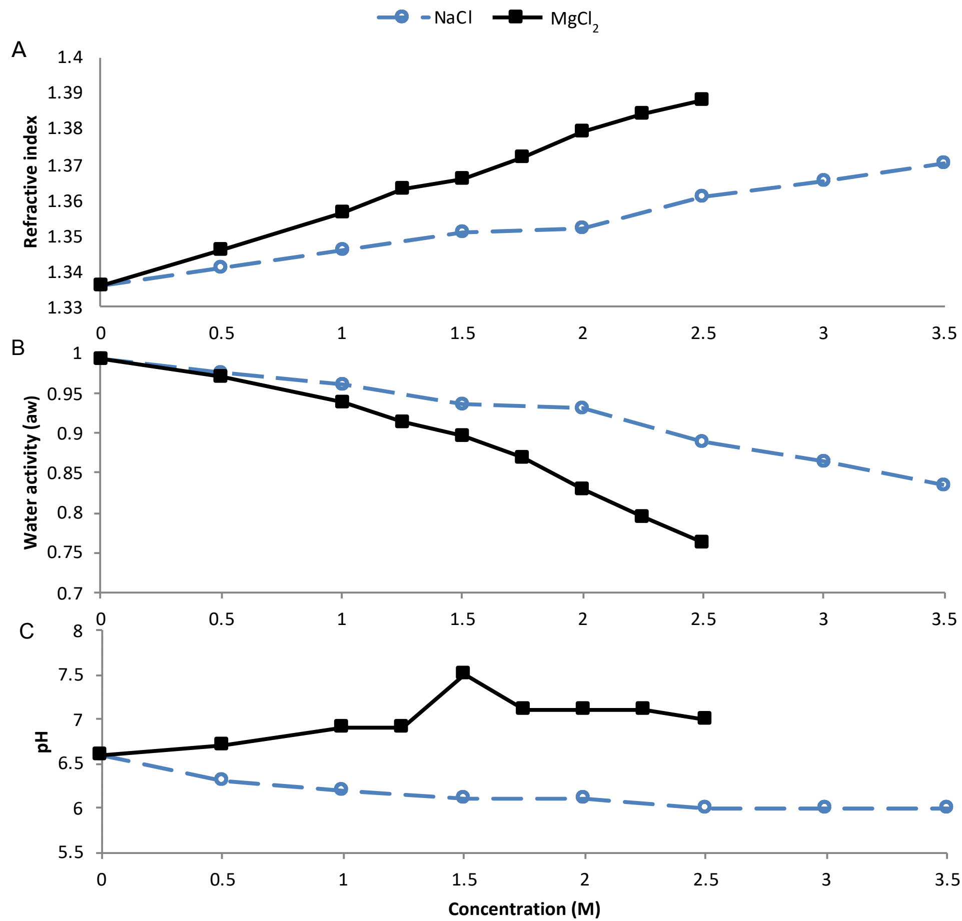


Figure 3. Physiochemical properties of magnesium chloride and sodium chloride solutions. A) refractive index, B) water activity and C) pH for sodium chloride (NaCl) and magnesium chloride ($MgCl_2$) across 0-3.5 M where 0 M is the no-solute control. Concentrations above 2.5 M $MgCl_2$ were not tested. n=1.

3.2 The effect of magnesium chloride and sodium chloride on microbes from salt marsh and agricultural soil environments

3.2.1 Viable counts from $MgCl_2$ and NaCl agar plates

In order to quantify the effect of a range of concentrations of magnesium chloride and sodium chloride on viable microbial counts, agar plates containing 1% yeast extract and with different concentrations of both solutes were inoculated with dilutions of samples from agricultural and salt marsh soils, and the resulting colonies were counted.

Figure 4 A-D shows that the viable counts decreased as concentrations of both solutes increased, which corresponded with a decrease in water activity. Growth was observed at the highest concentration of NaCl tested (3.5 M), but for $MgCl_2$ growth was not observed above a concentration of 1.25 M. Similarly, growth was observed at 0.84 a_w in NaCl but not below 0.90 a_w in $MgCl_2$, thus implying that a factor other than water activity was responsible for limiting growth.

In NaCl-supplemented media, viable counts from salt-marsh samples were significantly higher than those from agricultural soil only at 3 M NaCl (one-way ANOVA; $F_{1,4}$=33.7, p<0.05). There was no significant difference in viable counts between salt-marsh and agricultural soils when $MgCl_2$ was added to the media (see Appendix A1-A3 for One-way ANOVA analysis between the environments). Regression analysis showed that from both environments and with both salts, viable counts decreased significantly as salt concentrations increased (Table 8). Appendix B1-B8 contain the regression analysis between environment and salt.

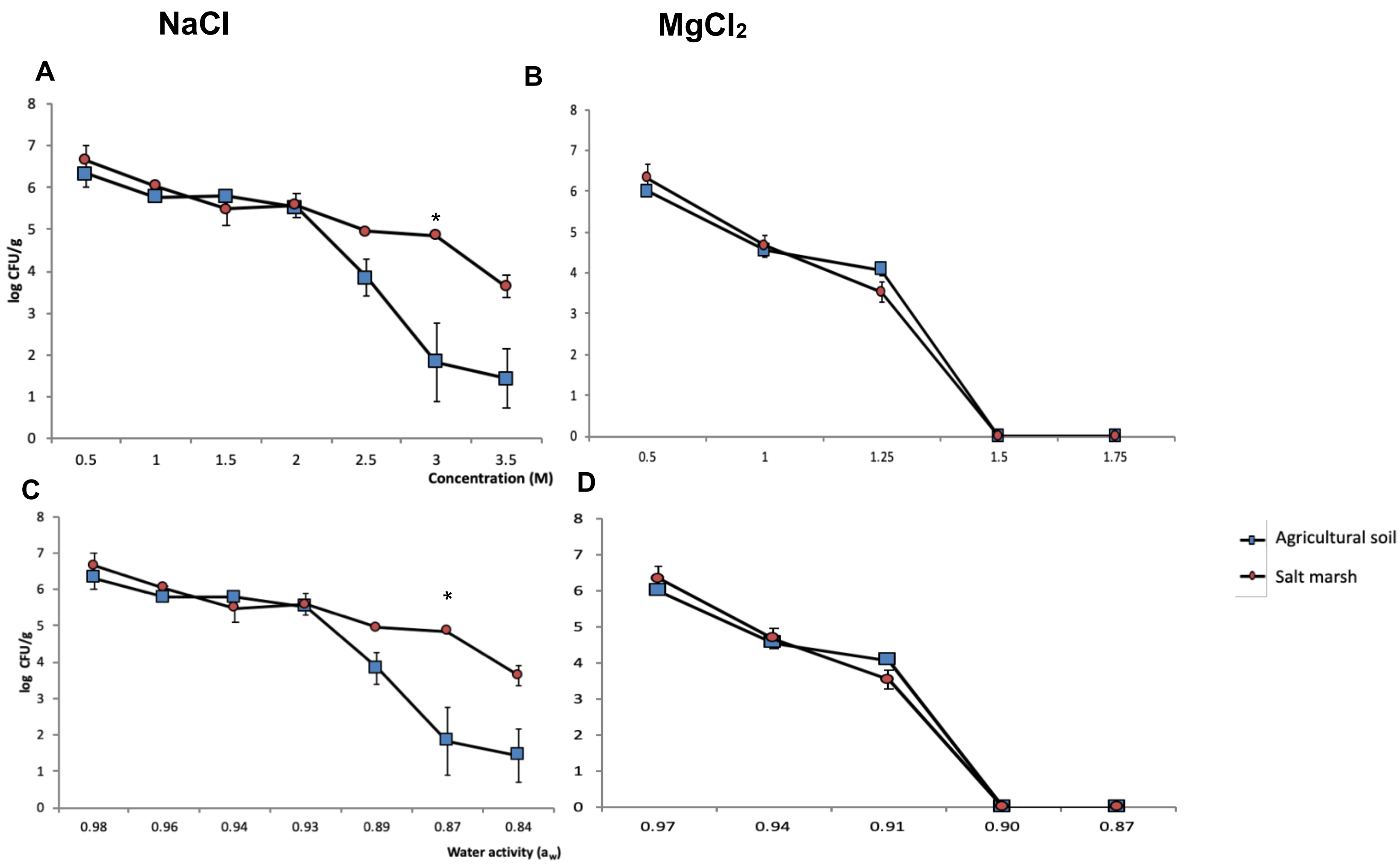


Figure 4. Mean colony forming units per gram (log CFU/g) from agricultural soil and salt marsh environments in sodium chloride (NaCl) and magnesium chloride ($MgCl_2$). A) and B) are 0.5-3.5 M NaCl concentrations and 0.5-1.75 M $MgCl_2$ concentrations and C) and D) water activity (a_w) values corresponding to salt concentrations. Error bars represent standard error, n=3. * represents statistical significant difference (one-way ANOVA test, $p<0.05$). Colonies in no-solute controls were confluent.

Table 8. R-squared, t-values and significance levels (p-value) from regression analysis of viable counts from the solute and environment type.

Environment	Solute	R-squared	t-value	p-value
Agricultural soil	NaCl	0.24	-2.48	0.023
Agricultural soil	$MgCl_2$	0.68	-5.20	0.000
Salt marsh	NaCl	0.35	-3.20	0.005
Salt marsh	$MgCl_2$	0.45	-3.26	0.006

Colonies on plates in no-solute control, NaCl concentrations from 0.5-2 M and $MgCl_2$ 0.5 M were observed to clump to one-another and grow over other colonies. Above these concentrations, colonies grew more distinctly, as seen in Figure 5 diversity appeared to decrease as concentrations for both solutes increased.

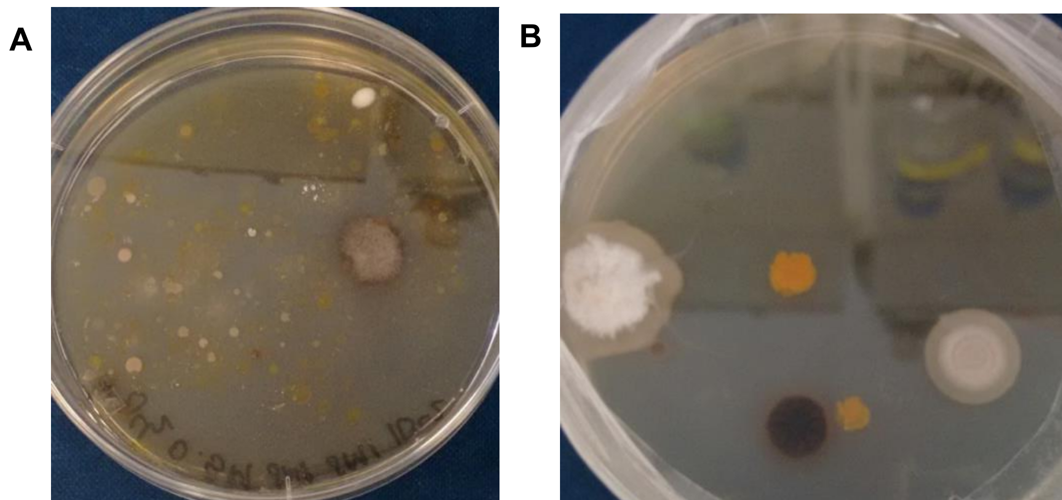


Figure 5. Examples of plates of salt marsh samples. A) is 0.5 M $MgCl_2$ and B) 1.25 M $MgCl_2$, where both plates are of 10^{-2} dilutions.

3.3 Amplification and sequencing of salt marsh and agricultural soil microbes in NaCl and $MgCl_2$ liquid enrichments

3.3.1 PCR amplification of DNA from liquid enrichments

In order to confirm growth in liquid enrichments and to perform microbial community analysis and sequencing, PCR was performed to amplify 16S rRNA and ITS genes from liquid enrichments. Table 9 shows that archaeal and bacterial DNA from both agricultural and salt-marsh soil enrichments were amplified in the majority of NaCl concentrations and amplified in 0.5 M $MgCl_2$ enrichments with faint amplification in some 1 M and 1.25 M $MgCl_2$ enrichments. Fungal ITS genes from agricultural soil enrichments also amplified in most NaCl enrichments but only had amplification in 0.5 M and 1.25 M $MgCl_2$ enrichments. Whereas in salt-marsh enrichments, fungal primers amplified ITS genes from one 1.5 M NaCl enrichment and in $MgCl_2$ enrichments from 0.5 M to 1.75 M.

Figure 6 shows example PCR products from bacterial, archaeal and fungal amplification using DNA from agricultural soil enrichments with 0.5 M NaCl and 1.25 M $MgCl_2$ and from the no-solute control.

3.3.2 Species (band) richness of agricultural soil and salt marsh enrichments based on denaturing gel gradient electrophoresis (DGGE)

Table 10 shows that species richness, based on the number of observed bands in DGGE analysis, in both agricultural-soil and salt-marsh enrichments in sodium chloride generally decreased until 1.5 M or 2 M and then increased. In magnesium chloride, species richness generally decreased as the concentrations increased for all agricultural soil and salt marsh samples, except for bacteria at 1.25 M and fungi at 1.75 M.

The highest species richness was seen in bacteria from both environmental samples whilst fungi were the least species rich. The highest average count of fungal species was 4.5 +/- 1.5

from salt marsh enrichments containing $MgCl_2$ 0.5 M but bacteria in the same enrichments had a higher average of 27.7 +/- 4.3 species. Similarly, agricultural soil enrichments with the same salt and concentration had on average 28.3 +/- 1.5 bacterial species and 2.67 +/- 0.9 fungal species.

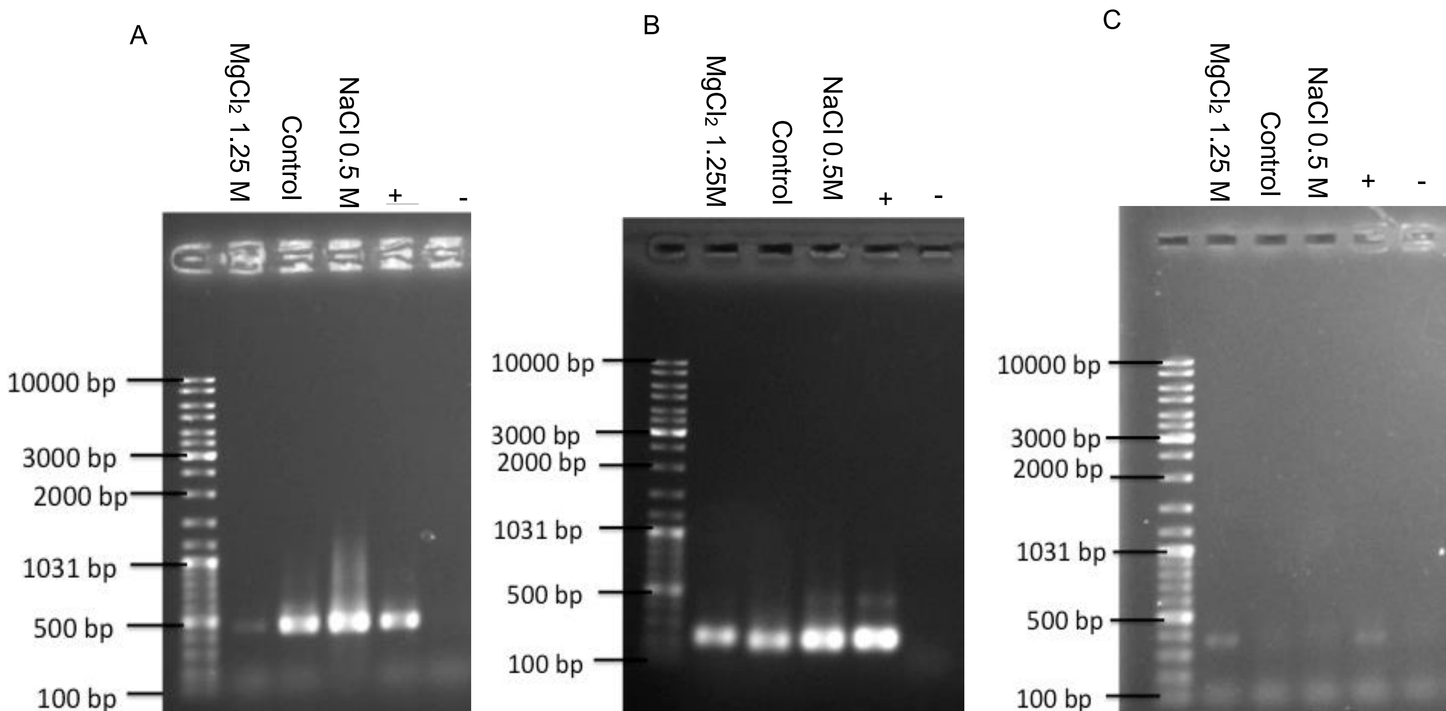


Figure 6. Example agarose gels following PCR on agricultural soil samples . A) Archaeal primers 968F (GC Clamp attached) and 1401R used, where positive control (+) is a no-solute control sample that amplified during test PCRs; B) Bacterial primers F341 (GC Clamp attached) and 534R where positive control (+) is *E.coli* and C) fungal primers ITS3 (GC Clamp attached) and ITS4, where positive control (+) is a *Cladosporium* species. Negative control (-) for all PCRs was sterile water. The left hand lanes show the Thermo Scientific GeneRuler DNA Ladder.

Table 9. Summary of PCR amplification results in enrichments containing agricultural soil and salt marsh samples, in solutes sodium chloride or magnesium chloride of different concentrations, or in the no-solute control.

	Agricultural soil									Salt marsh								
Sample	**Archaeal**			**Bacterial**			**Fungal**			**Archaeal**			**Bacterial**			**Fungal**		
Control	+	+	+	**+**	+	**+**	-	-	-	+	+	+	**+**	+	**+**	-	-	-
NaCl 0.5 M	+	+	+	+	+	+	-	+-	-	+	+	+	+	+	+	-	-	-
NaCl 1 M	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-
NaCl 1.5 M	+	+	+	**+**	+	**+**	+	+	+	+	+	+	**+**	+	**+**	-	-	+
NaCl 2 M	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-
NaCl 2.5 M	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-
NaCl 3 M	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+	-	-	-
$MgCl_2$ 0.5 M	+	+	+	+	+	+	+	+-	+	+	+	+	+	+	+	+	+	+
$MgCl_2$ 1 M	+	-	+-	**+**	-	-	-	-	-	+-	+-	+-	-	**+-**	-	-	+-	-
$MgCl_2$ 1.25 M	-	+-	-	-	**+**	-	-	+-	-	+-	-	+-	-	-	-	+-	+	-
$MgCl_2$ 1.5 M	-			-			-			-	-	-	-	-	-	-	+	+
$MgCl_2$ 1.75 M										-	-	-	-	-	-	-	+-	-

"+" represents position amplification, "-" represents no amplification and "+-" represents faint amplification.
Blank spaces represent no amplification performed for those samples due to no observed growth.
Samples in bold represent those that have been sequenced via Illumina MiSeq.

Table 10. Average bands counted per sample across all agricultural soil and salt marsh samples subjected to DGGE. Most samples have three replicates at each solute and concentration (n=3). Samples with 1 replicate (n=1) are represented by [a], samples with 2 replicates are represented by [b] and samples with 4 replicates (n=4) are represented by [c]. "X" represents samples not run for DGGE due to no PCR product. Figures have been rounded to 3 significant figures where appropriate.

	Community	Control	NaCl						$MgCl_2$				
			0.5 M	1 M	1.5 M	2 M	2.5 M	3 M	0. 5 M	1 M	1.25 M	1.5 M	1.75 M
Agricultural soil	**Archaeal**	18[c]	16.3	10.3	6	6.33	8.33	8.5[b]	5.33	5[b]	4	X	X
	Bacterial	23.7	26.3	24.7	24	18	9.33	13.7	28.3	9[a]	17[a]	X	X
	Fungal	X	2[a]	1[b]	2.33	2[b]	4[2]	3[b]	2.67	X	2[a]	X	X
Salt marsh	**Archaeal**	17.3	11	9.67	10.7	16.5[c]	14.3	14.7	14	7.33	6[a]	X	X
	Bacterial	30.5[c]	28	22.3	17.7	17.3	13.3	14	27.7	10[1]	X	X	X
	Fungal	X	X	X	1[a]	X	X	X	4.5[b]	3[a]	2[b]	1.33	4[a]

3.3.3 Community composition of microbes from DGGE

Cluster analysis of DGGE profiles based on presence/absence of bands was used to identify changes in community composition in response to different locations, salts and concentrations. Multiple dendrograms exist due to not all sample sets fitting on one gel – each dendrogram represents one gel (see Appendix C1-C10 for the DGGE profiles on the gels).

