Bacterial Ion Effects and their Relation to Salt Tolerance

Thesis submitted to the University of Strathclyde in fulfilment for the degree of Doctor of Philosophy

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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree

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Abstract

Extremophiles are organisms that are able to tolerate conditions that would otherwise inhibit or even kill non-extremophilic organisms – such extremes include acidity, high salt concentrations, high temperatures and high pressure. Specifically, halophiles are organisms that have a requirement for high concentrations of salt for growth. These organisms have been found to use either of two adaptation strategies, known as 'salt-in' (accumulation of inorganic ions) and 'salt-out' (removal of inorganic ions and accumulation of neutral molecules). In the current study, the relationship between the level of salt tolerance of an organism and its ion metabolism was investigated in order to gain insight into halo-adaptation and mechanisms of bacterial salt tolerance. This was accomplished by analysing the effects of a variety of salts (21 different combinations) on a halophile (*Salinibacter ruber*), non-halophile (*Escherchia coli*) and halotolerant (*Echinicola vietnamensis*) organism, which was achieved via an analysis of the effects of salts on bacterial growth, intracellular cation accumulation, enzymatic activity and and bioinformatics analysis. It was found that cation preferences were directly related to the level of salt tolerance of the organism, which is hypothesised to be a product of proteome acidity as well as the presence of specific membrane cation transporters. Specifically, the preference of *S. ruber* for the higher charge density Na^+ over K^+ may be rationalised based on the Hofmeister effect –i.e. this cation may provide better stabilisation of intracellular enzymes at the optimal salt concentrations for growth of *S. ruber*, but may be destabilising if accumulated at higher concentrations, and for non-salt adapted organisms. The ability of *E. vietnamensis* to tolerate and utilise many non-physiological ions supports this theory. Additionally, *E. vietnamensis* was postulated to use a 'hybrid' osmotic adaptation strategy – this organism may have industrial applications due to its large salt concentration tolerance range and high tolerance for non-physiological cations. Crucially, it was also found that *E. vietnamensis* and *S. ruber* contained membrane cation transporters that may be essential for their salt tolerance, giving insight into the essential nature of these proteins for the possession of salt resistance, which may have potential to be utilised for the transfer of salt-

tolerance to commercially important organisms. Finally, one specific salt combination tested, equimolar LiCl + KBr proved to totally inhibit bacterial growth and may show promise as an antimicrobial agent, for which a patent application has been initiated. The results of the current study can have various applications, including those within industry, medicine and astrobiology.

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Contents

Chapter 1: Introduction

1.1: Overview

In recent years there has been an upsurge in interest regarding extremophilic organisms [1]. These are organisms which are able to thrive in environments that 'normal' life cannot tolerate, i.e. most organisms would be unable to survive in such conditions. Extremophiles not only survive but positively prosper in these habitats which were once considered to be devoid of life, such as environments which are extremely hot, acidic or those that contain very high concentrations of salt [2]. The fact that these organisms survive at the so-called limits of life makes them particularly fascinating. The study of extremophiles not only has has direct applications for investigations into the boundaries of life but these organisms are also able to act as models within the expanding field of astrobiology [3]. The research area of astrobiology is concerned with investigations into life on other planets and the utilisation of extremophiles for these investigations is based upon the reconsideration of the limits of life and the finding of life in regions once considered to be inhospitable environments.

In addition to extremophiles in general having applications as models for astrobiology, those which can survive within hypersaline conditions (halophilic organisms) not only have applications for astrobiology but also may be particularly useful regarding applications of commercial and industrial value [4]. Such potential uses concern the treatment of agricultural soils as well as contaminated waters (such as industrial wastewaters) blighted with salt [5,6]. Since these organisms thrive in such conditions, they have considerable potential applications for the bioremediation of these areas.

Halophiles have been well studied and characterised in terms of their adaptation to hypersaline environments as well as their physiology [7]. These organisms adapt to hypersaline conditions by one of two strategies: the first strategy

involves the accumulation of neutral solute molecules, in order to maintain adequate cell water; the second strategy involves the accumulation of large quantities of inorganic ions, which organisms utilizing this strategy requiring adapted proteomes in order for this to be feasible [8]. However, even though research on halophiles has made significant progress in terms of our understanding of these organisms, there is a gap in the current literature regarding the effects of specific salts on the physiology of different bacteria. In particular, the effects of specific cations on bacteria have not been well studied. This is of central importance, as an understanding of general bacterial-ion effects will help to gain insight into mechanisms of salt tolerance as well as factors essential for survival in such conditions. This research will increase our knowledge of the limits of life (in terms of salt concentrations) and therefore has applications not only for astrobiology, but the insight gained into the effects of specific ions on bacteria has direct applications in terms of offering insight and potential solutions to the remediation of areas contaminated with these different ions.

Regarding current literature on the topic, no systemic studies have been carried out to compare the effects of different cations on bacteria, in terms of understanding differences between organisms as well as between different salts. Nostro *et al.* [9] carried out a study analyzing the effects of different anions on the growth of *Staphylococus aureus* and *Pseudomonas aeruginosa*, and found that these effects varied between the organisms and were also dependent upon the particular anion present. Additionally, Jensen *et al.* [10] analysed the effects of low levels of alkali cations on the halophile *Haloarcula marismortui*, and found that the effects of different cations on growth of the organism varied, which was dependent on the particular cation present. However, this latter study did not compare the effects of these cations to that of other organisms and only used lower concentrations of these cations, with only a very limited selection of salts. Therefore, the current literature is lacking in terms of an understanding of the differing effects that distinct ions have on microorganisms as well as how these effects vary between organisms. This is important regarding gaining insight into the mechanisms associated with survival in such conditions, as an overall

understanding of specific ion effects on bacteria and how this varies between organisms and in different conditions will help us to understand the physiological and molecular effects associated with survival in such environments.

Therefore, the current study is the most systemic study carried out on this topic, and took the form of a comparitive study, in terms of the characterisation of the different effects of a multitude of salts and salt combinations on 3 different bacteria (a halophile, non-halophile and halotolerant organism), in order to understand the specific effects between organisms and between salts. This study was carried out with the aim to gain insight into factors that are important for the salt tolerance of these organisms, as well as to gain a better understanding of general bacterial salt effects and life at the extremes.

1.2: Extremophiles and extreme environments

1.2.1: Extreme environments

Historically, extreme environments were thought to contain no life [2]. As paraphrased from Rothschild and Mancinelli, extreme environments can be defined as: “All physical factors are on a continuum, and extreme can be defined as the conditions at either end of this continuum that make it difficult for organisms to function” [11]. Many of the extreme environments on the planet, such as those with very high or low pH, high temperature, or a high salt concentration, result from the plate tectonic activities of the Earth, as well as from the activities of humans [12,13]. These plate tectonic activities (the collision between two tectonic plates) can result in volcano and mountain formation, and the environments that arise due to this tend to produce chemical and physical extremes, such as high temperatures or highly acidic environments. Human activities that can result in the formation of extreme environments include oil spillages, pollution or deep drilling of ocean floors [12]. Since plate

tectonic activities continuously occur, and have occurred throughout history, it is no surprise that the world is abounding with extreme environments. These environments have – contrary to the original thought - been found to contain a diverse array of life [1].

Life has the ability to occur within any region where water is present, and this includes extreme environments, which are at the boundary of what most life is able to tolerate, in terms of factors such as pH, temperature and salt concentration [12]. In terms of the absolute extremes, the temperature in the stratosphere can reach as low as -40^{o}C and temperatures on Earth can reach as high as 115^{o}C, with one example being within hydrothermal vents. Pressure can be as high as (or greater than) 120MPa (1184 atm) in the deep sea. In hypersaline lakes the salt concentration can be as high as 5M or greater (close to the limits of solubility) and the pH in acidic environments, such as acid mine drainages, can be below 1 [14].

Regarding temperature, an environment is considered to be 'thermophilic' if the temperature exceeds 55-60^{o}C [15]. Examples of thermophilic environments include hot springs (Figure 1.1.) and geysers, which are heated by the flow of lava underground from an extinct volcano. They contain extremely hot water and often also experience a low pH, while sometimes also contain chemicals that are normally harmful to most organisms [11]. Hydrothermal vents occur within the deep sea and have high pressures as well as high temperatures. A controversial theory exists stating that complex life could have arisen within a hydrothermal vent, due to all but thermophilic organisms being killed during the Late Heavy Bombardment period (4 billion years ago) [16]. In addition, another thermophilic environment is that of acidic solfatara fields (which are also acidic), which are located within volcanically active regions and are heated by magma [15].

Figure 1.1. Hot spring at Yellowstone National Park in the USA. The photo shows Grand Prismic Hot Spring at Yellowstone National Park (USA). The colours are due to Archaea growing in the spring. Adapted from Encyclopedia Britannica (2017).

Conversely, psychrophilic environments are those that are too cold to support most life, usually around 0^{o}C, and include polar areas as well as the deep sea [18]. These environments make up a large area of the planet as many oceanic regions are considered to be psychrophilic [19]. In addition, it has been stated that as much as 85% of the planet consistently has a temperature of 5^{o}C or lower [20]. Typically, temperatures in the deep sea are -1 to 4^{o}C, marine environments within the Arctic and Antarctic range from -35 to -1^{o}C, and the ice on glaciers can become as low as -5^{o}C [21].

In the deep sea organisms have to deal with high pressures as well as the very low temperatures [11]. Living organisms have been isolated from the ocean at a depth as great as 10900m [22]. The majority of the high pressure environments on the planet are in the deep sea, with pressures as considerable as 1100 atm/110 Mpa [23]. In the deepest ocean floor in the world (11000 metres) at the Mariana Trench, living bacteria have been isolated [13].

Another environmental extreme is that of pH. Alkaline environments include soda lakes and soda deserts [22]. These environments are the most abundant of the alkaline habitats and contain very high levels of sodium carbonate, sodium chloride and sodium sulphate [13]. The pH of soda lakes and soda deserts is usually above 10 and they are mostly found in sub-tropical regions, such as the Rift Valley in Kenya [15,24]. In soda lakes the surrounding rocks have a significant composition of sodium (Na^+)-silicates, and it is the mineral content of these surrounding rocks that is the main factor in determining the pH and chemical composition of these lakes [13]. In addition, high pH environments can also be a consequence of human activities, such as from mining and pulp and paper production [25].

Contrasting with environments with high pH levels, environments with low pH usually contain large levels of sulphur which - when exposed to oxygen - will create a highly acidic environment [15,25]. Consequentially, the presence of sulphuric acid is the main reason for such high acidity in these environments. An example of a type of acidic environment is that of acid mine drainages, which usually have a pH of around 3.6 [26]. These environments arise due to mining activities and involve the release of sulphur or other acidic minerals into the surrounding water [27]. Acidic environments caused by human mining activities are now one of the most common type of low pH environment in the world [28]. The Rio Tinto river in Spain is an example of a highly acidic environment that contains a large variety of organisms, shown in Figure 1.2 [29]. Acid solfatara fields (also thermophilic) are another example of an acidic environment, which are located within a volcanically active area and become acidic due to high levels of sulphur [30].

Figure 1.2. The Rio Tinto. The Rio Tinto river located in south western Spain is highly acidic - the red colouration arises due to a high concentration of ferric iron. Image adapted from Aguilera, 2013.

Hypersaline environments (i.e. those with very high salt concentrations) are generally found in areas with high temperatures and a high level of UV exposure, which will cause water in these lakes to evaporate, hence creating a hypersaline brine [15]. These areas also usually have relatively low levels of rainfall. Thalassohaline environments are formed from the evaporation of seawater, have a pH around neutral and contain mostly NaCl. Athassohaline environments, on the other hand, contain a salt composition different from seawater and the pH may deviate from neutral, mostly on the basic side [31]. Examples of hypersaline environments include the Great Salt Lake in the USA (thalassohaline) and the Dead Sea in the Middle East (athassohaline) [32,33]. Since organisms isolated from hypersaline regions are one of the main focuses of this thesis, this will be discussed further in section 1.1.3.

1.2.2: Extremophiles

The organisms that are able to tolerate these extreme environments can be grouped into two classes: those that can tolerate these conditions but grow best at moderate conditions are known as extremotolerant; whereas those that grow

optimally in the presence of these harsh conditions and often cannot tolerate more moderate conditions are known as extremophiles [1,13].

The interest in extremophiles has increased significantly in recent times due to their potential biotechnological uses as well as their potential for studying the evolution of life as well as life on other planets [34]. Moderate (non-extremophilic) conditions can be considered as being between 4 – 40^{o}C, a salt concentration below that of seawater (3.5%) and a pH between 5 – 8.5 [15,35]. Any deviations from these conditions would be considered extreme. It is generally the case that extreme environments contain less species diversity than more moderate environments, due to the pressures that organisms face in order to adapt to life within these environments [15,36].

Extremophilic organisms are of special interest since they live at the so-called 'limits of life'. The study of their adaptation to these environments will lead to a greater understanding of the absolute boundaries that determine the occurrence of life [37]. Because of this, the field of extremophile research is growing fast and is becoming increasingly popular, emphasized by the creation of a database dedicated entirely to extremophilic organisms [38].

The main categories of extremophiles are as follows [13]:

- Acidophiles: organisms that grow best at low (acidic) pH
- Alkaliphiles: organisms that grow best at high (basic) pH
- Halophiles: organisms that grow best in the presence of high salt concentrations
- Piezophiles: organisms that grow best at high pressures
- Psychrophiles: organisms that grow best at low temperatures
- Thermophiles: organisms that grow best at high temperatures
- Metallotolerant: organisms that are able to grow in the presence of high concentrations of metals
- Oligotrophs: organisms that are able to grow in the presence of very little nutrients

- Radioresistant: organisms that are able to grow in the presence of high levels of radiation (technically extremotolerant)
- Xerophiles: organisms that are able to grow in the presence of very low water activity
- Endoliths: organisms that are able to grow inside rocks

The Archaea contain the largest number of extremophiles out of the three domains of life [39]. Many Archaea are among some of the most ancient organisms known, and therefore many had to survive the harsh conditions that were present on early Earth [40]. In fact, Archaea were not classified as a separate domain until relatively recently, before which many archaeal organisms were classified as bacteria [41]. However, extremophiles exist from all three domains of life and bacterial and eukaryotic extremophiles also exist. The tardigrade (also known as 'water bears' - an animal that measures roughly half a millimeter in length) is an example of a eukaryotic extremophile [1,42]. These organisms have been found to be able to survive temperatures as low as 1^{o}C above absolute zero (-273.15^{o}C) and up to as high as 151^{o}C, and have even survived gamma-radiation, when they are in the so called 'hibernation' state (non-growing state, where the organisms are protected by cysts and show a lack of metabolism and usage of protectant molecules) [43].

Thermophiles

Mesophilic (i.e. non-thermophilic) organisms generally grow between temperatures of 20 – 45^{o}C [22]. Thermophilic organisms are separated into two categories: thermophiles and hyperthermophiles [44]. Thermophiles are characterised by having optimal growth between 60 – 80^{o}C, whereas hyperthermophiles are characterised by having optimal growth at temperatures above 80^{o}C - with some hyperthermophiles possessing the ability to grow at above 100^{o}C [45]. For example, thermophilic bacteria have been isolated from the hot springs at Yellowstone National Park in the USA [46]. One of these organisms is *Thermus aquaticus* (optimal growth at 70^{o}C and maximum growth

at 79°C), which was isolated from a hot spring at Yellowstone in 1969 [47]. An enzyme isolated from this organism is now one of the most widely used enzymes in biological research – Taq polymerase, used for the polymerase chain reaction (PCR) due to its extreme heat stability [48]. Figure 1.3 shows the spring at Yellowstone where this organism was originally isolated. Regarding hyperthermophilic organisms, *Pyrolobus fumarii* has the highest recorded growth temperature of all known organisms, being able to grow at as high as 113°C [49]. The controversial theory that life arose within a hydrothermal vent suggests that that complex life may have evolved from hyperthermophiles, due to their position at the shortest and deepest branches near the root of the phylogenetic tree of life, as well as the fact that early Earth is thought to have experienced extremely high temperatures [50,51].