Figures 7 and 8 show bacterial community compositions in NaCl, $MgCl_2$ and no-solute enrichments of differing concentrations from agricultural soil and salt marsh environments. The communities in NaCl enrichments of similar concentrations clustered together, such as NaCl 2.5 and 3 M in both Figures. Surprisingly, the majority of $MgCl_2$ enrichments did not show close relationships in community compositions to each other. Furthermore, not all replicates within the same dendrogram showed close community compositions; in Figure 7B, the community of one replicate of NaCl 2 M is clustered with the community in $MgCl_2$ 0.5 M, whilst the other replicate is clustered to the community in NaCl 1.5 M.

Archaeal community compositions are shown in Figure 9. NaCl enrichments of similar concentrations were clustered, and that the replicates of no-solute control enrichments were not closely related to each other within the same dendrograms. The difference between replicate dendrograms was that the relationships between $MgCl_2$ enrichments differed; in Figure 9A, $MgCl_2$ 1 and 1.25 M were most similar but replicates of 0.5 M enrichments were not. This also applied to salt marsh samples where $MgCl_2$ enrichments and replicates were not closely related to one another. There did not appear to be any differences in community composition between the two environments.

Fungal community compositions are shown in Figure 10. In enrichments from agricultural soil in Figure 10A, replicates from both NaCl and $MgCl_2$ enrichments did not show similarities in community compositions. This was also similar in replicates in $MgCl_2$ enrichments from salt marsh in Figure 10B.

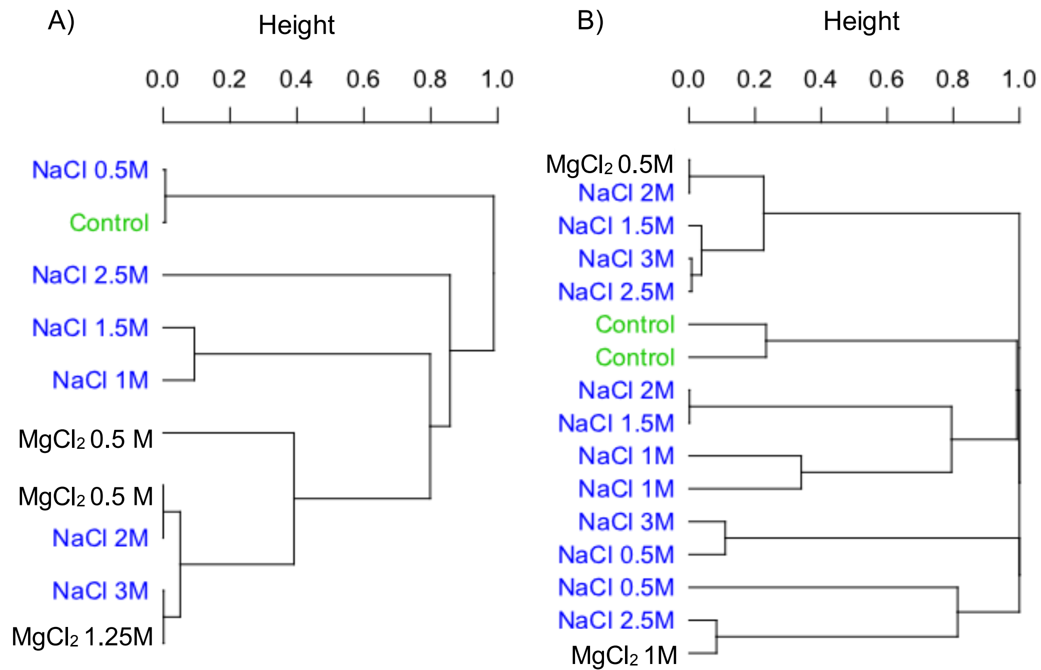


Figure 7. Hierarchal cluster analysis of DGGE profiles of bacterial 16S rRNA genes amplified from agricultural soil enrichments. A) and B) represent separate gels used in DGGE as all agricultural soil enrichments did not fit on one gel. Each dendrogram represents one gel and was produced using the "raup" method in R. Samples of the same name are biological replicates. No-solute control samples are in green, sodium chloride samples in blue and magnesium chloride samples in black.

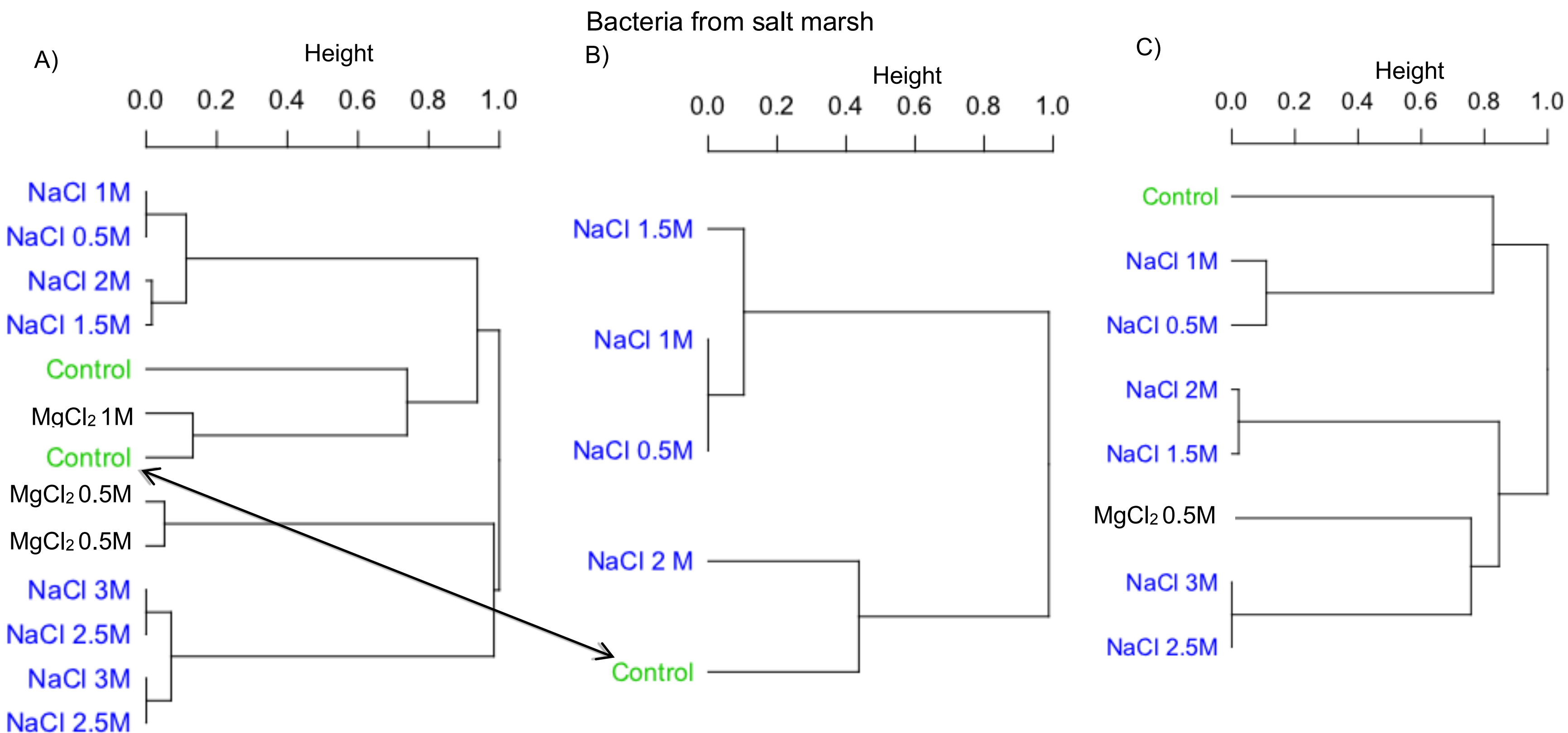


Figure 8. Hierarchal cluster analysis of DGGE profiles of bacterial 16S rRNA genes amplified from salt marsh enrichments. A), B) and C) represent separate gels used in DGGE as all salt marsh enrichments did not fit on one gel. Each dendrogram represents one gel and was produced from the “raup” method in R. Samples of the same name are biological replicates. No-solute control samples are in green, sodium chloride samples in blue and magnesium chloride samples in black. The black arrow represents the same sample present in both gels.

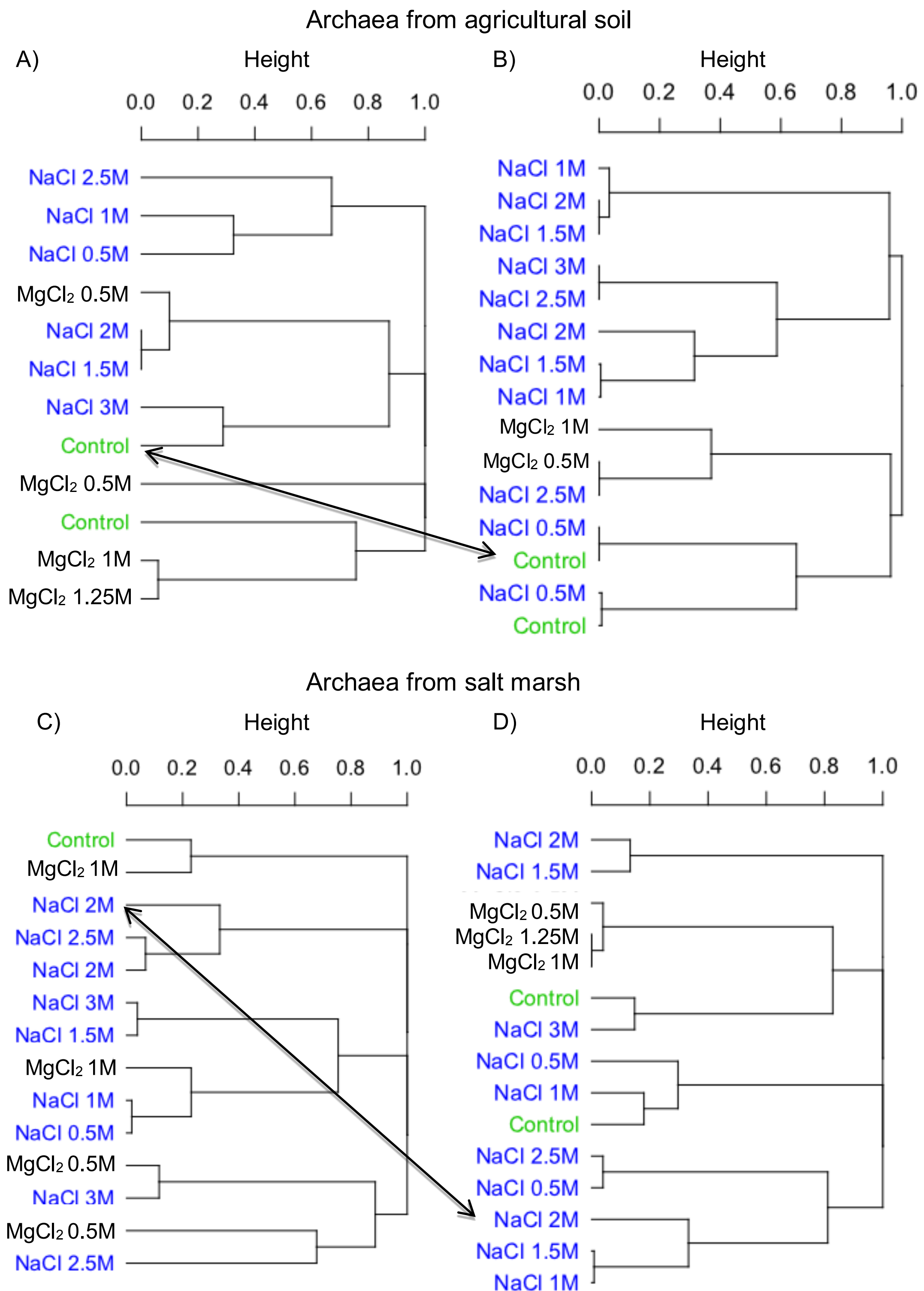


Figure 9. Hierarchal cluster analysis of DGGE profiles of archaeal 16S rRNA genes amplified from A) and B) agricultural soil enrichments and C) and D) salt marsh enrichments. Each dendrogram represents one gel and was created using the "raup" method in R. Samples of the same name are biological replicates. No-solute control samples are in green, sodium chloride samples in blue and magnesium chloride samples in black. The black arrow represents the same sample present in both gels.

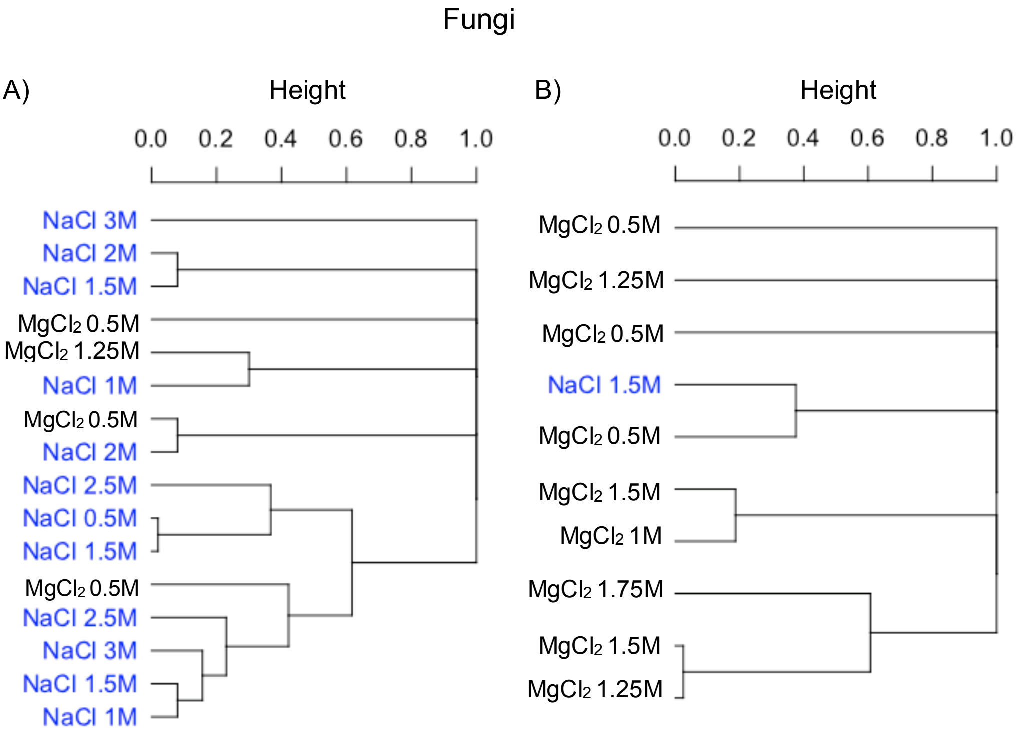


Figure 10. Hierarchal cluster analysis of DGGE profiles of fungal18S rRNA genes amplified from A) agricultural soil enrichments and B) salt marsh enrichments. Each dendrogram represents one gel and was created using the “raup” method in R. Samples of the same name are biological replicates. Sodium chloride samples are in blue and magnesium chloride samples in black.

3.3.4 Phylogenetic analysis of selected fungal DGGE bands

Fungal DGGE bands from high concentrations of NaCl and across concentrations of $MgCl_2$ were amplified and sequenced for phylogenetic analysis, as shown in Table 11, and the corresponding solute and concentrations shown in Figure 11. DGGE bands 1 and 2 (*Penicillium*) were identified from NaCl 3 M and 2.5 M respectively in Figure 11A. Similar migrating bands were also seen in NaCl 1.5 M and 2.5 M for band 1, and NaCl 2 M and 2.5 M for band 2. In Figure 11B Band 3 (*Saturnispora*) was identified in $MgCl_2$ 0.5 M but no similar migrating bands were seen. Band 4 (a fungal species closely matched to *Acremonium*) was identified in $MgCl_2$ 1.5 M. A similar migrating band was seen in $MgCl_2$ 1.25 M. Band 5 (*Baeospora*) identified in 1.75 M $MgCl_2$ also did not have similar migrating bands. Figure 12, visualising the evolutionary relationships between the bands and their closest matches, shows band 3 and *Saturnispora* species as an outgroup.

Table 11. Closest results from BLAST analysis of 18S rRNA gene sequences from DGGE bands against the Nucleotide collection. (Refer to Figure 11.)

DGGE band	Closest match	Sequence length (bp)	Sequence similarity (%)	Environments found in	GenBank sequence ID
1	*Penicillium canescens*	131	98	Twigs (plants)	KM519655.1
2	*Penicillium* sp.	128	100	Sediment, root	KY401119.1
3	*Saturnispora mendoncae*	210	93	Food, rotting wood	KY105322.1
4	Fungal sp.	123	89	Marine sediment	KX098125.1
5	*Baeospora myosura*	325	100	Spruce forest topsoil	LN714524.1

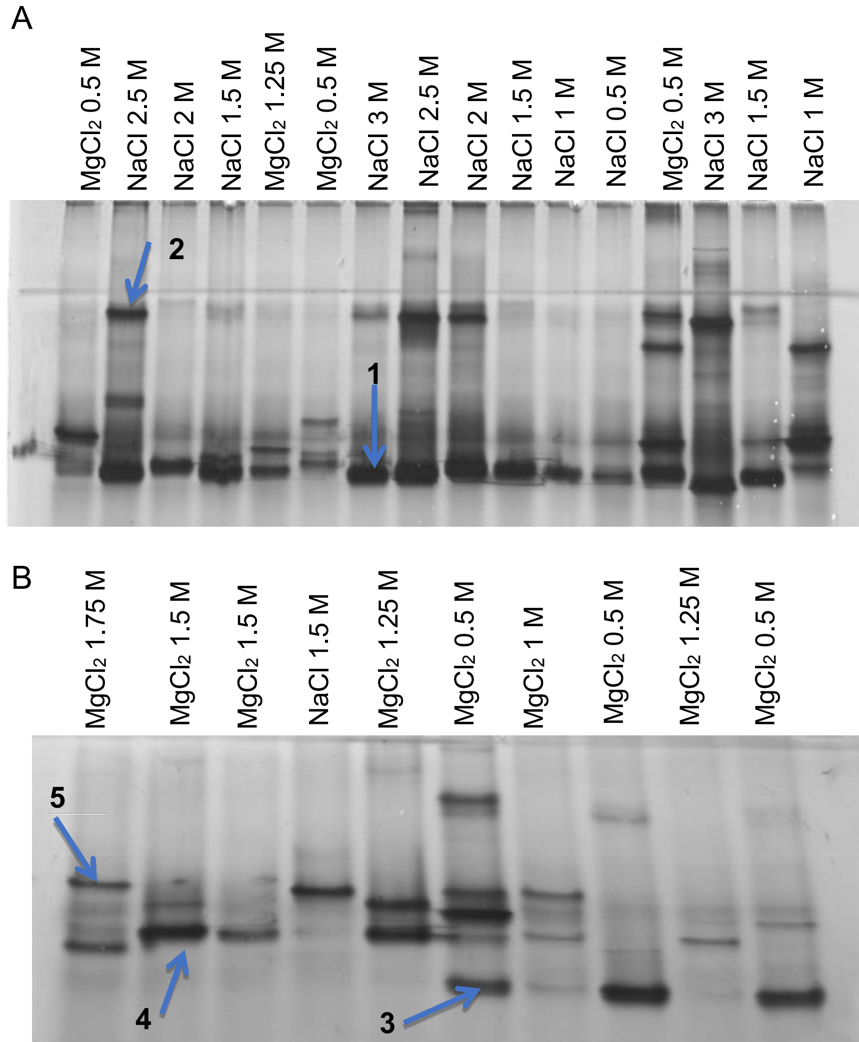


Figure 11. DGGE profiles of fungal PCR products amplified from enrichments inoculated with A) agricultural soil and B) salt marsh, containing sodium chloride or magnesium chloride.