Figure 1.3. Mushroom Spring at Yellowstone National Park. The photo shows the hot spring where the organism (*T. aquaticus*) that contains the Taq polymerase enzyme used within PCR was originally isolated by Brock and Hudson in 1969. Photo adapted from Rothschild and Mancinelli, 2001.

Thermophiles have a wide range of industrial applications, in addition to the significant application of *T. aquaticus*, as described above. These mostly stem from the fact that thermophilic enzymes are active at high temperatures, which are often optimal temperatures for various industrial processes (for example in food processing as well as in the pharmaceutical industry), as well as higher temperatures being associated with lower levels of microbial contamination

[44,52]. For this reason, thermophiles are one of the most widely utilized group of extremophiles.

Psychrophiles

In contrast to thermophiles, psychrophiles are organisms which can grow at extremely low temperatures [53]. Microbes growing at such low temperatures were first characterised in 1887 from a frozen bioluminescent fish [19]. Psychrophilic organisms can be defined as organisms that have an optimum growth temperature below 20^{o}C - some psychrophiles have been found to grow in as low temperatures as -16^{o}C [22,54]. Similar to psychrophilic organisms, psychrotolerant organisms are those that can grow at temperatures as low as 0^{o}C but grow optimally at more moderate temperatures, above 20^{o}C [55]. These organisms are usually found in the deep oceans or in polar regions of the world [56]. Psychrophiles have to contend with the slower rate of biochemical reactions at lower temperatures as well as the increase in viscosity of aqueous solutions associated with a decrease in temperature [53]. Enzymes from psychrophiles have applications within the cleaning and detergent industry, as they are active at lower temperatures (as compared with traditional enzymes) and hence could reduce costs and the ecological impact associated with hot water as well as being beneficial for people unable to access hot water [57,58].

Acidophiles

Acidophiles can be defined as organisms that have optimal growth at a pH of less than 4 [59]. The first acidophilic organism that was characterised was *Thiobacillus thiooxidans* in the 1920s [60]. In addition to the theory of life originating within a thermophilic environment, early Earth was thought to be acidic as well as hot, so acidophiles today may give additional insight into the first microbes on Earth [61]. Some acidophiles are able to grow at a pH of 2 or lower, most of which pump out protons in order to keep their cytoplasm around neutral pH [22]. Consequentially, acidophilic organisms generally maintain an

intracellular pH many times greater than that of the environment. For example, the acidophilic archaeon *Thermoplasma acidophilum* grows optimally at pH 1.4 but maintains an intracellular pH of 6.4, and the bacterial acidophile *Acidithiobacillus ferrooxidans* has a pH optimum for growth of 1.8 but an internal pH of 6.5 [62]. Acidophilic enzymes have a wide array of applications within industries that require acid-stable enzymes, such as in the manufacture of fruit-juice, animal feed or within the pharmaceutical industry [25].

Alkaliphiles

Alkaliphilic organisms are those which have optimal growth at pH 9 or above - growing slowly or not at all at lower pH: most have optimal growth between pH 10 – 12 [63]. Figure 1.4 shows a schematic of the pH growth range of a typical alkaliphilic organism. These organisms possess the ability to be able to grow at such high pH by increased levels of Na^+/H^+ antiporters in their membranes, allowing them to pump in protons to lower their internal pH [25]. Before 1968, alkaliphiles were not well known and only a few papers were published about them [13]. The earliest work on alkaliphilic organisms was published in 1922, and only a handful of papers were published between then and 1968 [64–68]. Since 1968, the Japanese extremophile researcher Koki Horikoshi has vastly improved the knowledge on alkaliphiles and uncovered the potential biotechnological applications of these organisms [63,69]. One of the major applications of alkaliphiles is the use of their enzymes within detergents as many commonly used detergents have a very high pH and so alkaliphilic enzymes have the ability to remain more stable and active in these conditions, as compared to 'traditional' enzymes [70].

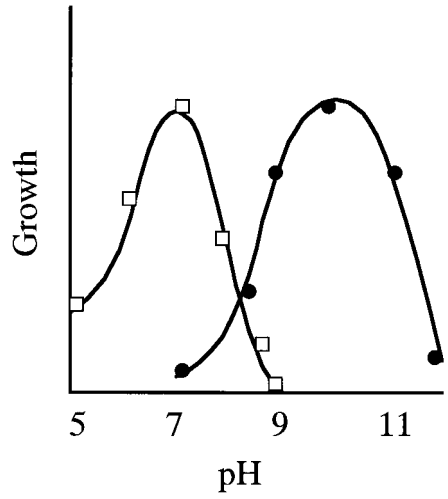

Figure 1.4. Schematic of the pH range of growth of an alkaliphile as compared to a neutrophilic organism. The curve on the right shows the pH range of growth of a typical alkaphiilic organism and the curve on the left shows that of a neutrophilic organism. Growth rates (h^{-1}) are hypothetical and do not represent exact values. Adapted from Horikoshi, 1999.

Piezophiles

Piezophilic organisms are those which grow best at high pressure, such as in the deep oceans [22]. Normal atmospheric pressure on Earth is around 0.1MPa, which is the usual pressure that most organisms are exposed to and grow optimally within [71]. Figure 1.5 shows the pressure growth range of a typical piezophile as compared with a non-pressure tolerant organism [72]. Due to the high percentage of the planet that is under high pressure, including the deep oceans (as mentioned in section 1.1.1), there is estimated to be as much as $1x10^{30}$ microbial cells living in such high pressure environments, many of which will be classed as piezophilic [73]. Organisms have been isolated from the Mariana Trench - which is the deepest ocean bottom in the world - growing at 80MPa [74]. However, research on piezophilic organisms has not gained as much momentum as research on other types of extremophile, mostly due to the difficulty in isolating organisms from the deep oceans and the problems associated with growing these organisms in the laboratory at high pressure [75]. The cell membranes of non-piezophilic organisms will become compacted at high pressure, which will inhibit growth and lead to the destruction of the cell

[76]. As an adaptive strategy, piezophilic organisms have been found to contain an altered membrane lipid composition (lower levels of monounsaturated fatty acids) as well as altered protein structures, to allow them to remain more rigid and stable at such high pressures [76,77].

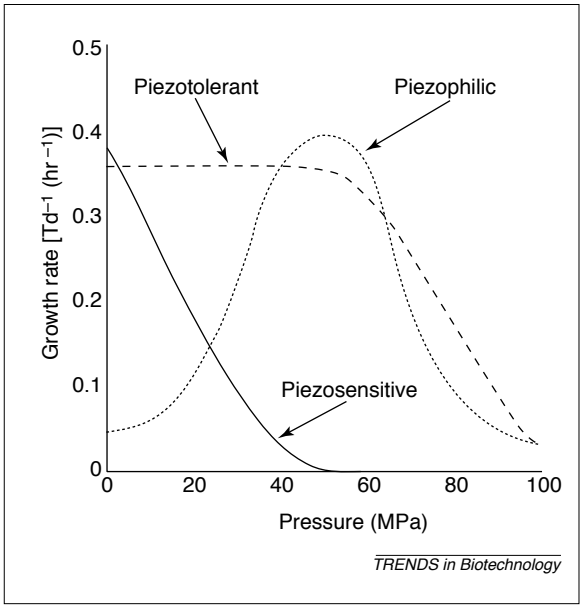

Figure 1.5. Pressure growth range of piezophilic and non-piezophilic organisms. Piezosensitive refers to non-extremophilic organisms, piezotolerant organisms are those that grow optimally at lower pressures but can tolerate higher pressures, and piezophiles are those that grow optimally at high pressures. Adapted from Abe and Horikoshi, 2001.

Halophiles

Halophiles are organisms that are able to grow at salt concentrations that would otherwise inhibit or even kill non-halophilic organisms [78]. They can be defined as having optimal growth at salt concentrations higher than that of seawater, and have an obligate requirement of salt for growth – being unable to grow in its absence [79]. These organisms have several unique characteristics that allow them to tolerate such conditions, which will be covered in detail in the following sections.

1.2.3: Halophiles and hypersaline environments

Until the 1940s it was thought that the Dead Sea (total dissolved salt content of 322.6g/L) was sterile. However microbial growth was discovered in this sea in the 1940s, after which many other halophiles were isolated from this area [80–82], although the existence of halophiles in general was first mentioned in 1919 [83]. True halophiles need to be distinguished from those that are merely halotolerant, and moderate halophiles in turn need to be distinguished from those that are extreme halophiles and borderline extreme halophiles [84]. The halophile classification system that is most common used (established by Don Kushner) defines the categorisation of halophiles as follows (salt concentration range that produces optimal growth): extreme halophiles (2.5-5.2M); borderline extreme halophiles (1.5 – 4M); moderate halophiles (0.5 – 2.5M). In addition, halotolerant organisms are able to grow in the presence of salt but do not require it - with extremely halotolerant organisms being able to tolerate in excess of 2.5M [85–87].

Halophilic organisms are dispersed throughout all three domains of life: Bacteria, Eukarya and Archaea [84]. Regarding bacteria, there are halophilic species within the following phyla: Cyanobacteria; Proteobacteria; Firmicutes; Actinobacteria; Spirochaetes and Bacteriodetes [84]. Archaeal halophiles are found in the Halobacteria and also the Methanococci, with the main genera of halophilic archaea being: *Halobacterium*; *Haloferax*; *Haloarcula*; *Halococcus*; *Natronobacterium* and *Natronococcus* [88]. Halophilic organisms can also be eukaryotic, such as halophytes, which are marsh plants that are able to grow within hypersaline conditions [89]. In addition to halophytes, the brine shrimp *Artemia* are able to tolerate high salt concentrations and are considered halophiles. These creatures feed on algal halophiles (mostly *Dunaleila spp.*) and are then eaten by flamingos, being responsible for the pink colour on these birds [90,91]. Bacterial and archaeal halophiles generally differ in their responses to salt. All but one (*Methanohalophilus mahii*) characterised archaeal halophile are able to tolerate greater than 4M salt (extreme halophiles), whereas the majority

of characterised bacterial halophiles are only able to tolerate somewhat lower salt concentrations (moderate halophiles) [85,92].

High salt environments include hypersaline lakes, deep sea hypersaline basins, evaporation ponds and salt flats [11]. Highly concentrated salt water environments are often considered to be among the most extreme, since not only is the salt concentration extremely high but these environments often contain high levels of UV radiation as well as high pH [93]. Hypersaline waters can be classified dependent upon their ionic composition: thalassohaline and athalassohaline [94]. Thalassohaline waters are highly saline environments that originated from the evaporation of seawaters and tend to have a similar ionic composition to that of seawater (large levels of Na^+ and Cl^-), although at a higher salt concentration, and with a pH of around neutral [85,95,96]. In contrast to thalassohaline environments, athalassohaline environments often have multiple stressors, such as high temperatures and high pH, and their ionic composition is generally different to that of seawater [95].

Salt marshes are found on the coast and lie between land and areas of open sea water [97]. Within these environments the salt concentrations change frequently as tides are responsible for diluting the water - hence decreasing the salt concentration - and during warm weather the evaporation of this water increases the salt concentration [98]. Salterns are formed from human activity, whereby a number of salt water pools are left to evaporate in order to produce salt, with some ponds having lower salinity (known as concentrators) and others having higher salinity (known as crystallisers) [15,99]. Rainfall will dilute hypersaline environments, but the overall salt concentration does not dramatically change as this increase in the level of water will cause salt crystals to dissolve into the water, hence keeping the overall salt concentration around the same level [100]. Two of the best known hypersaline environments are the Great Salt Lake (shown in Figure 1.6) and the Dead Sea [15].

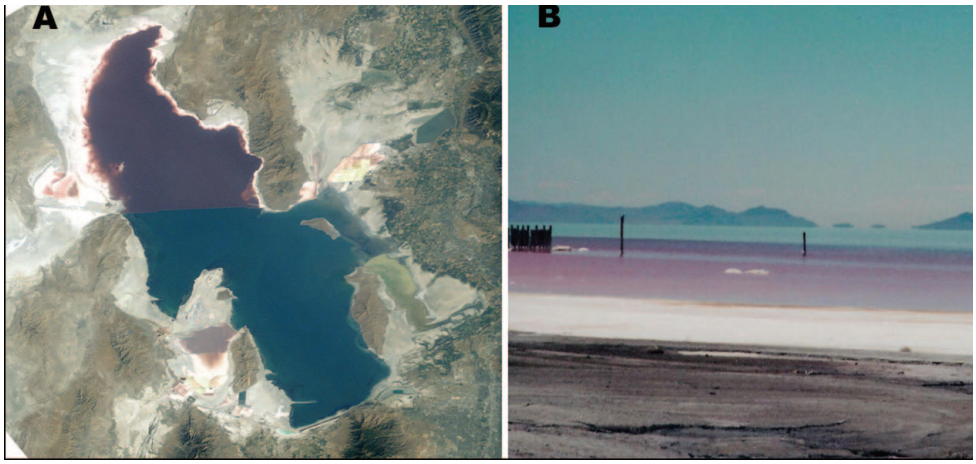

Figure 1.6. The Great Salt Lake in Utah (USA). A: view from space, B: view of the north part of the lake. The red colouration is due to the pigmentation of the halophilic organisms living in the water [101]. Figure adapted from DasSarma, 2006.

In comparison to other types of extremophile, which may require, for example, temperatures or pressures that are difficult to reproduce within a laboratory setting, halophiles are generally easier to cultivate, as they do not have complex growth requirements or require specialist equipment [95]. Therefore, a significant amount of knowledge has been gained on halophilic organisms regarding their adaptation strategies towards these extreme environments [84].

1.3: Bacterial salt effects

1.3.1: Bacterial Growth in Salts

To non-halophilic organisms, salt is toxic at moderate concentrations [102]. Growth in high salinity medium will result in a drop in cell turgor, hence slowing down growth, and if the salt concentration is high enough will result in cell death, due to destabilisation of intracellular proteins and plasmolysis [103,104]. The initial response of *Escherchia coli* (an example of a non-halophile) to an increase in the salt concentration of its environment is a drop in cell turgor as water is lost to the environment. The response to this turgor decrease is to accumulate ions and solutes from the environment in order to restore the cell

turgor to the appropriate level [105,106]. Non-halophilic bacteria need to maintain a solute concentration inside their cells slightly higher than the external concentration, in order to maintain a substantial enough turgor pressure, which is required for growth [107]. The preferred ion for this is usually potassium (K^+), after which 'osmotic solutes' are synthesized or taken up from the environment (refer to section 1.3.1). K^+ is usually accumulated in preference to sodium (Na^+) as it has a less disruptive effect on cellular activities (see section 1.5). In addition, the pH regulation of bacterial cells is dependent upon a tight control of the membrane ion permeability, not just to H^+ but also to K^+ and Na^+, and any deviations from this can lead to a decrease in the growth rate of the organism [108].

K^+ is required within bacterial cells for not only osmotic balance but also for enzyme activity, pH regulation and also as a mechanism of energy generation, via transmembrane K^+ gradients [109]. Bacteria require concentration gradients to be established at both sides of their membrane as this can be utilised for energy generation [100]. For a neutral molecule to cross a biological membrane, its movement is determined by the concentration gradient, i.e. how much of the molecule is inside the cell as compared to the outside. Similarly, the movement of a charged molecule (i.e. an ion) across a biological membrane is the result of the electrical potential, which is due to its concentration gradient as well as the charge difference between the inside and outside of the cell. The membrane potential across a biological membrane is the charge difference between the inside and outside of the membrane that results from the movement of ions across that membrane [110]. Figure 1.7 shows a schematic of the membrane potential in an acidophile, neutralophile and alkaliphile. This membrane potential can be used to drive chemical reactions, and often takes the form of a pH gradient over the membrane, due to the flow of H^+ (protons) across the membrane, known as the proton motive force (PMF) [39].