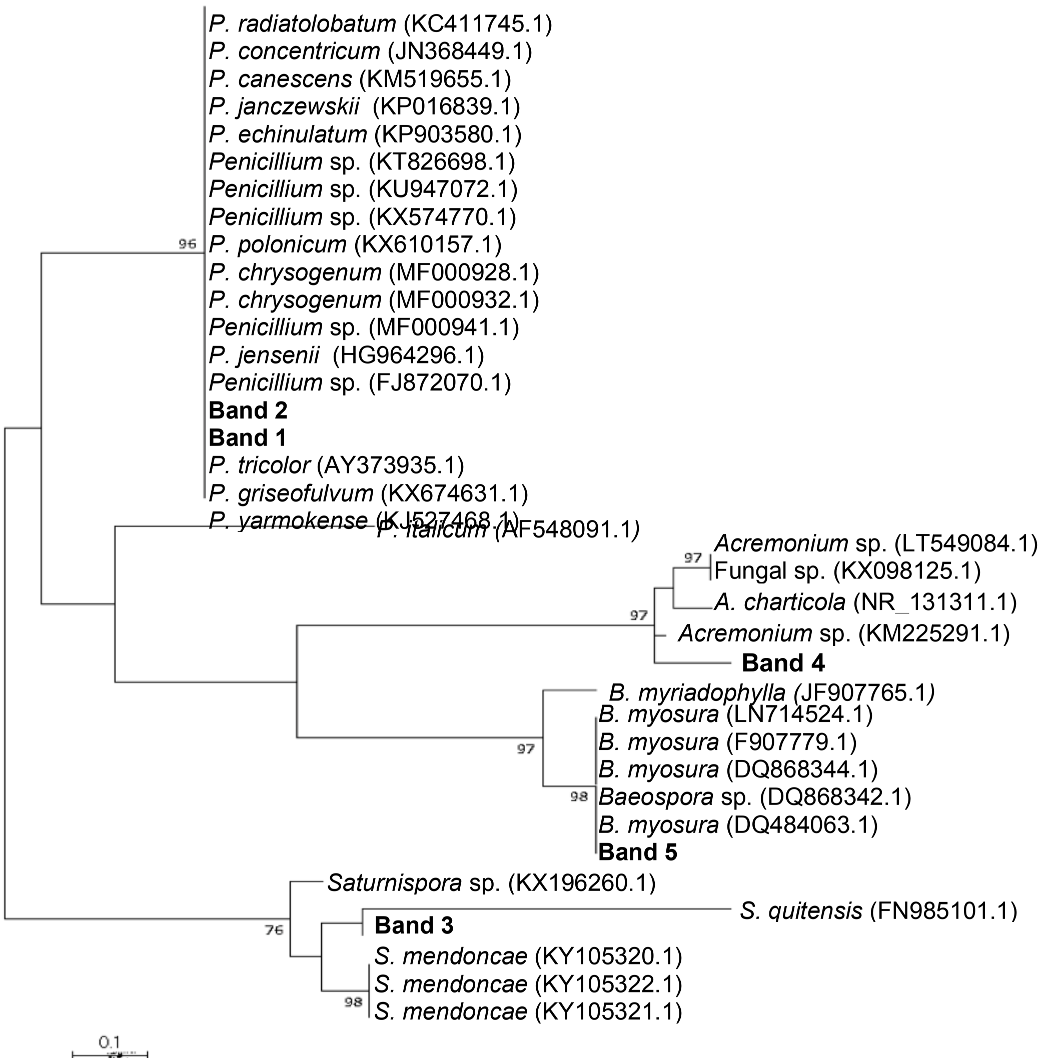


Figure 12. Phylogenetic tree based on 18S rRNA gene sequences of isolated DGGE bands 1, 2, 3, 4 and 5 from agricultural soil and salt marsh environments, and their closest relatives from the genera *Penicillium*, *Saturnispora*, *Baeospora* and *Acremoniun*. Sequences (38) of 110 base pairs were aligned using MUSCLE and the tree constructed from the Maximum Likelihood method and Tamura 3-parameter model. Accession numbers of reference strains are included in brackets. Bootstrap values (500 replicates) above 75% are shown. Scale bar represents the number of substitutions per site.

3.3.5 MiSeq Illumina sequencing of selected bacterial amplification products from liquid enrichments

MiSeq Illumina sequencing was performed in order to obtain and compare community profiles on bacterial samples from different solutes and locations. A PCA plot (Figure 13) shows close clustering of communities, with a few exceptions where the two communities in NaCl 1.5 M from salt marsh and the community in the no-solute control from agricultural soil were distant. Also, the communities in $MgCl_2$ 1 M from salt marsh and 1.25 M from agricultural soil clustered closely to the communities in the no-solute controls from salt marsh.

As seen in Figure 14 from agricultural soil samples, *Clostridia* and *Gammaproteobacteria* were more abundant in the no-solute control enrichments compared to in $MgCl_2$ and NaCl, whereas *Bacilli* were much more abundant in $MgCl_2$ and NaCl enrichments in comparison to the control. As shown in Figure 15 *Actinobacteria* and *Bacilli* in salt marsh were most abundant in the only $MgCl_2$ enrichment of 1 M. *Gammaproteobacteria* in salt marsh were more abundant in NaCl than in control enrichments.

Figures 16 and 17 show the mean proportion of sequences of genera in NaCl, $MgCl_2$ and the control for both environments. In agricultural soil, *Halobacillus* was the most abundant in $MgCl_2$ 1.25 M, followed by *Oceanobacillus* and *Halomonas*, but they were not present in $MgCl_2$ 1 M. *Bacillus* was present in $MgCl_2$ 1 M but not in 1.25 M. In salt marsh, *Sediminibacillus* and *Staphylococcus* were very abundant in 1 M $MgCl_2$ but not present in the no-solute control and NaCl. The presence of genera and proportion of sequences differed between environments.

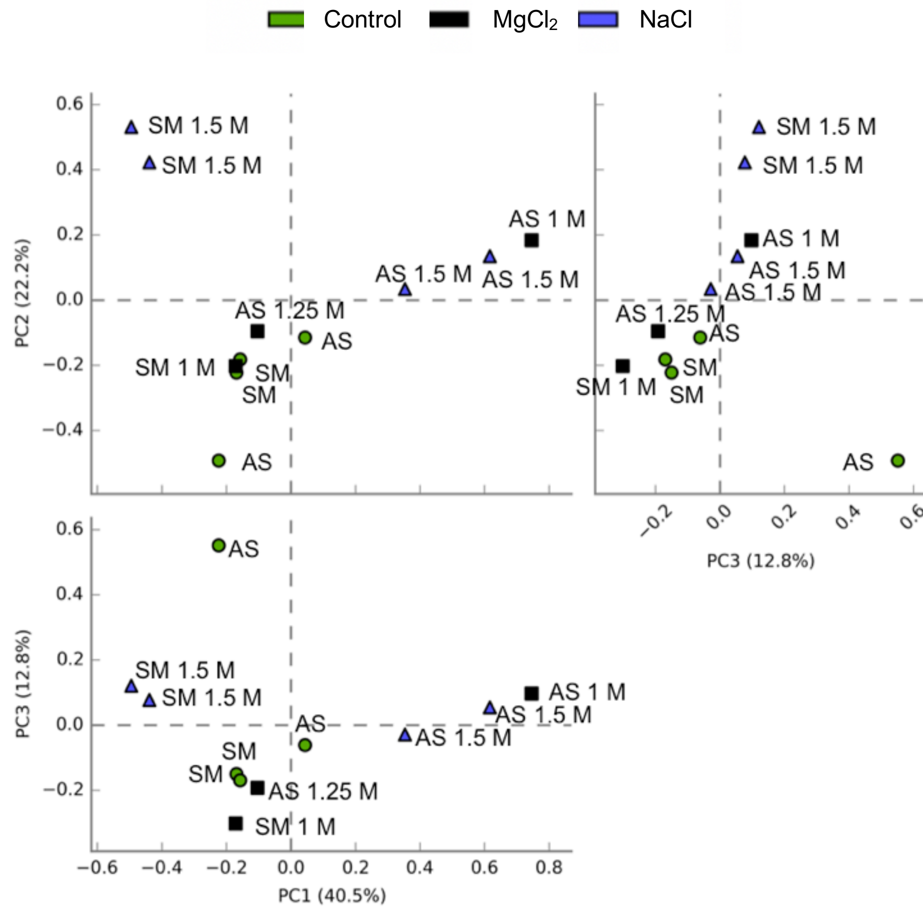


Figure 13. Principal component analysis (PCA) on bacterial communities in magnesium chloride ($MgCl_2$), sodium chloride (NaCl) and no-solute control enrichments from agricultural soil (AS) and salt marsh (SM) samples at the genera level. Enrichments of NaCl 1.5 M and $MgCl_2$ 1 M and 1.25 M were used, as shown on the figure with “AS” and “SM” representing location of sample (no concentration next to the location is the no-solute control). PC1, the first principle component, is the axis that found the highest level of variance through the 11 samples. The variance for each axis (principle component) is expressed as percentage (%). Plot produced in STAMP (Parks *et al.,* 2014).

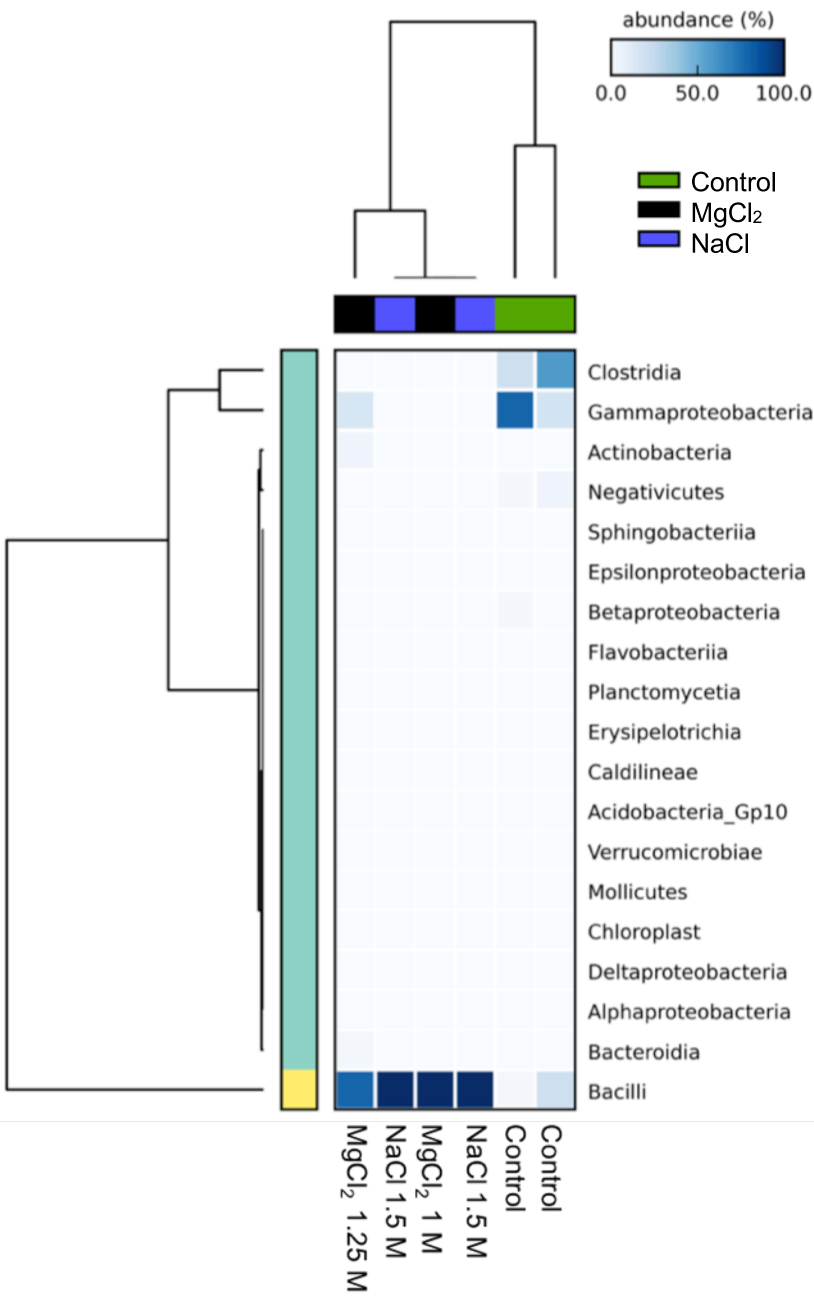


Figure 14. Comparison of bacterial communities in magnesium chloride ($MgCl_2$), sodium chloride (NaCl) and no-solute control enrichments from agricultural soil samples. Heatmap plot was generated using STAMP (Parks *et al.,* 2014).

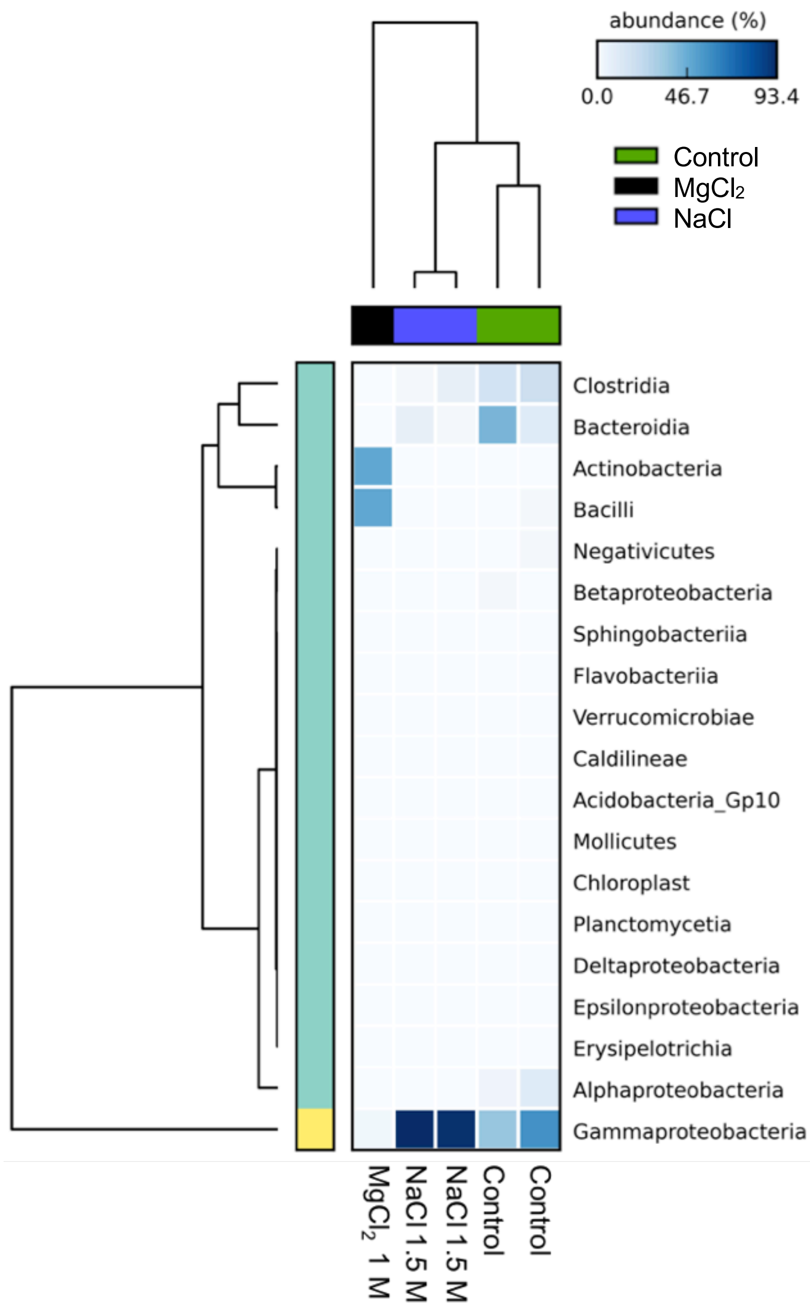


Figure 15. Comparison of bacterial communities in magnesium chloride ($MgCl_2$), sodium chloride (NaCl) and no-solute control enrichments from salt marsh samples. Heatmap plot was generated using STAMP (Parks *et al.,* 2014).

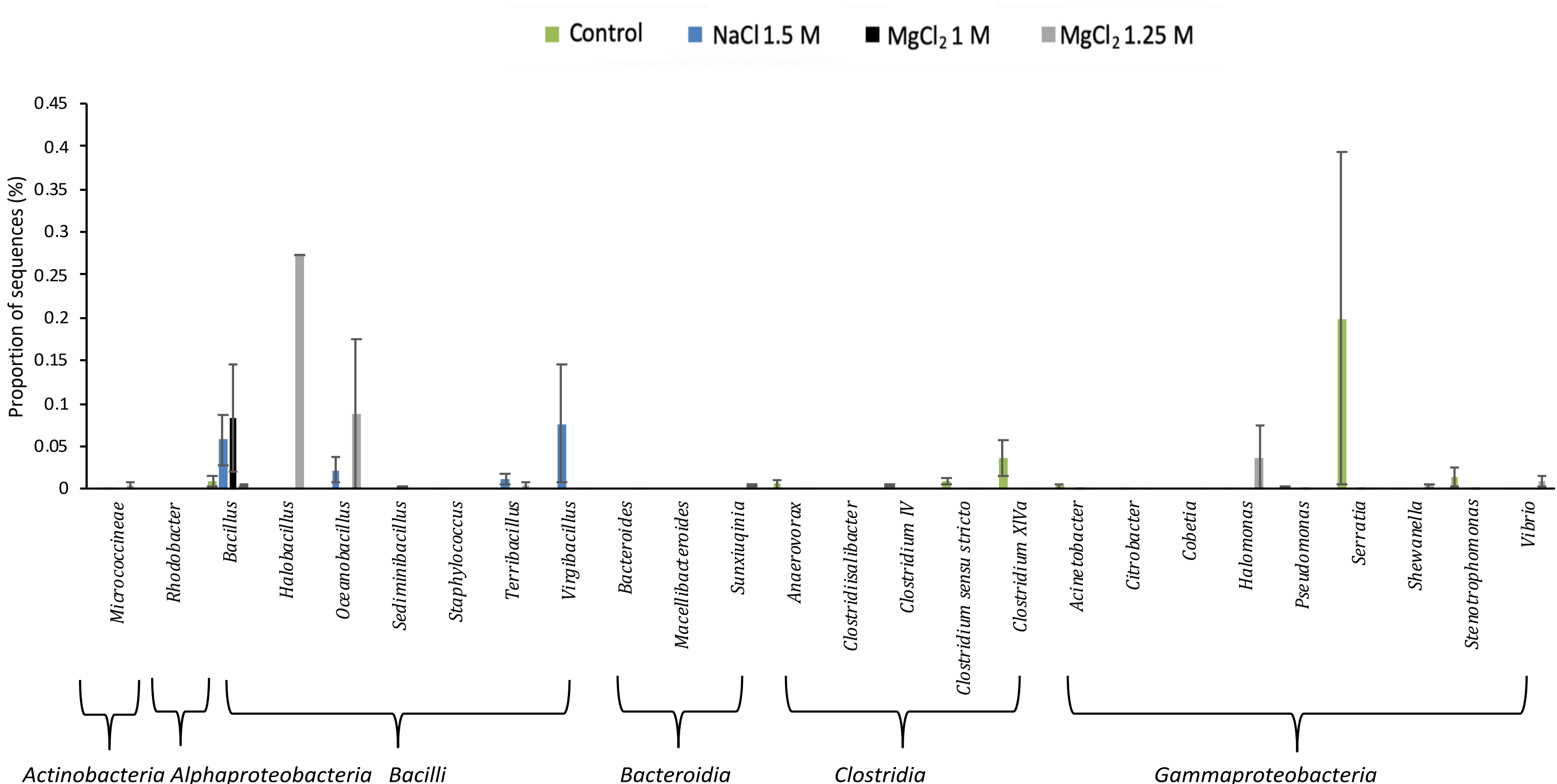


Figure 16. Mean proportion of sequences (%) of bacterial genera from agricultural soil in no solute control, sodium chloride and magnesium chloride. Error bars represent standard error, no control solute (n=2), sodium chloride 1.5 M (n=2), and magnesium chloride 1 M (n=1) and 1.25 M (n=1). Genera with a sum proportion of sequences below 5% were excluded from analysis.