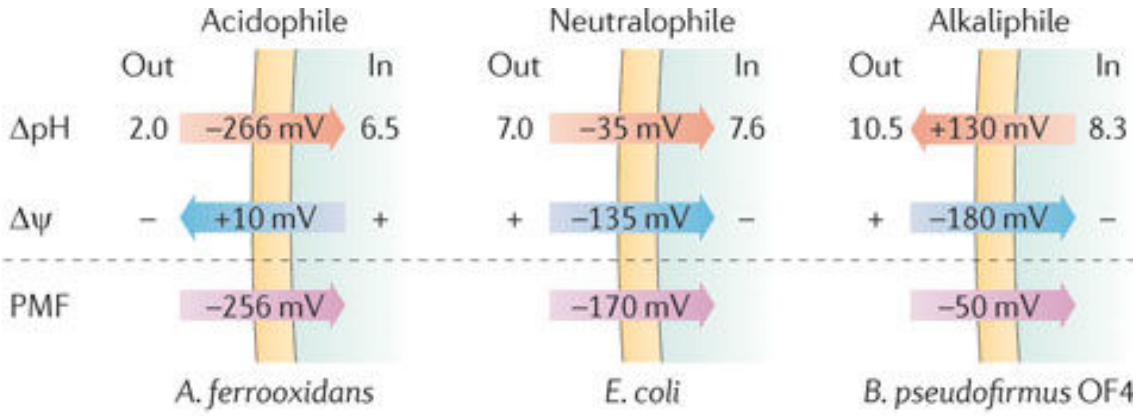

Figure 1.7. PMF in an acidophile, neutralophile and alkaliphile. ΔpH is the transmembrane pH gradient, ψpH is the transmembrane electrical potential, and PMF is the proton motive force. ΔpH is more alkaline inside the cell compared to the outside, except for alkaliphilic organisms, and the ψpH is usually more negative inside that outside, except for acidophilic organisms. Figure adapted from Krulwich *et al.*, 2011.

Cheung *et al.* reported that the specific solute encountered by *E. coli* has an effect on its response to osmotic stress. For example, 3 times more beta-galactosidase was produced when *E. coli* were exposed to NaCl as compared with sucrose [112]. This solute-specific effect has also been found for *S. aureus*, as NaCl and KCl were found to produce different levels of growth inhibition [113]. Similar solute-specific effects have been shown in other studies, with some solutes more likely to result in growth inhibition than others [9]. This suggests that different solutes have specific and varying effects on bacteria, and that the specific solute present may be a major factor in determining how a specific bacterium copes with the osmotic stress, as effects may be distinct for different organisms, as well as for different solutes. In addition, many organisms have a non-specific requirement for NaCl or KCl, meaning that other salts can replace these in culture medium.

It is important to note that water is not actively transported over the membrane as it moves by osmosis, so the movement of water across a biological membrane cannot be controlled directly [105]. For this reason, bacteria have to control their ion balance in order to indirectly control water flux across the membrane. As well as being responsible for maintaining an adequate cell turgor, proper

regulation of cellular ion content is essential for maintaining an electrochemical potential over the membrane [114]. In order for this to occur, cells have to be equipped with ion channels and pumps to regulate their intracellular ion concentrations, especially during conditions of osmotic stress. The membranes of bacterial cells only allow for the passage of water and certain specific solutes [112].

1.3.2: Bacterial ion transport

Bacterial cells generally maintain a cytoplasm with a K^+ concentration higher than the environment, but a Na^+ concentration that is lower than that of the environment [114]. The general preference of bacteria to accumulate K^+ over Na^+ may be due to the utilisation of an inward directed Na^+ transmembrane gradient for energy generation, i.e. to pump out Na^+ in order to generate energy for processes such as cell movement and solute transport, as well as K^+ being less disruptive towards intracellular proteins than Na^+ [97].

Bacteria contain three classes of ion transporters, as shown in Figure 1.8 [114]: uniporters, symporters and antiporters.

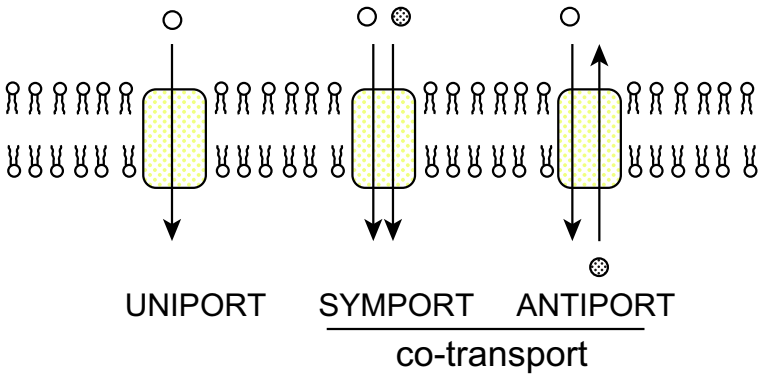

Figure 1.8. The three types of membrane ion transport systems. Uniporters transport one solute across the membrane, independently of any other solutes. Synporters use the energy generated from the transport of one solute across the membrane to transport another solute in the same direction. Antiporters transport two solutes into or out of the cell at same time in opposite directions. Adapted from Stillwell, 2013.

The ability of ion transporters to distinguish between Na^+ and K^+ is of vital importance for many cell types, as these are the most abundant cations that are physiologically relevant [116]. Some of the most well studied K^+ and Na^+ transporters are described briefly in sections 1.2.2.1 and 1.2.2.2. In terms of bacterial ion transport, those studied within the model organism *E. coli* are among the best characterised and therefore ion transport in terms of those studied within *E. coli* will be described in these sections. However, these transporters and their homologues/paralogues have been found to be present in many bacterial species and therefore apply to many organisms other than the well-studied *E. coli* [117–120].

1.3.2.1: K^+ transport

K^+ transport through cell membranes has been studied for many years, due to the high level of discrimination of these proteins between K^+ and the smaller Na^+ [121]. Before discussing potassium transport further it is important to clarify the difference between 'transporters' and 'channels': channels are involved in the movement of ions down their concentration gradient and rates are usually around the limits of diffusion, whereas transporters move ions against their

concentration gradient, which involves the expenditure of energy [122]. Potassium channels have been found to contain a selectivity filter (SF) which coordinates the binding of a K^+ ion but not a Na^+ ion – which is due to the positions of oxygen atoms in this filter that would make Na^+ ion binding energetically unfavourable, with similar selectivity filters also being found within other potassium transport systems [123–125]. In order to maintain a cytoplasm that has a greater K^+ concentration than the surroundings, bacteria will uptake K^+ against a concentration gradient. This K^+ concentration difference may be as great as 100-1000 times higher inside the cell than outside (in low osmolarity media) [126].

There are three main K^+ uptake systems within *E. coli*, which are among the most well studied of the bacterial cation transporters: [8]:

1. Kdp: high affinity K^+ transport, expressed when the turgor pressure decreases (i.e. a change in the stretch of the membrane)
2. Trk: low affinity for K^+ uptake but with a high uptake rate
3. kup (TrkD): low level of K^+ uptake

The kdp and Trk systems have been found to be widespread throughout many bacterial species [127]. The kdp system is driven by ATP (ATPase) and is also inhibited by higher K^+ concentrations [128]. The kdp system has the highest K^+ affinity out of the three K^+ transport systems in *E. coli* [129]. It is initiated when the K^+ concentration in the environment is low and also when the external salt concentration is high [130]. The kdp transporter is inactivated when the K^+ concentration in the external environment reaches above around 2mM. Therefore, this system is mostly important regarding low environmental K^+ concentrations. The Kdp transporter is a P-type ATPase - the energy used to hydrolyse ATP is coupled to the transport of K^+ across the membrane - and has a high degree of specificity for K^+ [130,131]. It consists of four inner membrane proteins, as is shown in Figure 1.9, (kdpA, kdpB, kdpC and kdpF), in addition to regulatory proteins kdpD (activated by low turgor, membrane-bound) and kdpE (cytoplasmic)– forming a signal-transduction system to activate the kdp complex [102,132,133].

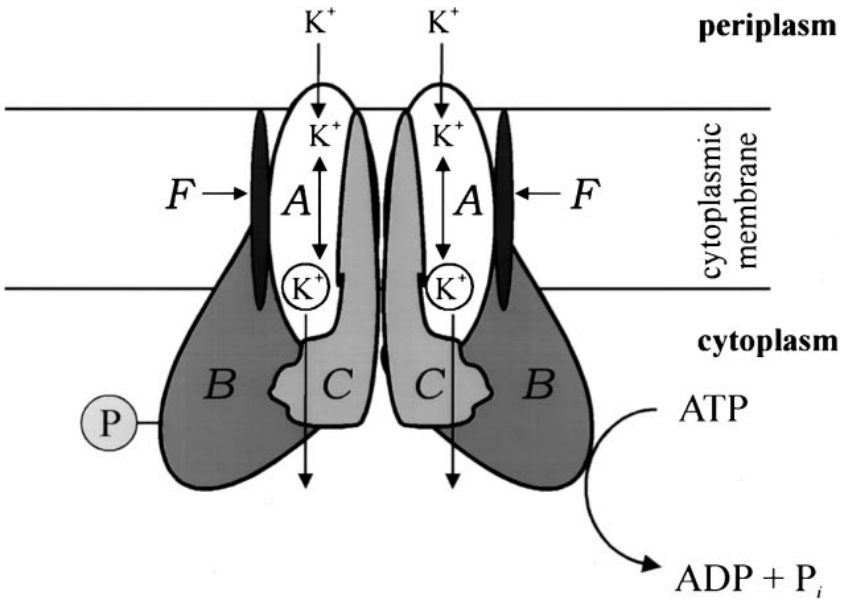

Figure 1.9. The kdp K^+ transport system. This system consists of four inner membrane proteins: kdpA, kdpB, kdpC and kdpF, as well as the regulatory proteins kdpD (membrane-bound) and kdpE (cytoplasmic), which form a signal-transduction system to activate the kdp complex. Adapted from Gabel *et al*., 1998.

When the external K^+ concentration increases above approximately 200μM, K^+ is imported into the bacterial cell via the Trk system [134]. Trk is composed of two separate systems - TrkG and TrkH. Both systems consist of one membrane spanning protein, either TrkG or TrkH (Figure 1.10), and TrkA, which is a peripheral membrane protein that can bind to NAD^+. The TrkH system also requires the ATP binding protein TrkE [135]. TrkA is essential to the function of both systems, but both systems are not needed for K^+ transport, although the knockout of either system significantly diminishes the rate of K^+ transport into the cell [136].

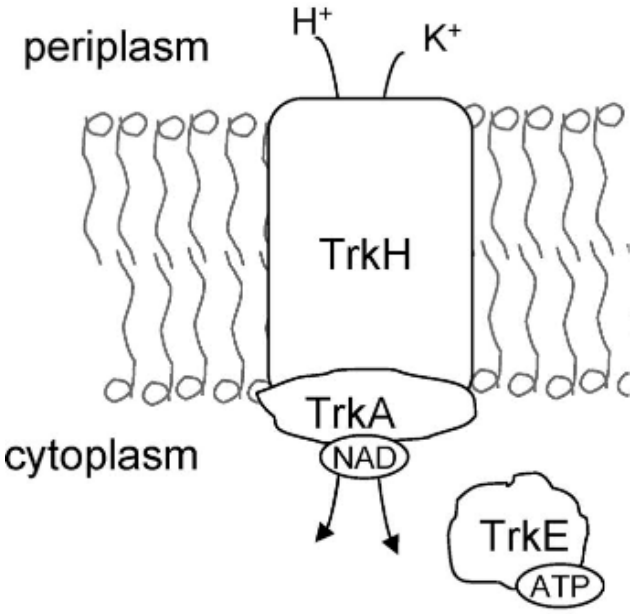

Figure 1.10. Schematic of the TrkH system. TrkH is the integral membrane protein, TrkA is the peripheral membrane protein and TrkE is the ATP-binding cytoplasmic component. Figure adapted from Johnson *et al*., 2009.

The 3rd main K^+ transport system present in *E. coli* is kup (TrkD), which is a single membrane spanning protein, composed of two domains, one of which spans the full membrane 12 times, the second of which is hydrophilic and is located at the cytoplasmic side of the membrane [102]. In addition, no ATP-binding regions have been found within the Kup protein [137]. Kup has a low specificity for K^+ and it has been suggested that kup is mostly responsible for K^+ uptake at lower pHs [138].

In addition to K^+ influx into cells, K^+ efflux is also crucial for appropriate osmoregulation. The Kef system is one of the main K^+ efflux pathways in *E. coli* and similar proteins are found in many other bacteria, which mostly functions in order to protect the cells from electrophiles [139]. The system is inhibited by glutathionine - this thiol containing tripeptide stops the ligand gated Kef channel from opening. When electrophiles are present in the environment, the glutathionine reacts with these and this causes the gate to open, allowing K^+ to leave the cell. This results in a decrease of the pH inside the cell, and the

subsequent protonation of proteins and DNA minimises the damage that electrophiles have on the cells [140,141].

1.3.2.2: Na^+ transport

Na^+/H^+ antiporters are of vital importance for the maintenance of an inward directed Na^+ gradient as well as for pH regulation [142]. For example, *E. coli* needs to maintain pH homeostasis of its cytoplasm at around 7.6, which is essential for appropriate membrane potential as well as enzymatic activities [111,143–145]. Na^+ efflux from the cell also generates the energy required for Na^+-coupled transport and can be used to assist in bacterial motility [146]. The NhaA and NhaB genes of *E. coli* encode two Na^+/H^+ antiporters, which are responsible for sodium extrusion from the cell [147]. Na^+/H^+ antiporters are widely distributed among bacteria and share many common features and mechanisms to the Nha system of *E. coli* [148]. The inward directed Na^+ gradient present in bacterial cells (a consequence of the earlier extrusion of Na^+ from the cell) can be used to drive many energy-requiring processes, such as solute transport and cell movement [149]. In addition to the Nha-family Na^+/H^+ transporters, ChaA is another Na^+/H^+ antiporter present within *E. coli*, which transports Ca^{2+}/H^+ but can also transport Na^+/H^+ [150]. This protein is regulated by the salt concentration in the environment as well as by pH.

The NhaA Na^+/K^+ antiporter is a member of the monovalent cation proton antiporter sub-family and has been found to be required for the survival of *E. coli* during growth at high salt concentrations, as well as at high pH [151,152]. This protein has been found to span the membrane 12 times, with both the N and C terminus located within the cytoplasm [153]. NhaA removes Na^+ from the cell via co-transport with H^+ - the H^+ are taken into the cell against their electrochemical gradient and the energy obtained from this is used for Na^+ export from the cell, transporting two protons to every one Na^+, with the rate at which this co-transport occurs largely dependent on the pH of the environment, increasing at higher pH [154]. The basic mechanism of this transport process is shown in

Figure 1.11. These transport events can occur (at optimal pH) up to as much as 89000 per minute and it has been reported that this may be one of the fastest membrane transporters characterised [143,155]. A change in the protonation of this protein leads to a change in conformation, hence regulating its activity at different pH [156].