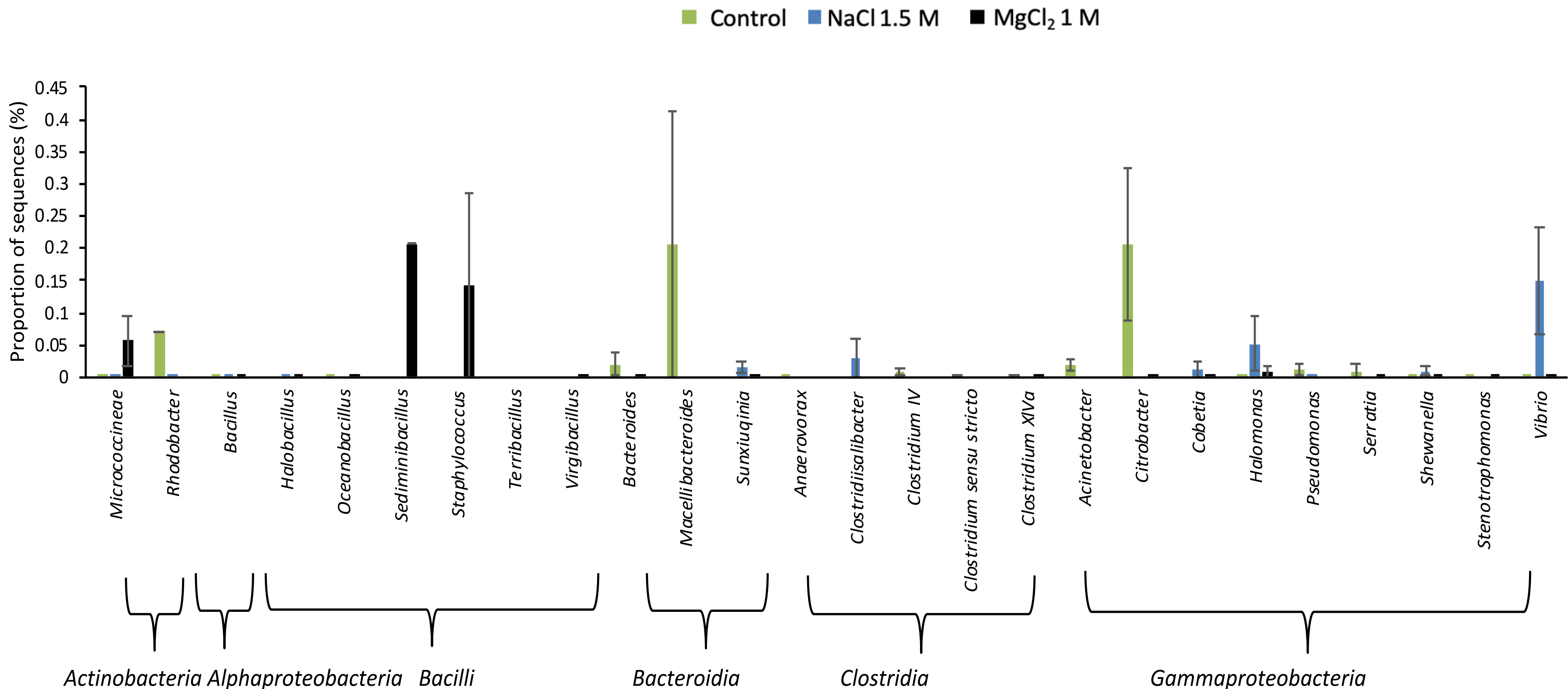


Figure 17. Mean proportion of sequences (%) of bacterial genera from salt marsh in no solute control, sodium chloride and magnesium chloride. Error bars represent standard error, no control solute (n=2), sodium chloride 1.5 M (n=2), and magnesium chloride 1 M (n=1). Genera with a sum proportion of sequences below 5% were excluded from analysis.

3.4 Isolated strains tested against magnesium chloride

3.4.1 Strain information

Sixteen strains from agricultural soil and salt marsh, and one strain from the Discovery basin were investigated by a range of different methods as summarised in Table 12. The highest magnesium chloride concentration to yield an isolate was 1.5 M, in which only fungal strains were isolated.

Table 12. Information of strains isolated from previous and current studies and the tests performed on each strain, where "a" refers to subculturing of strains, "b" sequencing of 16S rRNA and ITS sequences of strains, "c" high magnesium chloride experiment, "d" floating filters experiment and "e" microscopy.

Strain name[1]	**Bacteria or fungi[2]**	**Environment isolated from[3]**	**Solute and conc. of medium isolated from**	**Tests performed[4]**
Strains isolated from previous studies				
AS-Mg-Tm-1	Bacteria	Agricultural soil	$MgCl_2$ 1.1 M	a
AS-Mg-Tm-2	Bacteria	Agricultural soil	$MgCl_2$ 1.1M	a
SM-Mg-Tr-2	Bacteria	Salt marsh	$MgCl_2$ 1.1M	a
AS-Mg-Cn-3	Bacteria	Agricultural soil	$MgCl_2$ 1.1M	a
SM-Mg-Sn-2	Bacteria	Salt marsh	$MgCl_2$ 1.1M	a
DI-Mg-Ms-1	Fungi	Discovery basin	$MgCl_2$ 0.3 M	a
AS-Mg-Ml-1	Fungi	Agricultural soil	$MgCl_2$ 1.5M	a, c
SM-Mg-Pa-1	Bacteria	Salt marsh	$MgCl_2$ 1 M	a, b
SM-Mg-Pa-2	Bacteria	Salt marsh	$MgCl_2$ 1 M	a, b
Strains isolated in this study				
SM-Mg-Sv-1	Fungi	Salt marsh	$MgCl_2$ 1.5M	a, b, c, d, e
SM-Mg-Sv-2	Fungi	Salt marsh	$MgCl_2$ 1.5M	a, b, c
SM-Mg-Sv-3	Fungi	Salt marsh	$MgCl_2$ 1.5M	a, c, d
SM-Mg-Sv-4	Fungi	Salt marsh	$MgCl_2$ 1.5M	a, c, d, e
SM-Mg-Sv-5	Fungi	Salt marsh	$MgCl_2$ 1.5M	a, b, c
SM-Mg-Sv-6	Fungi	Salt marsh	$MgCl_2$ 1.25M	a, b, c, d, e
SM-Mg-Sv-7	Fungi	Salt marsh	$MgCl_2$ 1.25M	a
AS-Mg-Sv-1	Bacteria	Agricultural soil	$MgCl_2$ 1.25M	a, b, c

[1]where 'AS' represents agricultural soil, 'SM' represents salt marsh and 'DI' represents Discovery basin interface; 'Mg' represents magnesium chloride; 'Tm' and 'Sv' represent the initials of the person who isolated the strain.
[2]Confirmed by sequencing and/or PCR amplification.
[3]Agricultural soil and salt marsh environments in Wivenhoe, Essex, United Kingdom, and Discovery basin interface in the Mediterranean Sea (Hallsworth *et al.*, 2007).
[4] Please refer to sections 3.2.2 for sequencing results, 3.2.4 for high $MgCl_2$ concentration experiment, 3.2.5 for floating filter experiment and 3.2.6 for microscopy images.

3.4.2 Phylogenetic analysis of strains

Strains were analysed through BLAST in order to find closest relatives (Table 13) and a phylogenetic tree was constructed with bacterial strains and dominant OTUs from magnesium chloride enrichments (see Section 3.3.5) with their closest relatives to visualise evolutionary relationships, as shown in Figure 18. Strains SM-Mg-Pa-1 and SM-Mg-Pa-2 were closely related to *Arthrobacter arilaitensis* and *Planococcus maritimus* respectively, whereas the closest match for AS-Mg-Sv-1 was *Halobacillus hunanensis.* The remaining fungal isolates were closely matched to uncultured isolates, with the exception of SM-Mg-Sv-6 which had a close match to *Cladosporium*.

Table 13. BLAST results of isolated strains. Strains that have had their closest matches as clones have the clone's closest BLAST match and information directly underneath the clone.

Strain	Sequence length of strain (bp)	Closest match	Sequence length (bp)	Sequence similarity (%)	Environments found in	GenBank sequence ID
SM-Mg-Pa-1	1032	*Arthrobacter arilaitensis*	1519	100	Surface of cheese	NR_074608.1
SM-Mg-Pa-2	648	*Planococcus maritimus*	1507	99	Sea water of tidal flat	NR_025247.1
SM-Mg-Sv-1	91	Clone 321	358	96	Soil	MF568987.1
		Acremonium sp.	727	94	Plaster from a building	LT549084.1
SM-Mg-Sv-2	63	Isolate OTU 95	531	88	Infected coffee leaf	KT328827.1
		Kockovaella calophylli	522	95	Plant leaves	AB042227.1
SM-Mg-Sv-5	110	Clone Indiana_MOTU_LB_119 54	270	100	Soil	KT194725.1
		Rhodotorula sp.	559	89	Lichen	KU057818.1
SM-Mg-Sv-6	135	Cladosporium sp.	349	91	Wallpaper	HM172801.1
AS-Mg-Sv-1	694	*Halobacillus hunanensis*	1509	99	Subterranean brine from salt mine	NR_116655.1

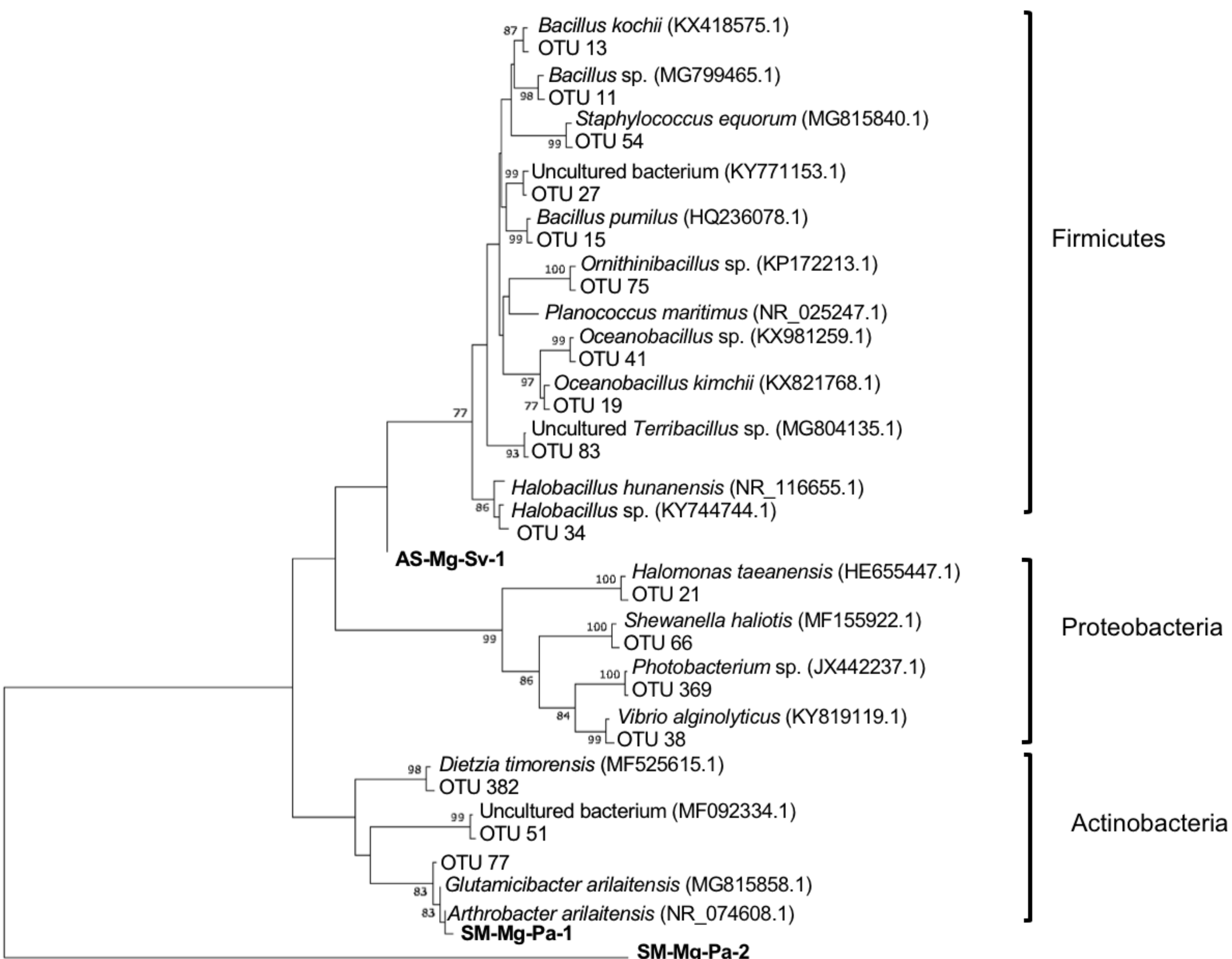


Figure 18. Phylogenetic tree based on 16S rRNA gene sequences of isolated strains AS-Mg-Sv-1, SM-Mg-Pa-1 and SM-Mg-Pa-2, dominant OTUs with more than 100 sequences in Magnesium chloride enrichments, and their closest relatives. Sequences (40) of 469 base pairs were aligned using MUSCLE and the tree constructed from the Neighbour-Joining method and Kimura 2-parameter model (gamma distribution where shape parameter= 1). Accession numbers of reference strains are included in brackets. Bootstrap values (500 replicates) above 75% are shown. Scale bar represents the number of substitutions per site.

3.4.3 Parameters of media used in further strain growth experiments

In order to determine the validity of the concentrations of media, pH, water activity and refractive index were measured for both sets of media used in the higher $MgCl_2$ concentration experiments and floating filter paper experiments. As shown in Figure 19 similar concentrations of media from both experiments appeared to be close in their figures and trends for water activity and refractive index, with the exception of the 2 M $MgCl_2$ media from the high $MgCl_2$ concentration experiments as seen in both parameters. There were differences in pH for the same concentrations between the two media, most noticeably at the no-solute control 0 M where the media for the high $MgCl_2$ concentration experiments had a pH of 5 in comparison to the pH of 7 in the floating filters media. Results involving the 2 M media in the high $MgCl_2$ concentration experiments had been excluded as the parameters tested indicate the assumed concentration of the media was not correct.

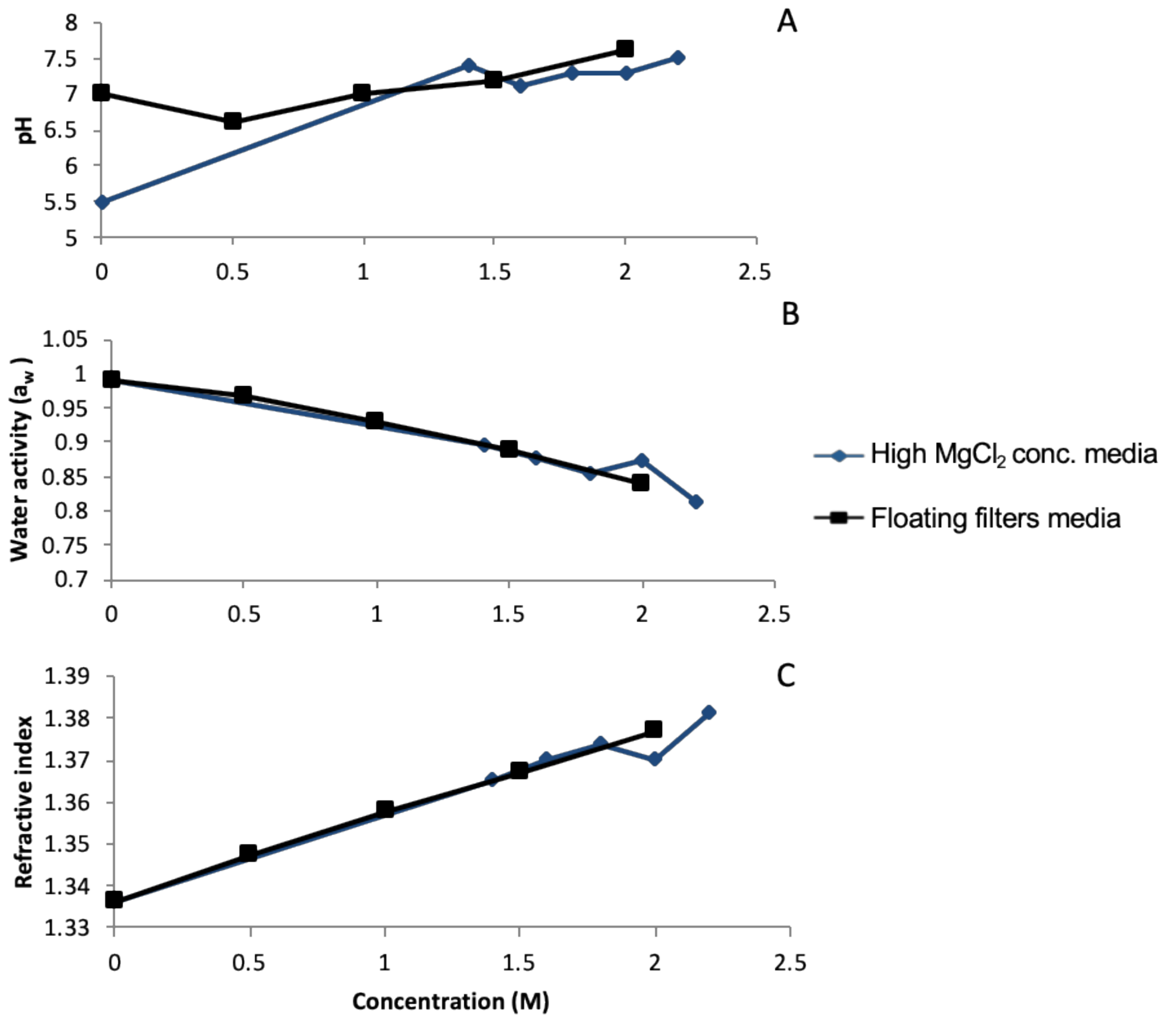


Figure 19. Physiochemical properties of magnesium chloride ($MgCl_2$). A) pH, B) water activity and C) refractive index for magnesium chloride ($MgCl_2$) across 0.5-2.2 M of media used in the high magnesium chloride ($MgCl_2$) and floating filter experiments. 0 M is the no-solute control. n=1.

3.4.4 Strains at high $MgCl_2$ concentrations in liquid enrichments

Strains were inoculated into liquid enrichments at high $MgCl_2$ concentrations to test whether they were able to grow beyond the concentrations they were isolated from and to determine if these strains could be classified as chaotolerant or chaophilic. Growth was confirmed from continuous observations of change in turbidity and/or the presence and growth of flocks after inoculation. As shown in Table 14, at least half of the strains tested did not show signs of growth. However, strain SM-Mg-Sv-1 grew in all concentrations, potentially including in 2.2 M, and strain SM-Mg-Sv-6 grew up-to and including $MgCl_2$ 1.8 M. Strains SM-Mg-Sv-4 and AS-Mg-Ml-1 grew consistently until 1.4 and 1.6 M respectively but strain SM-Mg-Sv-4 had one replicate growing at 1.6 M and 1.8 M. Figure 20 also show size and frequency of flocks of SM-Mg-Sv-1 decreased as concentrations increased.

Table 14. Signs of growth represented as "+" and no growth as "-" for strains in magnesium chloride ($MgCl_2$) concentrations from 1.4-2.2 M and a no-solute control, taken 28 days after inoculation with three replicates at each concentration.

							$MgCl_2$								
Strain	**Control**			**1.4 M**			**1.6 M**			**1.8 M**			**2.2 M**		
SM-Mg-Sv-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
SM-Mg-Sv-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SM-Mg-Sv-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SM-Mg-Sv-4	+	+	+	+	+	+	-	+	-	-	+	-	-	-	-
SM-Mg-Sv-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SM-Mg-Sv-6	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
AS-Mg-Sv-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AS-Mg-Ml-1	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-

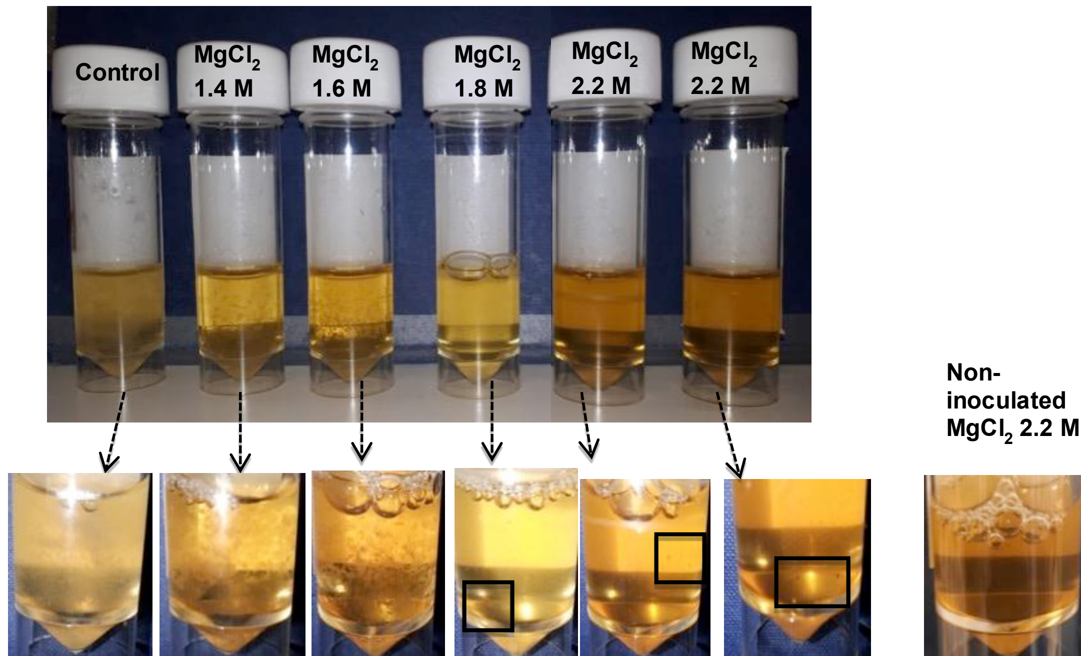


Figure 20. Images of strain SM-Mg-Sv-1 in magnesium chloride concentrations from no-solute control to 2.2 M magnesium chloride ($MgCl_2$). The boxes indicate the location of where fungal growth is suspected. A non-inoculated $MgCl_2$ 2.2 M is shown for reference.

3.4.5 Growth of strains on floating filter papers

Strains were grown on filter papers floating on $MgCl_2$ liquid media to quantitatively assess growth over increasing concentrations of $MgCl_2$. Figure 21 shows the mean diameter of colonies growing on filter papers from no-solute control to 2 M $MgCl_2$. All four strains decreased in diameter size as concentrations of $MgCl_2$ increased, however strains SM-Mg-Sv-1 and SM-Mg-Sv-6 grew in 1.5 and 2 M, unlike strains SM-Mg-Sv-3 and SM-Mg-Sv-4. Overall strain SM-Mg-Sv-6 showed the highest growth across the concentrations but grew the most in the no-solute control, which one-way ANOVA and Tukey HSD determined to be statistically significantly different compared to the diameters of the other strains at the control ($F_{3,8}$= 14.66, p<0.001). All three other strains had the largest diameters in $MgCl_2$ 0.5 M. The diameters of strains SM-Mg-Sv-1 and SM-Mg-Sv-6 at 1 and 1.5 M were determined as significantly different compared to the diameters of all other strains, but no significant difference was determined at 0.5 and 2 M for any strain. Appendix D1-8 contain One-way ANOVA and Tukey HSD analysis between

diameter of strains. Figure 22 demonstrates the decrease in colony size of SM-Mg-Sv-6 as $MgCl_2$ concentrations increased but still showed growth at 2 M.

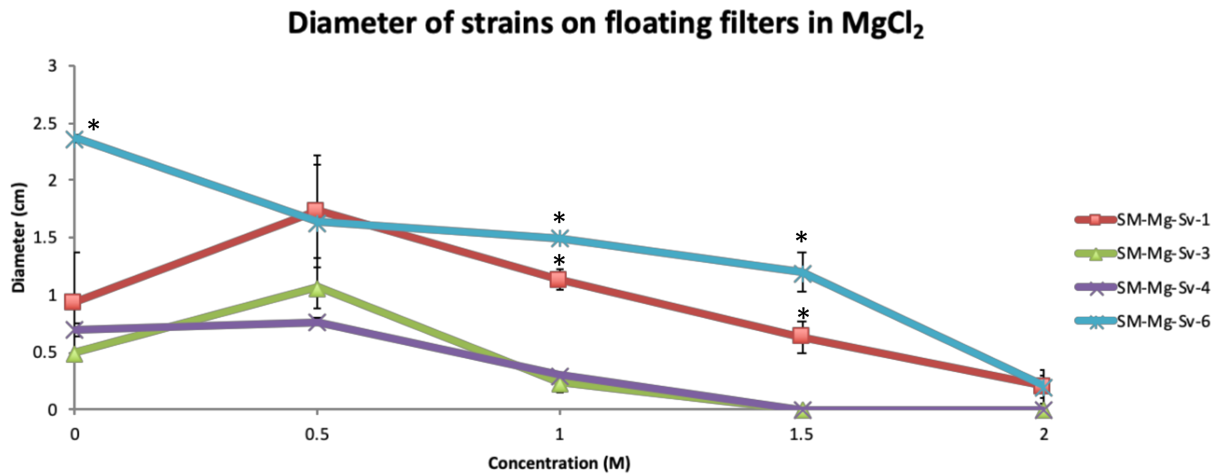


Figure 21. Mean diameters (cm) of strain colonies on floating filter papers in concentrations of magnesium chloride between 0.5-2 M, 14 days after inoculation. 0 M is the no-solute control. n=3. * above a strain represent significant difference (p<0.05) between that strain and the other strains at that concentration as determined by one-way ANOVA and Tukey HSD.

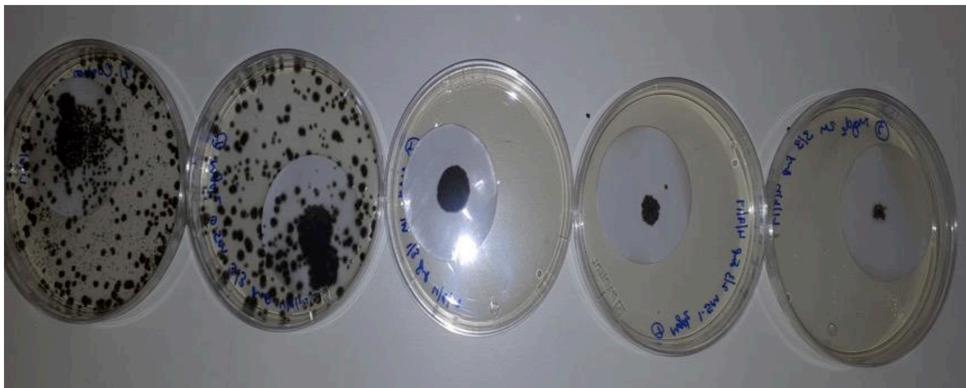


Figure 22. Images of SM-Mg-Sv-6 on floating filter papers in concentrations from left to right: no-solute control, magnesium chloride 0.5 M, 1 M, 1.5 M and 2 M, 2 weeks after inoculation.

3.4.6 Microscopy of strains

Fungal strains SM-Mg-Sv-1, SM-Mg-Sv-4 and SM-Mg-Sv-6 were viewed under a microscope and had hyphae widths respectively at approximately 10, 37 and 40 µm, shown in In Figures 23-25. At x1000 magnification, hyphae were observed for all strains with septa showing for SM-Mg-Sv-4 and SM-Mg-Sv-6. Sporangia were seen at x100 and x1000 magnification for SM-Mg-Sv-4.

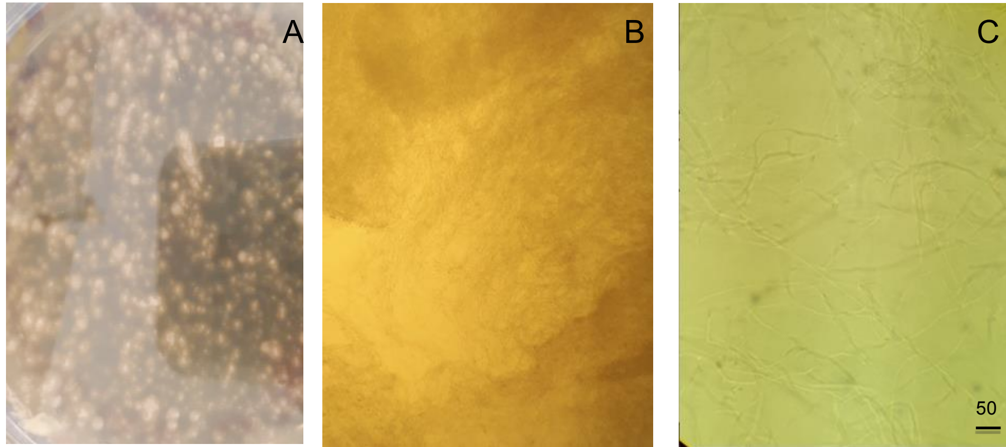


Figure 23. Strain SM-Mg-Sv-1 on A) a 1.5 M magnesium chloride ($MgCl_2$) plate, B) viewed under an Olympus BH2 microscope at x100 magnification and C) viewed at x1000 magnification, using colonies from a liquid enrichment of 1.25 M $MgCl_2$.

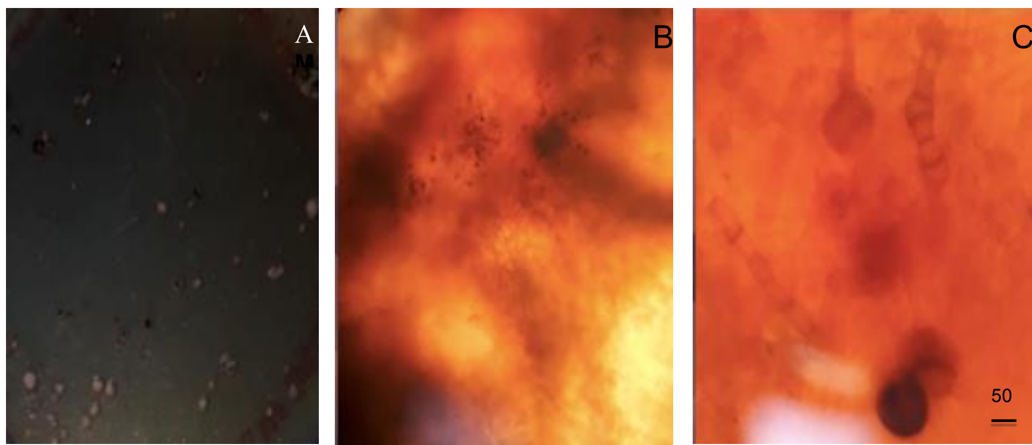


Figure 24. Strain SM-Mg-Sv-4 on A) a 1.5 M magnesium chloride ($MgCl_2$) plate, B) viewed under an Olympus BH2 microscope at x100 magnification and C) viewed at x1000 magnification, using colonies from 1.5 M $MgCl_2$ which were suspended in sterile 1.4 M $MgCl_2$.

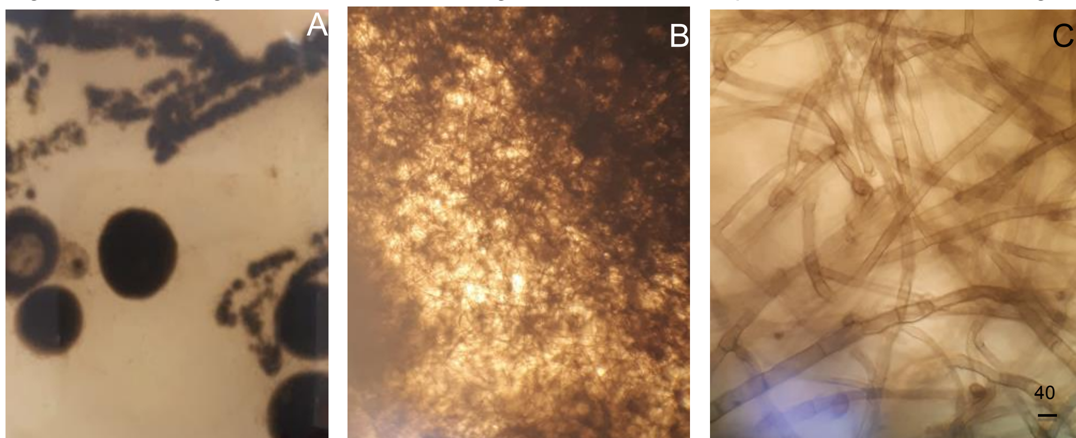


Figure 25. Strain SM-Mg-Sv-6 on A) a 1.25 M magnesium chloride ($MgCl_2$) plate, B) viewed under an Olympus BH2 microscope at x100 magnification and C) viewed at x1000 magnification, using colonies from a liquid enrichment of 1 M $MgCl_2$.

4 Discussion

Chaotropic agents are widespread in environments and encountered by microbes, but there is little focus on chaotropicity in microbiology. Chaotropic magnesium chloride in particular, severely limits growth at high concentrations but only a few studies have examined microbial growth in $MgCl_2$. This study was needed to further advance knowledge on the effect of magnesium chloride on microbial growth and community composition. Numerous methods ranging from sequencing to solid and liquid enrichments were employed to assess growth and analyse community composition at different magnesium chloride concentrations. The findings of this study are discussed here.

4.1 The physiochemical properties of $MgCl_2$ and NaCl

The pH, refractive index and water activity ensured the validity of the concentrations of the growth media, apart from 2 M $MgCl_2$ media of the high magnesium chloride concentration experiment which had to be omitted. This media may have been prepared incorrectly to not have achieved the desired concentration.

Testing the physiochemical properties were also useful in showing the effect of concentration change and the differences between the two salts. Water activity and pH are also factors affecting microbial growth and thus how the salts affect them are important to consider.

4.2 Effect of concentration of $MgCl_2$ on growth

Results from the viable counts, PCR amplifications, species richness and strain experiments accepted the hypotheses that increased concentrations of $MgCl_2$ would lead to a decrease in microbial growth and diversity, and that $MgCl_2$ would have less growth and diversity in comparison to NaCl at the same concentrations. However, analysis following Illumina sequencing showed surprising results where the genera *Halobacillus, Oceanobacillus* and *Halomonas* from agricultural soil were abundant in 1.25 M $MgCl_2$ but not present in the lower

tested $MgCl_2$ concentration of 1 M. These genera also had low abundance in the 1 M $MgCl_2$ salt marsh enrichment. One possible explanation behind this is that these genera could be outcompeted by others such as *Bacillus* in 1 M, but these microbes could not tolerate or thrive in higher chaotropic stress, leading to less competition and more nutrient availability, for *Halobacillus, Oceanobacillus* and *Halomonas* to exploit, who have a higher chaotolerance than their competitors.

Alternatively, it could be suggested that the species from the genera required a certain level of chaotropicity achieved at 1.25 M and potentially above for growth, but with only two tested concentrations and without replicates, this cannot be concluded. Furthermore, Tregoning *et al.* (2015) discovered that isolated strains related to *Halomonas* from the Lake Vanda brine in Antarctica, a highly chaotropic environment rich in $CaCl_2$ and $MgCl_2$, tolerated $CaCl_2$ and did not require the salt for growth. Future work should involve sequencing enrichments over a range of $MgCl_2$ concentrations with replicates for a more reliable analysis of change in community composition. In addition, since species from these genera have been identified as halophiles (Amoozegar *et al.*, 2003; Kim *et al.*, 2015), it may be of interest to study the specific *Halobacillus, Oceanobacillus* and *Halomonas* species identified from Illumina in $MgCl_2$ growth experiments to determine if they prefer $MgCl_2$ over NaCl, and whether any require $MgCl_2$ for growth.

4.3 Chaotropicity, not water activity, is a limiting factor on growth in $MgCl_2$

Water activity has achieved a lot of recognition due to its effect on microbial growth and consequently, its important application in food preservation (reviewed in Sperber, 1983). Studies over the past few decades have shown different minimal water activity limits, depending on the organism (Fontana, 2007; Stevenson *et al*., 2015b; Stevenson *et al*., 2017).

In this study, magnesium chloride has shown its ability in reducing water activity, in comparison to sodium chloride. More importantly, results from the viable counts showed water activity is not

a factor in limiting growth in $MgCl_2$ but suggested the chaotropic property of the salt is. Considering $MgCl_2$ has a greater chaotropicity value at +54 kJ kg^{-1} $mole^{-1}$ than NaCl (-11 kJ kg^{-1} $mole^{-1}$) (Cray *et al*., 2013a), these findings support Hallsworth *et al.* (2007) and Williams and Hallsworth (2009) who argue the chaotropic property of $MgCl_2$ limits growth rather than water activity.

4.4 DGGE did not show expected results in community compositions

Enrichments and replicates of similar concentrations of the same solute were expected to have shown similar community compositions based on the assumptions that large differences would not exist between biological replicates and that the nature of the solute would favour specific organisms. However, the results from the DGGE did not show similar community compositions between replicates or similar concentrations of the same solute and the results were inconclusive. This may be explained by stochasticity where variations in communities are caused by drift or birth and death, unlike deterministic processes such as environmental variables and interspecies interactions (Zhou *et al*., 2013; Vellend *et al*., 2014; reviewed in Zhou and Ning, 2017). Another explanation is that the methodology of manually creating presence/absence matrices using the eye is prone to human error. Software for analysing DGGE profiles could be used instead which would also be less time-consuming, but this may not be an available option due to the cost of the software. Nevertheless, the DGGEs were still useful in indicating and comparing species richness between salts and concentrations.

4.5 Are there any differences between salt marsh and agricultural soil microbes in $MgCl_2$?

Viable counts suggested no significant difference between location, with the exception at 3 M NaCl. However, PCR amplification specifically showed fungal distribution differed between the two environmental samples, and all seven fungal strains that had been isolated in this study for their ability to grow above $MgCl_2$ 1.25 M came from salt marsh. This suggests fungi in salt marsh are more chaotolerant than agricultural soil fungi. Since Bacteria and Archaea from salt

marsh did not outperform their counterparts from agricultural soil in $MgCl_2$, it may be argued the difference in fungi is not due to the factors in the environment that otherwise would have shown more consistent results.

Furthermore, principal component analysis from Illumina sequencing showed that the bacterial community from agricultural soil in 1.25 M $MgCl_2$ was more closely related to that of salt marsh in 1 M, than to the community from agricultural soil in 1 M. An explanation for this could be that the communities between the replicates from agricultural soil varied at the time of sampling and thus the chaotropic conditions selected for different organisms between the replicates. Future Illumina analysis should include testing and comparing communities from the same biological sample first, and then between replicates.

In addition, bacterial community profiles revealed the community compositions differed between locations for all solutes but huge variations at the OTU level and a limited number of replicates question the reliability of these findings.

The hypothesis that there would be more salt marsh microbes in high $MgCl_2$ concentrations is partially supported due to salt marsh fungi, but overall results do not show differences between community compositions from the two locations in the presence of magnesium chloride.

4.6 Chaophilic and chaotolerant fungi

Fungi had the lowest species richness compared to Bacteria and Archaea, yet fungi from salt marsh dominated in $MgCl_2$ enrichments and were undetected by PCR in no-solute control or sodium chloride enrichments, suggesting these fungi are chaophiles through showing a clear preference for chaotropic conditions. Sequencing of DGGE bands from salt marsh $MgCl_2$ enrichments revealed an *Acremonium* related species in 1.5 M and *Baeospora myosura* in 1.75 M. Very little literature exists on *Baeospora myosura*, and the species has not so far been

examined under different stress conditions. It may be of interest to specifically study strains of *Baeospora myosura* in $MgCl_2$ to determine whether the species is chaophilic.
Further testing of fungal strains showed growth in high $MgCl_2$ concentrations and in the no-solute controls, which describe chaotolerance as the strains did not show a clear preference for chatropic conditions. Despite this, the chaotolerance of two strains, SM-Mg-Sv-1 (Clone 321 closely related to *Acremonium*) and SM-Mg-Sv-6 (closely related to *Cladosporium*), stood out in particular. Zajc *et al*. (2014a) also showed chaotolerance in fungal strains, however only tested one *Acremonium* species in comparison to 23 *Cladosporium* strains, out of an overall of 135 tested strains. The chaotolerance observed in SM-Mg-Sv-6 were consistent with their tested *Cladosporium* strains, however SM-Mg-Sv-1 grew at higher $MgCl_2$ concentrations than their only tested *Acremonium* strain. Therefore, this study argues the genus *Acremonium* should have a higher focus and importance in future research on the chaotolerance of fungi, based on the findings of chaophilic and highly chaotolerant *Acremonium* species in this study.

Fungal chaotolerance may be explained by the similar use of adaptations in halophilic fungi, such as the production of compatible solutes and cell-wall melanisation (Kogej *et al*., 2007; reviewed in Gostinčar *et al*., 2011). Studying these strategies of chaotolerant fungi in chaotropic conditions against non-chaotropic conditions may offer insight into the specific adaptations behind chaotolerance, which is currently not known.

4.7 The limits of life defined by $MgCl_2$

Previous research by van der Wielen *et al.* (2005), Hallsworth *et al.* (2007) and Zajc *et al*. (2014a) contributed significantly through redefining the limits of life set by magnesium chloride. Strains were further tested in this study to challenge the upper concentration limits of $MgCl_2$.

In the absence of compatible solutes, strains SM-Mg-Sv-1 and SM-Mg-Sv-6 grew in 2 M and SM-Mg-Sv-1 had potential growth in two replicates in 2.2 M. Growth became more difficult to confirm in the liquid enrichments as the size of the flocks were decreasing with increasing

concentration. Growth was easier to visualise and measure on the floating filter papers, but the highest concentration tested was 2 M and thus cannot corroborate the growth in 2.2 M in liquid enrichments. Further testing, such as subcultures, amplification, higher concentrations for filter paper experiments, is needed to confirm growth in 2.2 M. This would be a significant contribution if confirmed as 2.2 M would be the highest $MgCl_2$ concentration where growth is possible and would also be the closest finding yet to the current 2.3 M upper limit of life.

4.8 Implications

The findings from this study contribute to a field that is not well understood, by identifying new chaophilic and chaotolerant microbes, and highlighting potential directions for future research. The study of chaotropicity on the growth and abundance of microbes is important and relevant as many environments on Earth are chaotropic, and this stress limits life at high concentrations. Identifying and improving understanding on microbes able to tolerate or thrive in high chaotropic conditions would not only redefine the set limits of where life is possible but would also have vast implications in other fields.