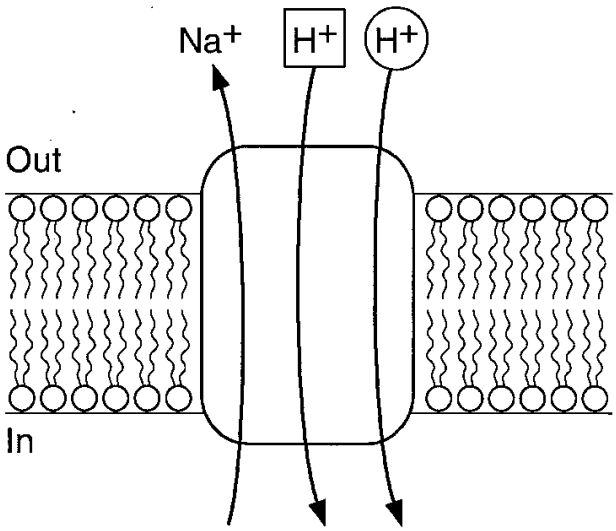

Figure 1.11. Schematic of a Na^+/H^+ antiporter. One Na^+ ion is removed from the cell for every two H^+ ions imported into the cell. Adapted from Southworth *et al.*, 2001.

In addition to NhaA, *E. coli* contains another Nha-family protein, NhaB - although only NhaA has been found to be essential to *E. coli* growth [158]. NhaB has been found to only be essential for growth when there is a lack of NhaA expression/activity [144]. The NhaB protein consists of 9 membrane spanning segments, where the N- terminus is located within the cytoplasm and the C-terminus is located in the periplasm (the area between the inner and outer membrane) [159].

In addition to the above, the coupling of Na^+ transport with certain solutes, such as melibiose and glutamate is also of importance, although Na^+/H^+ antiporters are thought to be more significant, regarding Na^+ balance [160]. This involves utilising the energy generated from Na^+ transport into the cell to also transport solutes into the cell [161].

It has been reported that exponentially growing bacterial cells contain a higher K^+ concentration and a lower Na^+ concentration than that of the environment, but as the cells reach stationary phase this changes, with the K^+ concentration decreasing and the Na^+ concentration increasing [162]. This is thought to be due to the changing energy status of the cell: when the culture is young there is a plentiful energy supply for K^+ to be pumped into the cell against a concentration gradient and for Na^+ to be extruded. However, as the energy supply dwindles, this may not be possible as less energy will be available in order to drive these processes, resulting in a change in the ion balance of the cell.

1.4: Halophilic adaptation

Non-halophilic bacteria will respond to salt in their environment according to similar osmoadaptation mechanisms [163]. However, for halophilic organisms salt is not toxic but is instead stimulatory towards growth [84]. There are two strategies that halophilic organisms use in order to permit their growth in the presence of such highly concentrated environments: the 'salt-out' strategy and the 'salt-in' strategy. The former strategy involves exporting inorganic ions from the cell and accumulating neutral solute molecules at higher concentrations than the salt concentration of the environment. The latter strategy involves accumulating inorganic ions (such as K^+) - with a proteome-wide adaptation being required, due to the potentially disruptive effects of such high levels of ionic species being present within the cell [164]. These two strategies will be covered in detail in sections 1.3.1 and 1.3.2. However, halophilic organisms, independent of which strategy they use, have been found to share some key features. For example, halophilic membranes have the ability to remain stable in high salt concentrations, in contrast to the membranes of non-halophiles [39]. Such adaptations involve an increased level of negative charges on the external portion of the membrane, in order to protect the membrane from the high external cation concentrations [165].

Due to the high levels of sunlight within hypersaline environments (refer to section 1.1.3), many halophiles have evolved various pigment molecules, such as carotenoids, in order to protect themselves against the potential damage that can be caused by UV radiation [100,166]. These pigments have been found to be linked with proton pumps (bacteriorhodopsin/ halorhodopsin) in order to utilize photochemical energy from this high level of UV light for energy generation, as well as binding to potentially damaging free-radicals [167–169]. In addition, a large number of halophilic species have been found to contain a high level (greater than 60%) of G-C base pairs in their DNA – this is thought to have evolved from the increased level of UV radiation that many halophiles encounter: the triple bond between the G-C base pair is more stable and so will be less likely to break when exposed to high levels of UV radiation [170]

1.4.1: Salt out strategy

The 'salt-out' strategy of halo-adaption is based on the fact that these organisms exclude salt from their cells. This involves the accumulation of small uncharged molecules in order to balance the internal and external osmolarities [171]. This adaptation strategy is the most common adaptation strategy used by moderately halophilic bacteria and allows for a greater flexibility in terms of the concentrations that they are able to tolerate, i.e. they can grow at lower salt concentrations than the salt-in halophiles are able to, as they do not have to adapt their proteomes towards accumulating potentially disruptive ions [97]. The salt-out adaptation method tends to be utilized by mostly halophilic bacteria and eukaryotes, as opposed to the halophilic archaea [172]. Most non-halophilic prokaryotic organisms will also use this method of osmoadaptation, although non-halophiles will not be able to tolerate the high salt concentrations that the halophiles which use this strategy are able to [8,173]. From an evolutionary perspective, the fact that this strategy appears to be more widespread than the salt-in strategy seems justified as it does not require an adaptation of the intracellular environment [174], as will be covered in section 1.3.2.

Organisms which utilise this salt-out adaptation strategy only accumulate these solutes at concentrations which lead to osmotic stress and due to the fact that these solutes do not interfere with enzymatic activity, their cellular proteins do not need to be adapted to their presence [84]. However, it is more energetically costly as the organisms have to either synthesise or actively uptake compatible solutes – both of which require energy [84]. Furthermore, most organisms are able to synthesise compatible solutes but due to energy requirements it is often more favourable to uptake them from the environment [173].

Before compatible solutes (also known as osmolytes) are either synthesized or taken up from the environment, the organism will accumulate K^+ to a particular concentration, which will then trigger the expression either of compatible solute synthesis genes or increase the expression of membrane proteins for compatible solute uptake [8]. These solutes are so-called due to the fact that they generally do not strongly interact with enzymatic processes and therefore are 'compatible' with cellular function [175]. This was originally defined by Brown and Simpson who stated that a compatible solute is a solute that can be accumulated to high concentrations inside a cell and yet cellular functions such as enzymatic activity are not disrupted [176]. The concentration of compatible solutes accumulated within the cell is determined by the external salinity, and any changes to the external salt concentration results in a change in the internal concentration of compatible solutes [177]. The compatible solutes used by halophilic organisms for osmotic balance (see Figure 1.12 for examples of some of the most widely used compatible solutes) can be grouped into the following categories [174]:

- Polyols and derivatives
- Sugars and derivatives
- Amino acids and derivatives
- Betaines
- Ectoines

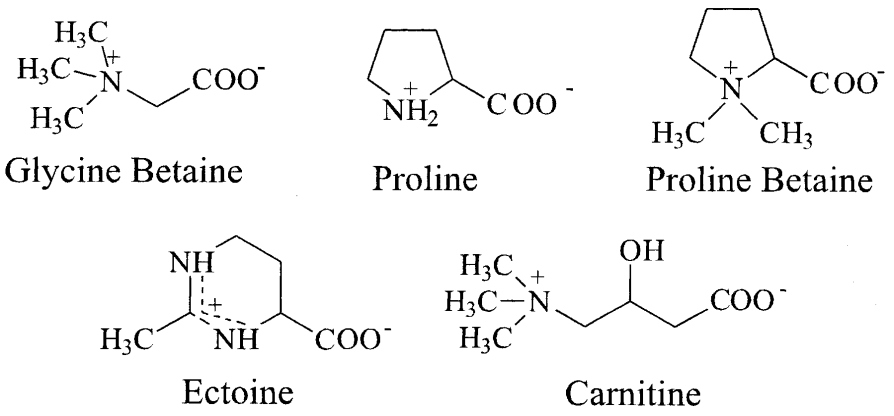

Figure 1.12. Chemical structures of various compatible solutes. The chemical structures are shown of some of the most common compatible solutes in halophilic organisms. Adapted from Wood *et al.*, 2001.

Regarding the actual compatible solutes accumulated, bacteria and methanogens (domain Archaea) tend to mostly accumulate amino acids and amino acid derivatives, as well as sugars and peptides. Fungi and yeasts mostly accumulate polyols and algae mostly accumulate sugar derivatives and polyols [173].