Astrobiology is one such example where research has focused on the potential habitability of planets and growing microbes under stimulated planetary conditions (Fajardo-Cavazos *et al.*, 2018; Stevens *et al.*, 2019). It could be possible that salty bodies of water, such as martian brines (Jones *et al.*, 2011), contain chaotropic salts, where arguably chaophiles and chaotolerant organisms could exist. This would be significant as it could prove the existence of alien life, or the possibility of Earth lifeforms surviving on other planetary bodies. In addition, the growth of chaophiles could be tested under stimulated planetary conditions. Furthermore, chaophiles or chaotolerant microbes may also have to be considered as contamination risks, from and to Earth.

Chaotropicity may also be considered in food microbiology in the interests of food preservation. Known chaotolerant fungi may be able to survive in low water-activity environments without the

presence of chaotropes, and therefore could be tested to predict the shelf life of certain food (Gock *et al*., 2003). Alternatively, chaotropes may be used to inhibit the growth of microbes that cause spoilage, such as ethanol extract from cranberry pomace (Tamkutė *et al*., 2019), which may be appealing to consumers who prefer natural preservatives.

Alternatively, studying chaotolerant organisms from non-extreme environments, as was done in this study with salt marsh and agricultural soil, may offer insights into how these organisms survive in their own environments when faced with chaotropic stress.

4.9 Concluding remarks

This study shows the effect of chaotropic magnesium chloride on microbial growth where growth and diversity decrease as concentrations of magnesium chloride increase. Whilst conclusions could not be drawn regarding change in community composition, and the growth between environments overall did not differ in $MgCl_2$, it was discovered salt marsh fungi are able to tolerate high concentrations of magnesium chloride, with a strain potentially growing in 2.2 M, but this needs to be confirmed with more testing. Further studies are needed for a better understanding of the adaptations and mechanisms behind the survival of chaotolerant species in high $MgCl_2$ concentrations.

References

Adam, P. 1993. *Saltmarsh Ecology.* Cambridge: Cambridge Studies in Ecology, [pagination unknown].

Albano, E. 2006. Alcohol, oxidative stress and free radical damage. *Proceedings of the Nutrition Society*, **65**, 278-290.

Alcaide, M., Stogios, P. J., Lafraya, A., Tchigvintsev, A., Flick, R., Bargiela, R., Chernikova, T. N., Reva, O. N., Hai, T., Leggewie, C. C., Katzke, N., La Cono, V., Matesanz, R., Jebbar, M., Jaeger, K. E., Yakimov, M. M., Yakunin, A. F., Golyshin, P. N., Golyshina, O. V., Savchenko, A., Ferrer, M. & The MAMBA Consortium. 2015. Pressure adaptation is linked to thermal adaptation in salt-saturated marine habitats. *Environmental Microbiology*, **17**, 332-345.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, **215**, 403-410.

Amoozegar, M. A., Malekzadeh, F., Malik, K. A., Schumann, P. & Spröer, C. 2003. *Halobacillus karajensis* sp. nov., a novel moderate halophile. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 1059-1063.

Baetz, U. & Martinoia, E. 2014. Root exudates: the hidden part of plant defense. *Trends in Plant Science*, **19**, 90-98.

Ball, P. & Hallsworth, J. E. 2015. Water structure and chaotropicity: their uses, abuses and biological implications. *Physical Chemistry Chemical Physics*, **17**, 8297-8305.

Bhaganna, P., Volkers, R. J. M., Bell, A. N. W., Kluge, K., Timson, D. J., McGrath, J. W., Ruijssenaars, H. J. & Hallsworth, J. E. 2010. Hydrophobic substances induce water stress in microbial cells. *Microbial Biotechnology*, **3**, 701-716.

Bhaganna, P., Bielecka, A., Molinari, G., Hallsworth, J. E. 2016. Protective role of glycerol against benzene stress: insights from the *Pseudomonas putida* proteome. *Current Genetics*, **62**, 419-429.

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W. & Stetter, K. O. 1997. *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. *Extremophiles*, **1**, 14-21.

Bridges, J. C. & Schwenzer, S. P. 2012. The nakhlite hydrothermal brine on Mars. *Earth and Planetary Science Letters*, **359-360**, 117-123.

Brock, T. D. & Freeze, H. 1969. *Thermus aquaticus* gen. n. and sp. n., a Nonsporulating Extreme Thermophile. *Journal of Bacteriology*, **98**, 289-297.

Chien, A., Edgar, D. B. & Trela, J. M. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *Journal of Bacteriology*, **127**, 1550-1557.

Collins, K. D. 1997. Charge density-dependent strength of hydration and biological structure. *Biophysical Journal*, **72**, 65-76.

Cray, J. A., Russell, J. T., Timson, D. J., Singhal, R. S. & Hallsworth, J. E. 2013a. A universal measure of chaotropicity and kosmotropicity. *Environmental Microbiology*, **15**, 287-296.

Cray, J. A., Bell, A. N. W., Bhaganna, P., Mswaka, A. Y., Timson, D. J. & Hallsworth, J. E. 2013b. The biology of habitat dominance; can microbes behave as weeds? *Microbial Biotechnology*, **6**, 453-492.

Cray, J. A., Stevenson, A., Ball, P., Bankar, S. B., Eleutherio, E. C. A., Ezeji, T. C., Singhal, R. S., Thevelein, J. M., Timson, D. J. & Hallsworth, J. E. 2015. Chaotropicity: a key factor in product tolerance of biofuel-producing microorganisms. *Current Opinion in Biotechnology*, **33**, 228-259.

Dartnell, L. 2011. Biological constraints on habitability. *Astronomy & Geophysics*, **52**, 1.25–1.28.

Domínguez-Cuevas, P., González-Pastor, J. E., Marqués, S., Ramos, J. L. & de Lorenzo, V. 2006. Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. *The Journal of Biological Chemistry*, **281**, 11981-11991.

Duda, V. I., Danilevich, V. N., Suzina, N. E., Shorokhova, A. P., Dmitriev, V. V., Mokhova, O. N. & Akimov, V. N. 2004. Changes in the Fine Structure of Microbial Cells Induced by Chaotropic Salts. *Microbiology*, **73**, 341-349.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

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

Elleuche, S., Schäfers, C., Blank, S., Schröder, C. & Antranikian, G. 2015. Exploration of extremophiles for high temperature biotechnological processes. *Current Opinion in Microbiology*, **25**, 113-119.

Fajardo-Cavazos, P., Morrison, M. D., Miller, K. M., Schuerger, A. C. & and Nicholson, W. L. 2018. Transcriptomic responses of *Serratia liquefaciens* cells grown under simulated Martian conditions of low temperature, low pressure, and CO_2-enriched anoxic atmosphere. *Scientific Reports*, **8**, 14938.

Fontana, A. J. 2007. Appendix D: minimum water activity limits for growth of microorganisms. In Water Activity in Foods: Fundamentals and Applications, edited by G.V. Barbosa-Canovas, A.J. Fontana, S.J. Schmidt, & T.P. Labuza. Oxford: Blackwell Publishing, 405.

Fox-Powell, M. G., Hallsworth, J. E., Cousins, C. R. & Cockell, C. S. 2016. Ionic Strength Is a Barrier to the Habitability of Mars. *Astrobiology*, **16**, 427-442.

Gock, M. A., Hocking, A. D., Pitt, J. I., & Poulos, P. G. 2003. Influence of temperature, water activity and pH on growth of some xerophilic fungi. *International Journal of Food Microbiology*, **81**, 11–19.

Gostinčar, C., Lenassi, M., Gunde-Cimerman, N., & Plemenitaš, A. 2011. Fungal Adaptation to Extremely High Salt Concentrations. *Advances in Applied Microbiology*, **77**, 71–96.

Glass, N.L. & Donaldson, G.C. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology*, **61**, 1323–1330.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G. & Bailey, M. J. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Applied and Environmental Microbiology*, **66**, 5488-5491.

Hallsworth, J. E. 1998. Ethanol-induced water stress in yeast. *Journal of Fermentation and Bioengineering*, **85**, 125-137.

Hallsworth, J. E., Heim, S. & Timmis, K. N. 2003. Chaotropic solutes cause water stress in *Pseudomonas putida*. *Environmental Microbiology*, **5**, 1270-1280.

Hallsworth, J. E., Yakimov, M. M., Golyshin, P. N., Gillion, J. L. M., D'Auria, G., de Lima Alves, F., La Cono, V., Genovese, M., McKew, B. A., Hayes, S. L., Harris, G., Giuliano, L., Timmis, K. N. & McGenity, T. J. 2007. Limits of life in $MgCl_2$-containing environments: chaotropicity defines the window. *Environmental Microbiology*, **9**, 801-813.

Hamaguchi, K. & Geiduschek, E. P. 1962. The effect of electrolytes on the stability of the deoxyribonucleate helix. *Journal of the American Chemical Society*, **84**, 1329-1338.

Hanson, C. A., Fuhrman, J. A., Horner-Devine, M. C. & Martiny, J. B. H. 2012. Beyond biogeographic patterns: processes shaping the microbial landscape. *Nature Reviews Microbiology*, **10**, 497-506.

Hofmeister, F. 1888. *Archiv für Experimentelle Pathologie und Pharmakologie*, **24**, 247-260.

Hua, L., Zhou, R., Thirumalai, D. & Berne, B. J. 2008. Urea denaturation by stronger dispersion interactions with proteins than water implies a 2-stage unfolding. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 16928-16933.

Hubert, C., Loy, A., Nickel, M., Arnosti, C., Baranyi, C., Brüchert, V., Ferdelman, T., Finster, K., Christensen, F. M., Rosa de Rezende, J., Vandieken, V. & Jørgensen, B. B. 2009. A constant flux of diverse thermophilic bacteria into the cold Arctic seabed. *Science*, **325**, 1541-1544.

Illumina. [Date unknown]. *16S Metagenomic Sequencing Library Preparation*. Retrieved from https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

Jančič, S., Zalar, P., Kocev, D., Schroers, H. J., Džeroski, S. & Gunde-Cimerman, N. 2016. Halophily reloaded: new insights into the extremophilic life-style of *Wallemia* with the description of *Wallemia hederae* sp. nov. *Fungal Diversity*, **76**, 97-118.

Jones, E. G., Lineweaver, C. H. & Clarke, J. D. 2011. An Extensive Phase Space for the Potential Martian Biosphere. *Astrobiology*, **11**, [pagination unknown].

Joshi, N. A., Fass, J. N. 2011. Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files. Retrieved from https://github.com/najoshi/sickle

Kallmeyer, J., Pockalny, R., Adhikari, R. R., Smith, D. C. & D'Hond, S. 2012. Global distribution of microbial abundance and biomass in subseafloor sediment. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 16213-16216.

Kearns, P. J., Angell, J. H., Howard, E. M., Deegan, L. A., Stanley, R. H. R. & Bowen, J. L. 2016. Nutrient enrichment induces dormancy and decreases diversity of active bacteria in salt marsh sediments. *Nature Communications*, **7**, 12881.

Kim, W., Siamphan, C., Kim, J. H. & Sukhoom, A. 2015. *Oceanobacillus arenosus* sp. nov., a moderately halophilic bacterium isolated from marine sand. *International Journal of Systematic and Evolutionary Microbiology,* **65**, 2943–2948.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, **41**, e1.

Kogej, T., Stein, M., Volkmann, M., Gorbushina, A. A., Galinski, E. A. & Gunde-Cimerman, N. 2007. Osmotic adaptation of the halophilic fungus *Hortaea werneckii*: role of osmolytes and melanization. *Microbiology*, **153**, 4261-4273.

Kumar S., Stecher G., and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, **33**, 1870-1874.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 6955–6959.

Lievens, B., Hallsworth, J. E., Pozo, M. I., Belgacem, Z. B., Stevenson, A., Willems, K. A. & Jacquemyn, H. 2015. Microbiology of sugar-rich environments: Diversity, ecology and system constraints. *Environmental Microbiology*, **17**, 278–298.

de Lima Alves, F., Stevenson, A., Baxter, E., Gillion, J. L. M., Hejazi, F., Hayes, S., Morrison, I. E. G., Prior, B. A., McGenity, T. J., Rangel, D. E. N., Magan, N., Timmis, K. N. & Hallsworth, J. E. 2015. Concomitant osmotic and chaotropicity-induced stresses in *Aspergillus wentii*: compatible solutes determine the biotic window. *Current Genetics*, **61**, 457-477.

Lo Nostro, P., Ninham, B. W., Lo Nostro, A., Pesavento, G., Fratoni, L. & Baglioni, P. 2005. Specific ion effects on the growth rates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *Physical Biology*, **2**, 1-7.

Low-Décarie E., Fussmann, G. F., Dumbrell, A. J. & Bell, G. 2016. Communities that thrive in extreme conditions captured from a freshwater lake. *Biology Letters*, **12**, 20160562.

Marion, G. M., Fritsen, C. H., Eicken, H. & Payne, M. C. 2003. The search for life on Europa: limiting environmental factors, potential habitats, and Earth analogues. *Astrobiology*, **3**, 785-811.

Masella, A. P., Bartram, A. K., Truszkowski, J. M., Brown, D. G. & Neufeld, J. D. 2012. PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics*, **13**, 31.

McGenity, T. J. & Oren, A. 2012. Hypersaline environments. In Life at Extremes: Environments, Organisms and Strategies for Survival, edited by E. M. Bell. Wallingford: CABI International, 402–437.

McKay, C. P., Anbar, A. D., Porco, C. & Tsou, P. 2014. Follow the Plume: The Habitability of Enceladus. *Astrobiology*, **14**, 352-355.

MEDRIFF Consortium. 1995. Three brine lakes discovered in the seafloor of the eastern Mediterranean. *Eos, Transactions American Geophysical Union*, **76**, 313–318.

Mesbah, N. M. & Wiegel, J. 2008. Life at extreme limits: the anaerobic halophilic alkalithermophiles. *Annals of the New York Academy of Sciences*, **1125**, 44-57.

Mesbah, N. M. & Wiegel, J. 2012. Life under Multiple Extreme Conditions: Diversity and Physiology of the Halophilic Alkalithermophiles. *Applied and Environmental Microbiology*, **78**, 4074-4082.

Müller, A. L., Rosa de Rezende, J., Hubert, C. R. J., Kjeldsen, K. U., Lagkouvardos, I., Berry, D., Jørgensen, B. B. & Loy, A. 2014. Endospores of thermophilic bacteria as tracers of microbial dispersal by ocean currents. *The ISME Journal: Multidisciplinary Journal of Microbial Ecology*, **8**, 1153-1165.

Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction- amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, **59**, 695–700.

Nikolenko, S. I., Korobeynikov, A. I. & Alekseyev, M. A. 2013. BayesHammer: Bayesian clustering for error correction in single-cell sequencing. *BMC Genomics*, **14**, S7.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W. & Backhaus, H. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology*, **178**, 5636-5643.

Nucleobytes. [Date unknown]. 4peaks. Retrieved from https://nucleobytes.com/4peaks/index.html

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A. A., Korobeynikov, A., Lapidus, A., Prjibelski, A. D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., Clingenpeel, S. R., Woyke, T., McLean, J. S., Lasken, R., Tesler, G., Alekseyev, M. A. & Pevzner, P. A. 2013. Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. *Journal of Computational* Biology, **20**, 714-37.

Ojha, L., Wilhelm, M. B., Murchie, S. L., McEwen, A. S., Wray, J. J., Hanley, J., Massé, M. & Chojnacki, M. 2015. Spectral evidence for hydrated salts in recurring slope lineae on Mars. *Nature Geoscience*, **8**, 829-832.

Omta, A. W., Kropman, M. F., Woutersen, S. & Bakker, H. J. 2003. Negligible effect of ions on the hydrogen-bond structure in liquid water. *Science*, **301**, 347-349.

Oren, A. 2005. A hundred years of *Dunaliella* research: 1905–2005. *Saline Systems*, **1**, 2.

Oren, A. 2010. Industrial and environmental applications of halophilic microorganisms. *Environmental Technology*, **31**, 825-834.

Parks, D. H., Tyson, G. W., Hugenholtz, P. & Beiko, R. G. 2014. STAMP: Statistical analysis of taxonomic and functional profiles. *Bioinformatics*, **30**, 3123-3124.

R Core Team. 2013. R: A language and environment for statistical computing. Retrieved from http://www.r-project.org/

Rognes, T., Flouri, T., Nichols, B., Quince, C. & Mahé, F. 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, **4**, e2584.

Roy, P., Dutta, A. & Deen, B. 2015. Greenhouse gas emissions and production cost of ethanol produced from biosyngas fermentation process. *Bioresource Technology*, **192**, 185–191.

Russo, A., Palumbo, M., Scifo, C., Cardile, V., Barcellona, M. L. & Renis, M. 2001. Ethanol-induced oxidative stress in rat astrocytes: role of HSP70. *Cell Biology and Toxicology*, **17**, 153-168.

Salvi, G., De Los Rios, P. & Vendruscolo, M. 2005. Effective interactions between chaotropic agents and proteins. *PROTEINS: Structure, Function, and Bioinformatics*, **61**, 492–499.

Sass, A. M., McKew, B. A., Sass, H., Fichtel, J., Timmis, K. N. & McGenity, T. J. 2008. Diversity of *Bacillus*-like organisms isolated from deep-sea hypersaline anoxic sediments. *Saline Systems*, **4**, 8.

Sawyer, W. H. & Puckridge, J. 1973. The Dissociation of Proteins by Chaotropic Salts. *The Journal of Biological Chemistry*, **248**, 8429-8433.

Sperber, W. H. 1983. Influence of Water Activity on Foodborne Bacteria — A Review. *Journal of Food Protection*, **46**, 142–150.

Stetter, K.O. 2006. History of discovery of the first hyperthermophiles. *Extremophiles*, **10**, 357-362.

Stevens, A. H., Childers, D., Fox-Powell, M., Nicholson, N., Jhoti, E. & Cockell, C. S. 2019. *Astrobiology*, 19, [pagination unknown].

Stevenson, A., Burkhardt, J., Cockell, C. S., Cray, J. A., Dijksterhuis, J., Fox-Powell, M., Kee, T. P., Kminek, G., McGenity, T. J., Timmis, K. N., Timson, D. J., Voytek, M. A., Westall, F., Yakimov, M. M. & Hallsworth, J. E. 2015a. Multiplication of microbes below 0.690 water activity: implications for terrestrial and extraterrestrial life. *Environmental Microbiology*, **17**, 257-277.

Stevenson, A., Cray, J. A., Williams, J. P., Santos, R., Sahay, R., Neuenkirchen, N., McClure, C. D., Grant, I. R., Houghton, J. D. R., Quinn, J. P., Timson, D. J., Patil, S. V., Singhal, R. S., Antón, J., Dijksterhuis, J., Hocking, A. D., Lievens, B., Rangel, D. E. N., Voytek, M. A., Gunde-Cimerman, N., Oren, A., Timmis, K. N., McGenity, T. J., & Hallsworth, J. E. 2015b. Is there a common water-activity limit for the three domains of life? *The ISME Journal: Multidisciplinary Journal of Microbial Ecology*, **9**, 1333-1351.

Stevenson, A., Hamill, P. G., O'Kane, C. J., Kminek. G., Rummel, J. D., Voytek, M. A., Dijksterhuis, J. & Hallsworth, J. E. 2017. *Aspergillus penicillioides* differentiation and cell division at 0.585 water activity. *Environmental Microbiology*, **19**, 687-697.

Tamkutė, L., Gil, B. M., Carballido, J. R., Pukalskienė, M., & Venskutonis, P. R. 2019. Effect of cranberry pomace extracts isolated by pressurized ethanol and water on the inhibition of food pathogenic/spoilage bacteria and the quality of pork products. *Food Research International*, **120**, 38-51.

Tregoning, G. S., Kempher, M. L., Jung, D. O., Samarkin, V. A., Joye, S. B. & Madigan, M. T. 2015. A Halophilic Bacterium Inhabiting the Warm, $CaCl_2$-Rich Brine of the Perennially Ice-

Covered Lake Vanda, McMurdo Dry Valleys, Antarctica. *Applied and Environmental Microbiology*, **81**, 1988–1995.

van der Waals, M. J., Atashgahi, S., da Rocha, U. N., van der Zaan, B. M., Smidt, H. & Gerritse, J. 2017. Benzene degradation in a denitrifying biofilm reactor: activity and microbial community composition. *Applied Microbiology and Biotechnology*, **[volume unknown]**, 1-14. Advance online publication.

van der Wielen, P. W. J. J., Bolhuis, H., Borin, S., Daffonchio, D., Corselli, C., Giuliano, L., D'Auria, G., de Lange, G. J., Huebner, A., Varnavas, S. P., Thomson, J., Tamburini, C., Marty, D., McGenity, T. J., Timmis, K. N. & BioDeep Scientific Party. 2005. The enigma of prokaryotic life in deep hypersaline anoxic basins. *Science*, **307**, 121-123.

Vellend, M., Srivastava, D. S., Anderson, K. M., Brown, C. D., Jankowski, J. E., Kleynhans, E. J., Kraft, N. J. B., Letaw, A. D., Macdonald, A. A. M., Maclean, J. E., Myers-Smith, I. H., Norris, A. R. & Xue, X. (2014). Assessing the relative importance of neutral stochasticity in ecological communities. *Oikos*, **123**, 1420–1430.

Wang, Q., Garrity, G. M., Tiedje, J. M. & Cole, J. R. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied Environmental Microbiology*, **73**, 5261-5267.

Washabaugh, M. W. & Collins, K. D. 1986. The systematic characterization by aqueous column chromatography of solutes which affect protein solubility. *The Journal of Biological Chemistry*, **261**, 12477-12485.

White, T. J., Bruns, T. D., Lee, S. B., Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: a guide to methods and applications, edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, 315–322.

Williams, J. P. & Hallsworth, J. E. 2009. Limits of life in hostile environments: no barriers to biosphere function? *Environmental Microbiology*, **11**, 3292-3308.

Yakimov, M. M., La Cono, V., Spada, G. L., Bortoluzzi, G., Messina, E., Smedile, F., Arcadi, E., Borghini, M., Ferrer, M., Schmitt-Kopplin, P., Hertkorn, N., Cray, J. A., Hallsworth, J. E., Golyshin, P. N. & Giuliano, L. 2015. Microbial community of the deep-sea brine Lake Kryos seawater–brine interface is active below the chaotropicity limit of life as revealed by recovery of mRNA. *Environmental Microbiology*, **17**, 364-382.

Zajc, J., Džeroski, S., Kocev, D., Oren, A., Sonjak, S., Tkavc, R. & Gunde-Cimerman, N. 2014a. Chaophilic or chaotolerant fungi: a new category of extremophiles? *Frontiers in Microbiology*, **5**, 708.

Zajc, J., Kogej, T., Galinski, E. A., Ramos, J. & Gunde-Cimerman, N. 2014b. Osmoadaptation Strategy of the Most Halophilic Fungus, *Wallemia ichthyophaga*, Growing Optimally at Salinities above 15% NaCl. *Applied and Environmental Microbiology*, **80**, 247-256.

Zhang, J., Kobert, K., Flouri, T. & Stamatakis, A. 2014. PEAR: a fast and accurate Illumina Paired-End reAd merger. *Bioinformatics*, **30**, 614–620.

Zhou, J., Liu, W., Deng, Y., Jiang, Y.-H., Xue, K., He, Z., Van Nostrand, J. D., Wu, L., Yang, Y. & Wang, A. 2013. Stochastic Assembly Leads to Alternative Communities with Distinct Functions in a Bioreactor Microbial Community. *mBio*, **4**, e00584-12.

Zhou, J., & Ning, D. 2017. Stochastic Community Assembly: Does It Matter in Microbial Ecology? *Microbiology and Molecular Biology Reviews*, **81,** e00002-17.

APPENDIX A

Appendix A1. One-way ANOVA analysis between agricultural soil and salt marsh enrichments in sodium chloride 3 M.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7898881666.667	1	7898881666.667	33.737	.004
Within Groups	936526666.667	4	234131666.667		
Total	8835408333.333	5			

Appendix A2. One-way ANOVA analysis between agricultural soil and salt marsh enrichments in sodium chloride 3.5 M.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	60801666.667	1	60801666.667	2.382	.198
Within Groups	102106666.667	4	25526666.667		
Total	162908333.333	5			

Appendix A3. One-way ANOVA analysis between agricultural soil and salt marsh enrichments in magnesium chloride 1.25 M.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	101681666.667	1	101681666.667	3.948	.118
Within Groups	103026666.667	4	25756666.667		
Total	204708333.333	5			

APPENDIX B

Appendix B1. The model summary of regression analysis of agricultural soil enrichments in sodium chloride.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.494[a]	.244	.205	1656630.24366

a. Predictors: (Constant), conc

Appendix B2. The coefficients in regression analysis of agricultural soil enrichments in sodium chloride.

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2528480.952	808352.788		3.128	.006
	conc	**-896221.429**	**361506.357**	-.494	**-2.479**	**.023**

a. Dependent Variable: Growth

Appendix B3. The model summary of regression analysis of agricultural soil enrichments in magnesium chloride.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.822[a]	.675	.650	236689.66380

a. Predictors: (Constant), Concentration

Appendix B4. The coefficients in regression analysis of agricultural soil enrichments in magnesium chloride.

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1089261.261	181123.366		6.014	.000
	Concentration	**-738562.162**	**142084.858**	-.822	**-5.198**	**.000**

a. Dependent Variable: Growth

Appendix B5. The model summary of regression analysis of salt marsh enrichments in sodium chloride.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.592[a]	.350	.316	2338660.90273

a. Predictors: (Constant), conc

Appendix B6. The coefficients in regression analysis of salt marsh enrichments in sodium chloride.

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	4583914.286	1141149.673		4.017	.001
	conc	-1633614.286	510337.648	-.592	-3.201	.005

a. Dependent Variable: growth

Appendix B7. The model summary of regression analysis of salt marsh enrichments in magnesium chloride.

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.671[a]	.450	.408	1284317.22102

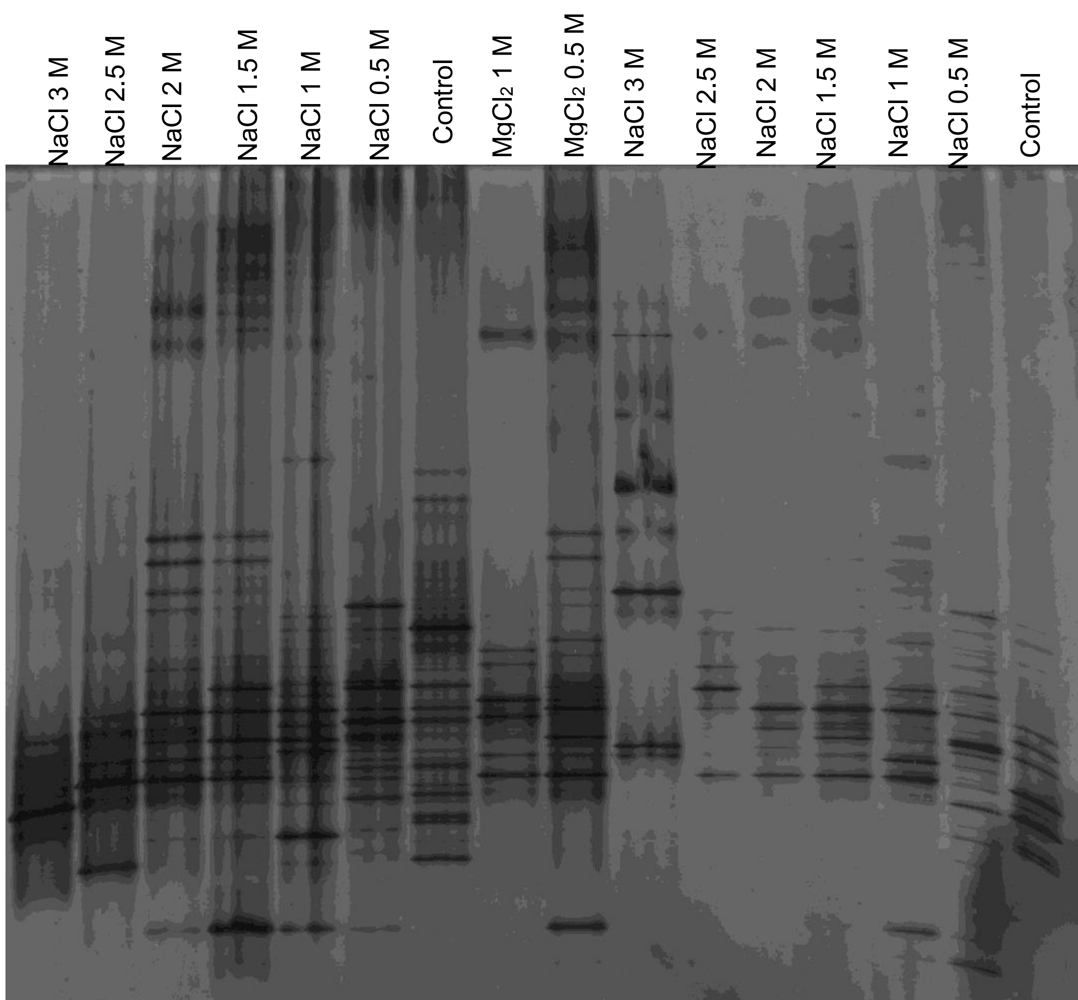
a. Predictors: (Constant), Conc

Appendix B8. The coefficients in regression analysis of salt marsh enrichments in magnesium chloride.

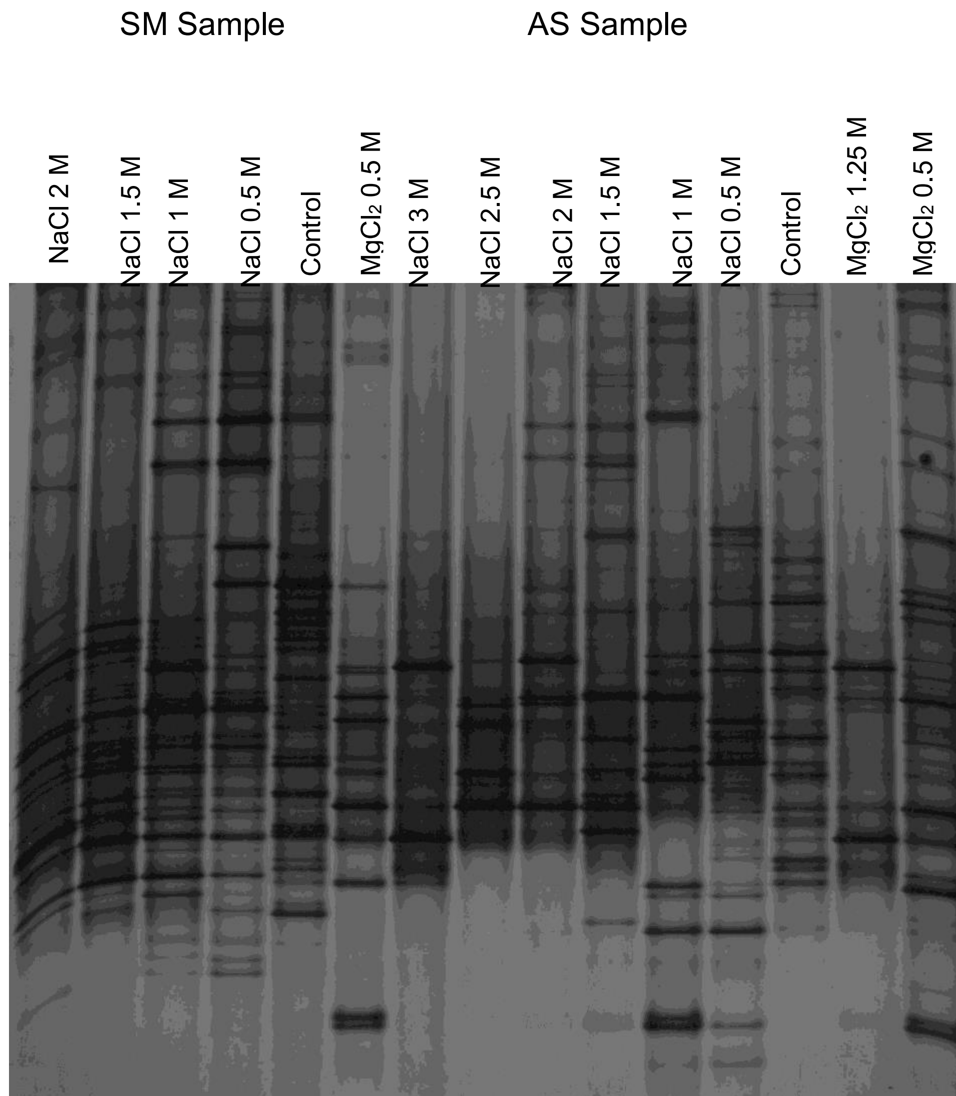
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	3691590.090	982805.311		3.756	.002
	Conc	-2514430.631	770975.917	-.671	-3.261	.006

a. Dependent Variable: Growth

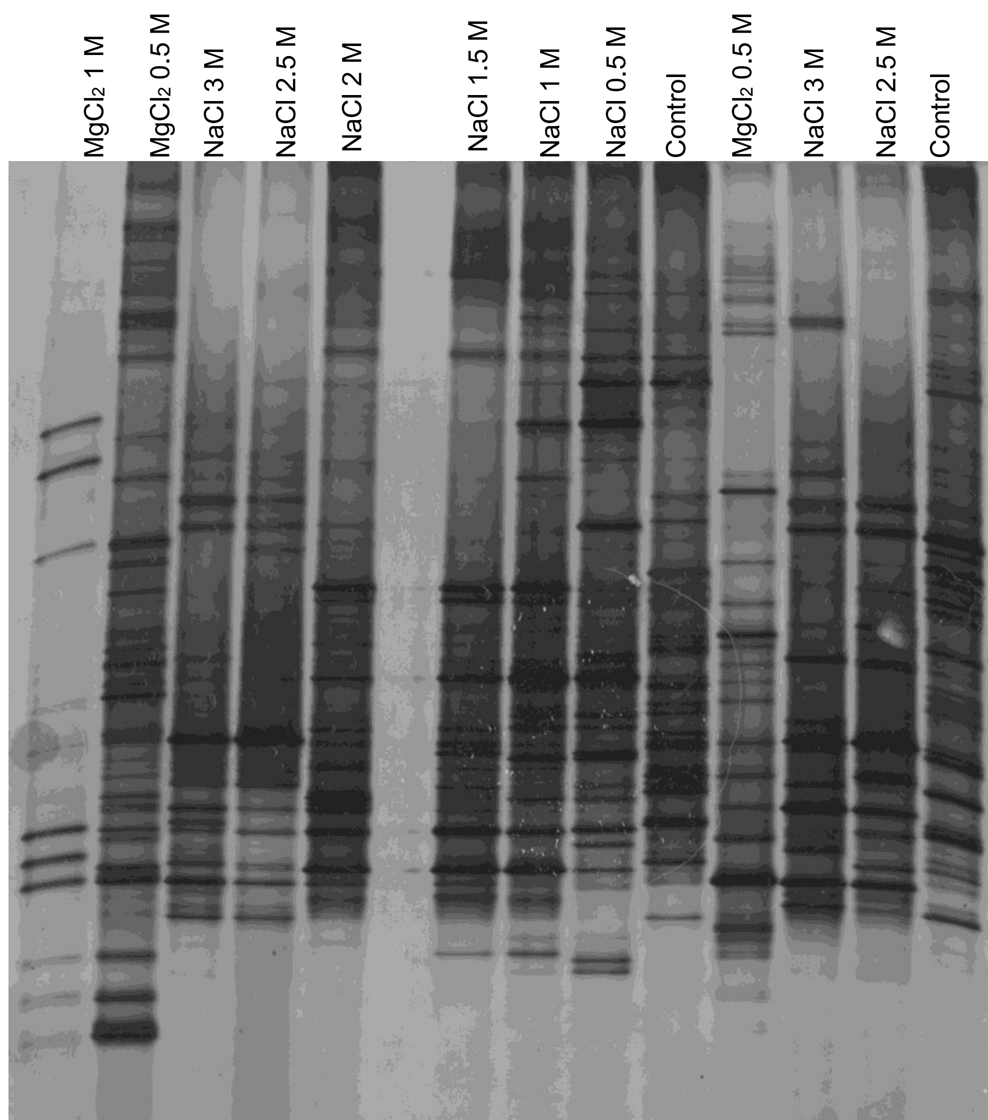
APPENDIX C



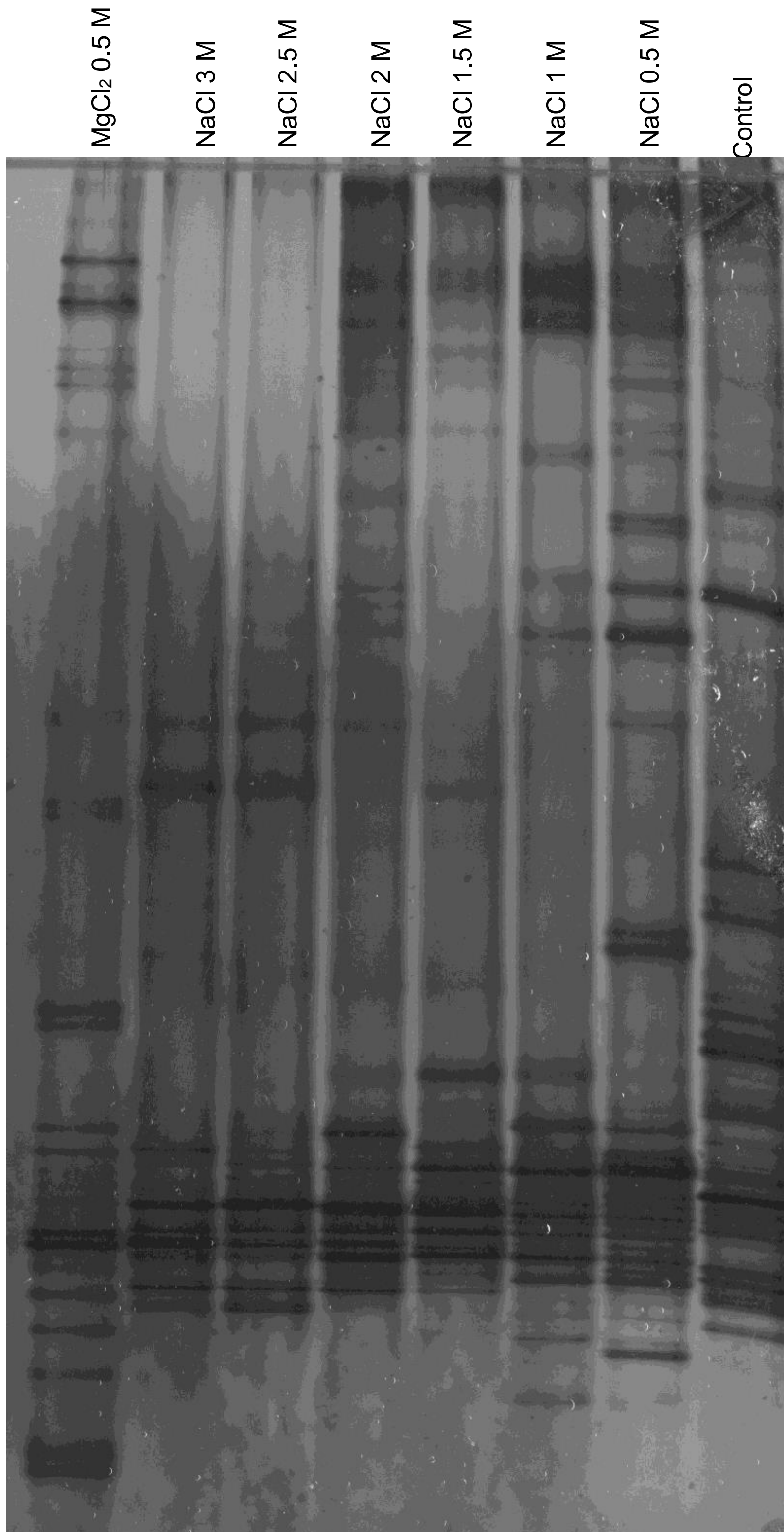
Appendix C1. DGGE profiles of bacterial PCR products amplified from agricultural soil samples.



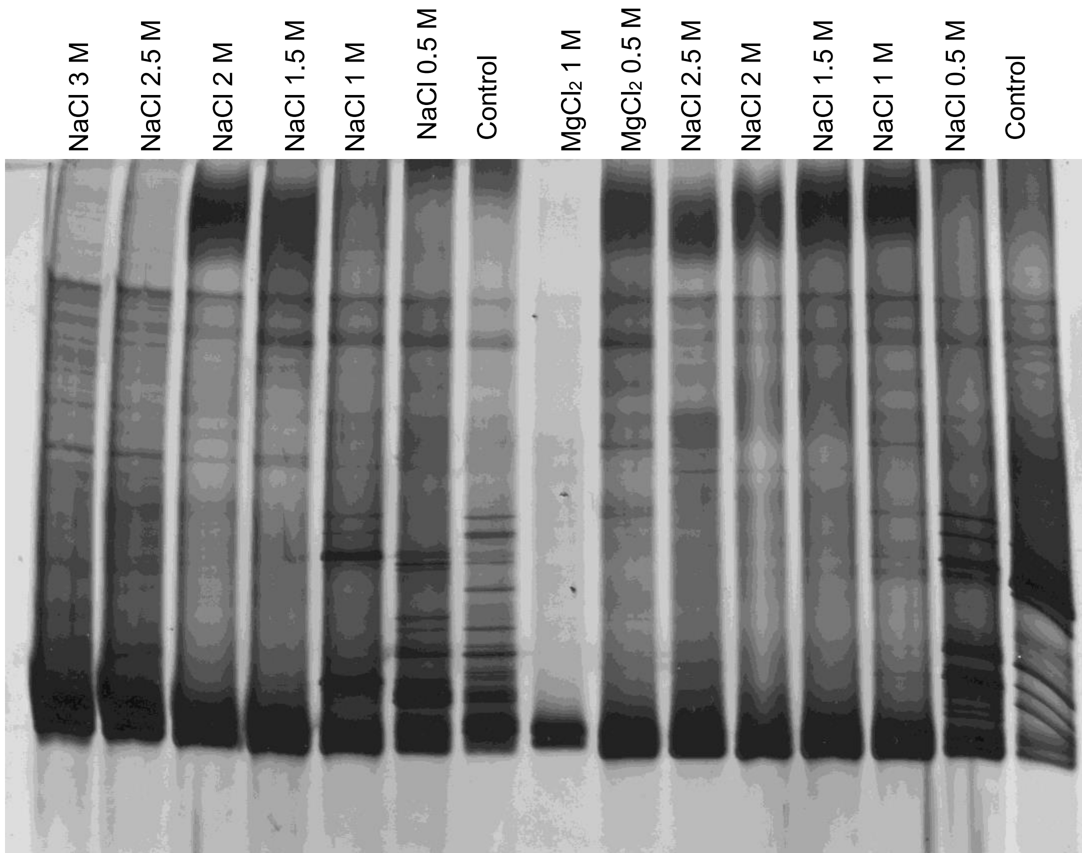
Appendix C2. DGGE profiles of bacterial PCR products amplified from agricultural soil and salt marsh samples.



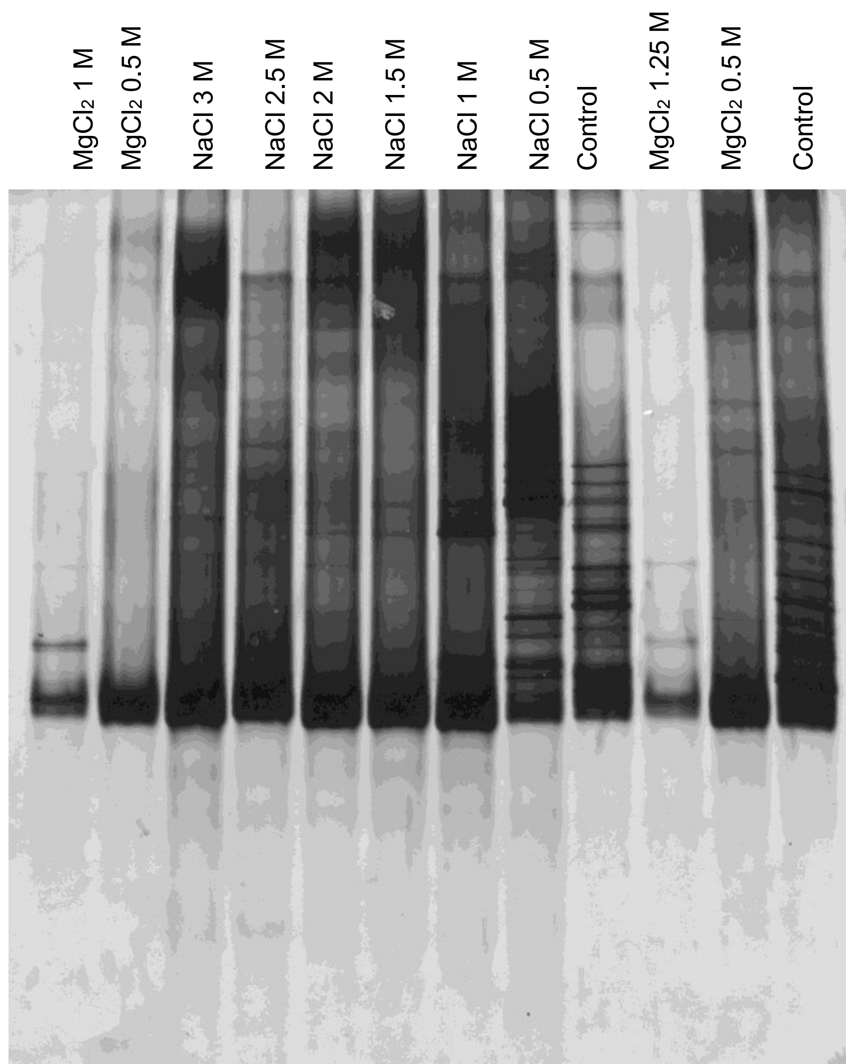
Appendix C3. DGGE profiles of bacterial PCR products amplified from salt marsh samples.



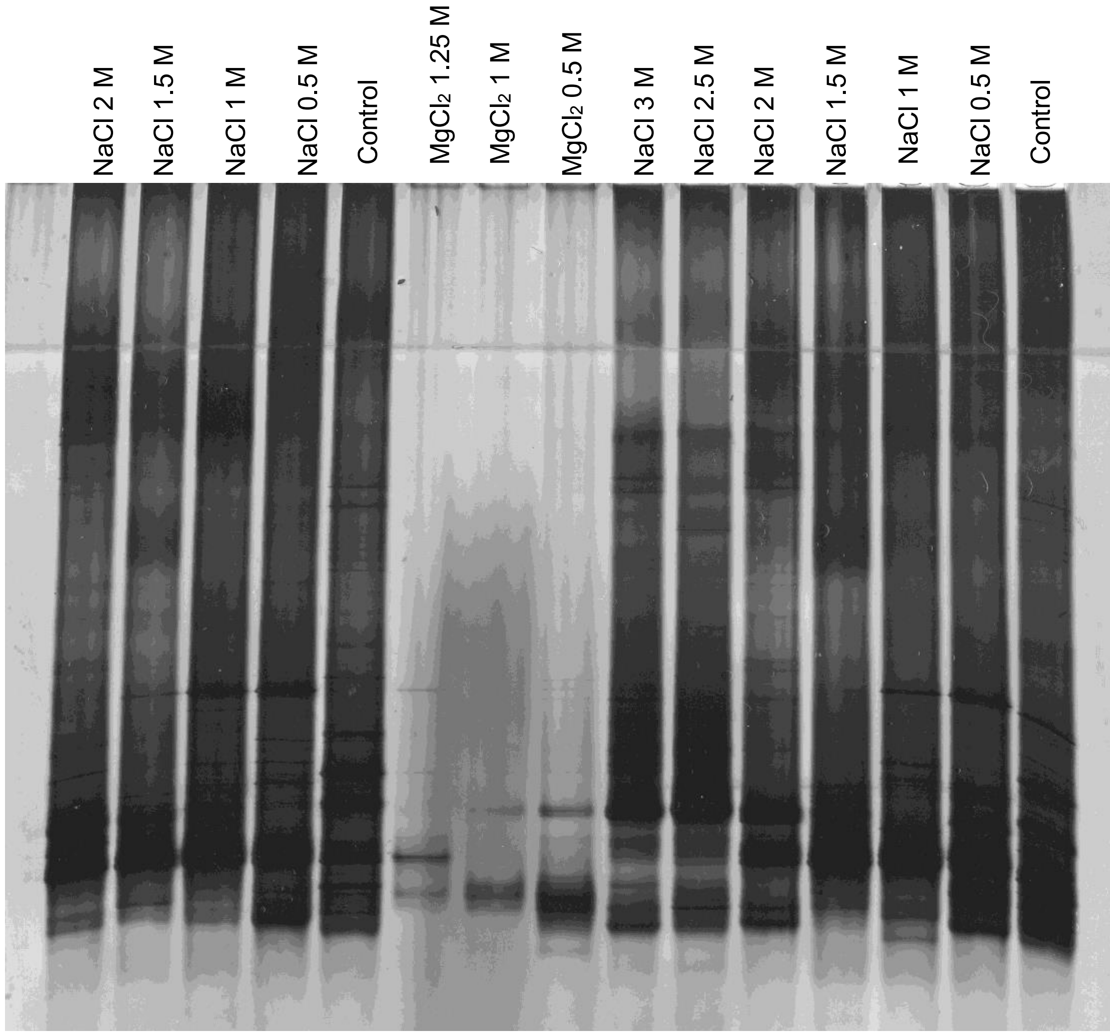
Appendix C4. DGGE profiles of bacterial PCR products amplified from salt marsh samples.



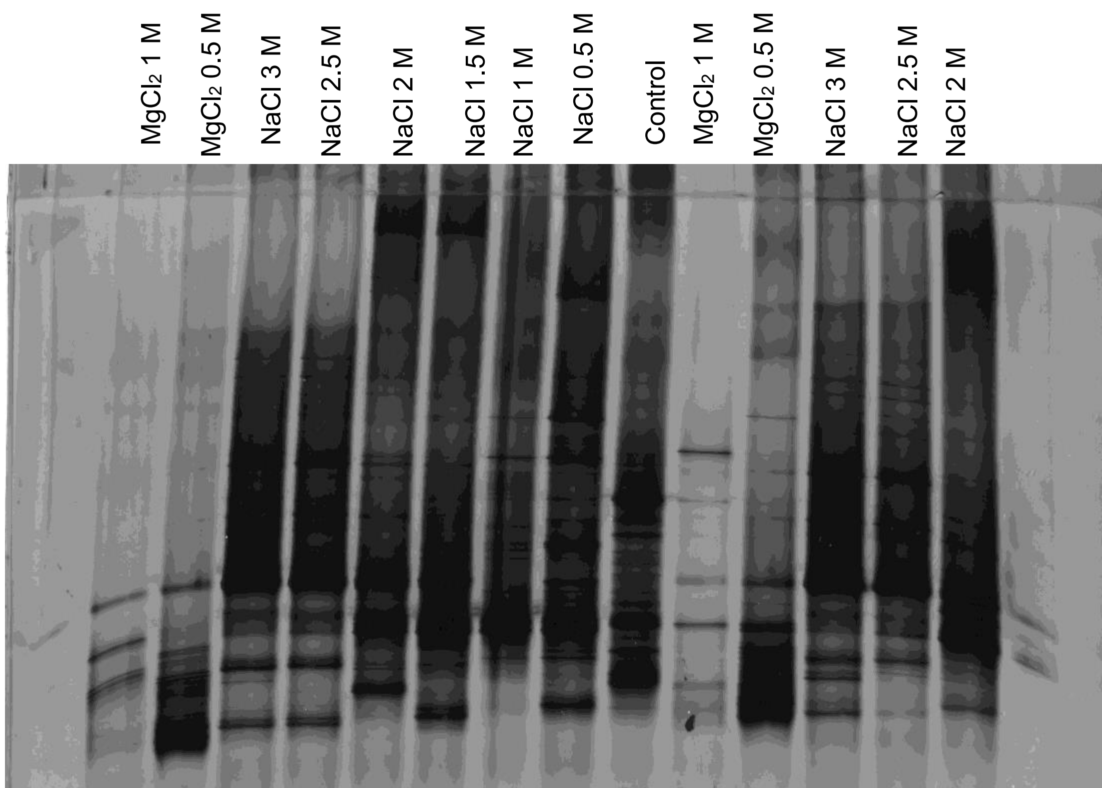
Appendix C5. DGGE profiles of Archaeal PCR products amplified from agricultural soil samples.



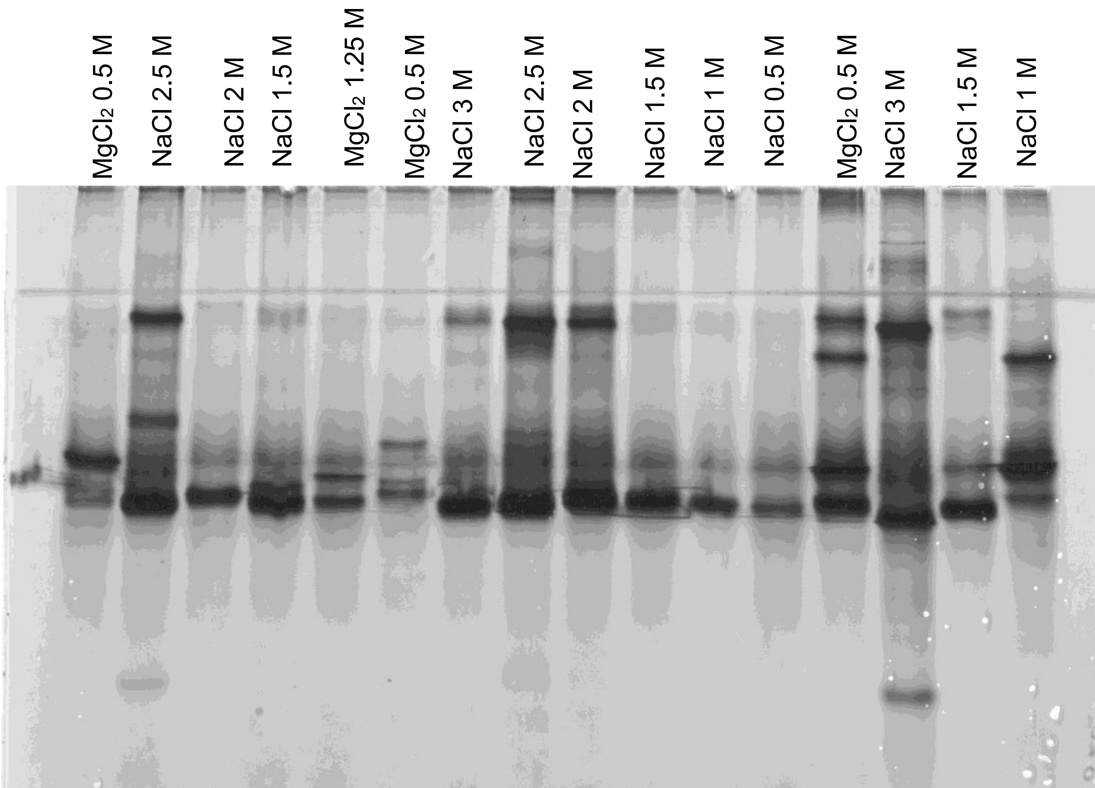
Appendix C6. DGGE profiles of Archaeal PCR products amplified from agricultural soil samples.



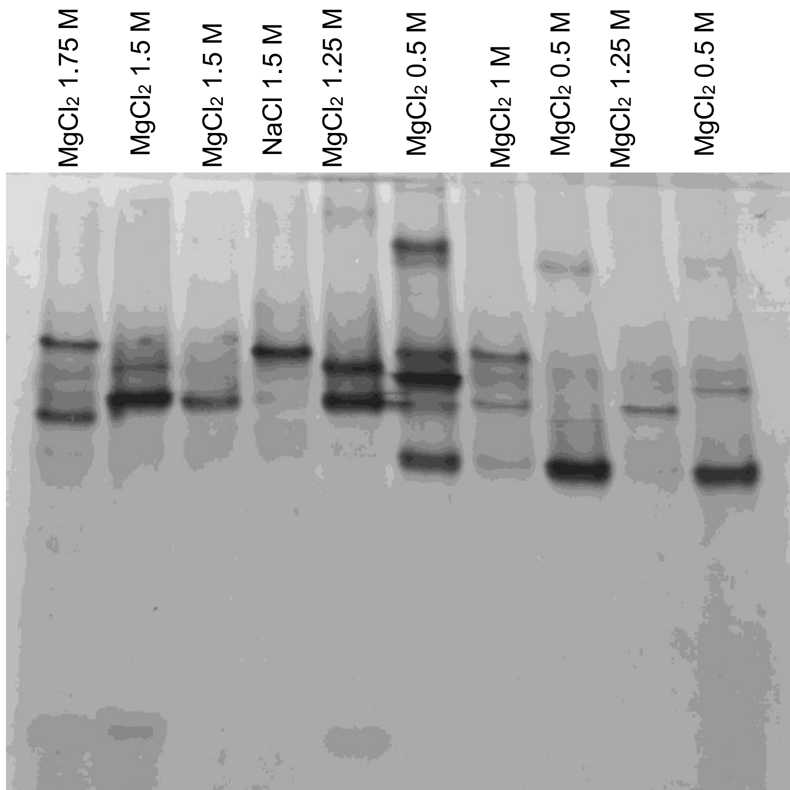
Appendix C7. DGGE profiles of Archaeal PCR products amplified from salt marsh samples.



Appendix C8. DGGE profiles of Archaeal PCR products amplified from salt marsh samples.



Appendix C9. DGGE profiles of fungal PCR products amplified from agricultural soil samples.



Appendix C10. DGGE profiles of fungal PCR products amplified from salt marsh samples.

APPENDIX D

Appendix D1. One-way ANOVA analysis between diameter (cm) of fungal strains in the no-solute control.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6.449	3	2.150	14.657	.001
Within Groups	1.173	8	.147		
Total	7.623	11			

Appendix D2. Tukey HSD analysis between diameter (cm) of fungal strains in the no-solute control.

(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Strain 1	Strain 3	.4333	.3127	.541	-.568	1.435
	Strain 4	.2333	.3127	.876	-.768	1.235
	Strain 7	-1.4333*	.3127	.008	-2.435	-.432
Strain 3	Strain 1	-.4333	.3127	.541	-1.435	.568
	Strain 4	-.2000	.3127	.916	-1.201	.801
	Strain 7	-1.8667*	.3127	.002	-2.868	-.865
Strain 4	Strain 1	-.2333	.3127	.876	-1.235	.768
	Strain 3	.2000	.3127	.916	-.801	1.201
	Strain 7	-1.6667*	.3127	.003	-2.668	-.665
Strain 7	Strain 1	1.4333*	.3127	.008	.432	2.435
	Strain 3	1.8667*	.3127	.002	.865	2.868
	Strain 4	1.6667*	.3127	.003	.665	2.668

Appendix D3. One-way ANOVA analysis between diameter (cm) of fungal strains in magnesium chloride 0.5 M.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.913	3	.638	1.552	.275
Within Groups	3.287	8	.411		
Total	5.200	11			

Appendix D4. One-way ANOVA analysis between diameter (cm) of fungal strains in magnesium chloride 1 M.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.516	3	1.172	100.452	.000
Within Groups	.093	8	.012		
Total	3.609	11			

Appendix D5. Tukey HSD analysis between diameter (cm) of fungal strains in magnesium chloride 1 M.

					95% Confidence Interval	
(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
1	3	.9000*	.0882	.000	.618	1.182
	4	.8333*	.0882	.000	.551	1.116
	7	-.3667*	.0882	.013	-.649	-.084
3	1	-.9000*	.0882	.000	-1.182	-.618
	4	-.0667	.0882	.872	-.349	.216
	7	-1.2667*	.0882	.000	-1.549	-.984
4	1	-.8333*	.0882	.000	-1.116	-.551
	3	.0667	.0882	.872	-.216	.349
	7	-1.2000*	.0882	.000	-1.482	-.918
7	1	.3667*	.0882	.013	.084	.649
	3	1.2667*	.0882	.000	.984	1.549
	4	1.2000*	.0882	.000	.918	1.482

*. The mean difference is significant at the 0.05 level.

Appendix D6. One-way ANOVA analysis between diameter (cm) of fungal strains in magnesium chloride 1.5 M.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.003	3	1.001	27.930	.000
Within Groups	.287	8	.036		
Total	3.289	11			

Appendix D7. Tukey HSD analysis between diameter (cm) of fungal strains in magnesium chloride 1.5 M.

(I) Strain	(J) Strain	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	3	.6333*	.1546	.015	.138	1.128
	4	.6333*	.1546	.015	.138	1.128
	7	-.5667*	.1546	.026	-1.062	-.072
3	1	-.6333*	.1546	.015	-1.128	-.138
	4	.0000	.1546	1.000	-.495	.495
	7	-1.2000*	.1546	.000	-1.695	-.705
4	1	-.6333*	.1546	.015	-1.128	-.138
	3	.0000	.1546	1.000	-.495	.495
	7	-1.2000*	.1546	.000	-1.695	-.705
7	1	.5667*	.1546	.026	.072	1.062
	3	1.2000*	.1546	.000	.705	1.695
	4	1.2000*	.1546	.000	.705	1.695

*. The mean difference is significant at the 0.05 level.

Appendix D8. One-way ANOVA analysis between diameter (cm) of fungal strains in magnesium chloride 2 M.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.120	3	.040	1.600	.264
Within Groups	.200	8	.025		
Total	.320	11			