The fact that there are only a few groups of chemicals that are able to provide osmotic balance within a cell implies the difficulty in finding substances that do not adversely affect cellular processes [179]. Compatible solutes are generally either un-charged or zwitterionic (charges cancel out to create an overall neutral molecule) [180]. The differences in the maximum salt concentration salt-in and salt-out organisms are able to grow at may be explained by the limited solubility of some of the commonly used compatible solutes, resulting in salt-out organisms being able to grow only up to a certain threshold [181].

Compatible solutes are accumulated after the initial accumulation of K^+ [182]. These solutes are able to stabilise proteins by the osmophobic effect - the protein will preferentially interact with water over the solute and so the solute will be excluded from the protein (preferential exclusion) and water will be included,

hence allowing the correct folding and solvation of the protein while also making protein denaturation less entropically favourable [183–185].

Whether an osmolyte has stabilising or destabilizing effects on a protein is dependent on the environment, as this will influence the charge on the osmolyte and hence its interactions with a given protein [186]. It has also been found that destabilizing osmolytes bind directly to the protein surface whereas stabilising osmolytes promote proper protein folding by becoming excluded from the protein surface [187]. For example, polyols tend to be particularly effective at stabilising proteins at low pH: proteins become more hydrophobic at low pH as the COO^- groups are protonated and this means the polyols will be excluded from the protein surface (due to the OH- groups present in the polyols), therefore polyol osmolytes usually stabilise proteins at the lower end of the pH spectrum [186].

1.4.2: Salt in strategy

Organisms that use the 'salt-in' adaptation strategy accumulate molar concentrations of (usually) KCl, in order to maintain a cytoplasm with an ion concentration slightly greater than that of the environment [172]. This adaptation strategy is widely used by the halophilic archaea as well as the halophilic bacterium *Salinibacter ruber* [97]. The cation of choice tends to be K^+ (as opposed to Na^+), while Cl^- has also been found to accumulate to high concentrations within many of these organisms [34]. Na^+ can be pumped out of the cell via Na^+/H^+ antiporters, with the energy for this is supplied via a proton gradient (refer to section 1.2.2.2). However, due to the exceptionally high concentrations of K^+ within the halophilic cytoplasm, ATP must be utilised in order to drive the active uptake of K^+ into the cell. A K^+ transporter like the ATP-powered Trk K^+ transporter in *E. coli* has been found in various halophiles [10]. In addition, recent research suggests that the only halophilic organism known to contain a homologuous version of the kdp K^+ transport system is *Halobacterium spp.* The high levels of Cl^- found in many of the organisms which use this

adaptation strategy is thought to be transported into the cell via co-transport with Na^+ and also via light-induced Cl^- pumps, such as halorhodopsion [188].

Due to the accumulation of high cation concentrations, halophilic proteins require to be adapted to remain stable in such conditions [88]. It has been found that halophilic proteins contain an excess of acidic (negatively charged) amino acid residues on their surfaces, which help them to remain stable in the presence of high cation concentrations [189]. The high positive ion concentrations within the cells of salt-in halophiles may help to 'shield' the repulsions between the high level of negative charges contained on the halo-adapted proteins, thus assisting in their stabilisation at elevated cytoplasmic cation concentrations [34]. Moreover, the stabilisation of halophilic proteins at high salt concentrations is thought to be aided by the fact that the acidic amino acids are able to become more strongly hydrated than non-acidic amino acids, and due to the fact that halophilic proteins contain an increased level of acidic amino acids a 'salt ion network' can be formed around the protein, which helps in its stabilisation [172]. A high salt concentration within a cell can cause the cellular proteins to aggregate, as water is excluded from their surfaces due to the lower water activity [190]. The high negative charges on halophilic proteins may aid in avoiding this aggregation by keeping the amino acid residues apart, due to the repulsive interactions between these negative charges.

Halophilic proteins have been found to be particularly unstable at low salt concentrations, which is thought to be caused - at least in part - by the fact that the negatively charged amino acids will be screened from one another at high internal cation concentrations by the association of the salt cations with the carboxyl groups on the acidic residues, whereas at lower salt concentrations these charges will repel one another, hence causing the protein to destabilize [191]. This has been suggested to be the case from studies on the malate dehydrogenase of the extreme halophile *Haloarcula marismortui* [34]. These adaptations will be discussed further in section 1.4.

1.3.3: Some examples of notable halophilic organisms

Halobacillus halophilus

H. halophilus is a moderately halophilic bacterium that can grow from 0.5M up to as high as 3M NaCl. This organism has been found to use a so-called hybrid adaptation strategy: it accumulates compatible solutes in order to maintain its cellular turgor but also accumulates large concentrations of Cl^-. The 'unusual' nature of this bacterium increases due to the fact that it contains a slightly acidic proteome (i.e. acidic proteins) - higher than that of salt-in halophiles but lower than 'salt-out' halophiles, i.e. its proteome acidity sits in the middle of these [97].

Halorhodospira halophila

H. halophila has also been found to use a hybrid osmotic adaptation strategy: it does not accumulate K^+ at lower salt concentrations - at these salt concentrations it has a similar K^+ content in its cell to that of non-halophiles [190]. This observation would suggest that its proteome is not acidic, or else it would have a strict requirement for K^+ within its cytoplasm at all salinities. However, it has been found, by using isoelectric focusing (IEF) techniques that this organism contains an acidic proteome, while in the same study they also found that there is a substantial intracellular accumulation of K^+ when the organism is grown at higher salt concentrations [190].

Halomonas elongata

H. elongata is a salt-out organism and has been used at an industrial scale for the production of ectoine (which is a compatible solute that it requires for growth at high salt concentrations, recently utilised for various medicinal and cosmetic products), via a process known as 'bacterial milking' (refer to section 1.6) [192]. This bacterium has been reported to be able to tolerate in excess of 5M NaCl, which is exceptionally high for an organism that uses the salt-out strategy, as salt-out organisms are usually moderate halophiles [193,194]. The proteome of *H. elongata* has an acidic nature, somewhere in between that of non-halophiles and the salt-in halophiles [172], which again is unusual for salt-out organisms.

Haloarcula marismortui

This extremely halophilic archaeon which was originally isolated from the Dead Sea has been reported to be able to tolerate up to 5.1M NaCl, and has optimal growth between 3.5M – 3.9M NaCl [195]. The adaptation of the proteins from this organism has been studied in detail (salt-in organism) and it has a highly acidic proteome [196,197]. The malate dehydrogenase from this organism is the most extensively studied halophilic protein - with much of the early knowledge of halophilic protein adaptations being gained from studies on this enzyme [191,198]

Salinibacter ruber

At the end of the 1990s there was only one known group of halophilic bacteria that were thought to adapt via the salt-in strategy - the anaerobic Haloanaerobiales (Firmicutes) [180]. Upon its isolation from a crystallizer pond in Spain in 1999, *S. ruber* was the most halophilic bacterium known [199]. Until this time, it was thought that hypersaline environments with saturated NaCl concentrations only consisted of extremely halophilic archaea, but the discovery of *S. ruber* modified this viewpoint. The extremely halophilic bacterium *S. ruber* has subsequentially been found worldwide in salt lakes from Spain to Australia [200]. It is thought that *S. ruber* could make up to as much as 25% of the total prokaryotic cell density of salt-ponds within the salterns it was originally isolated from in Spain [201]. *S. ruber* cells have a slow rate of growth and this is perhaps the reason it took so long to isolate them, whereas many of the archaea that live within the same environments were isolated much sooner [199]. In addition, the shape and colour of *S. ruber* colonies, when cultured on agar plates, look very similar to that of halophilic archaea, which could be another reason why it took so long for this organism to be isolated [202].

S. ruber are rod-shaped, aerobic Gram-negative, red-pigmented, motile extremely halophilic bacteria (Figure 1.13) [199]. The pigment of *S. ruber* is thought to function in order to protect the organism from the high level of UV

exposure within these environments. Phylogenetically, *S. ruber* belongs to the Flavobacterium/Cytophaga branch of the domain Bacteria [203]. Like many halophilic prokaryotes, *S. ruber* contains a relatively high G-C content of just over 66%, in common with the archaeal halophiles [170,200]. In addition, *S. ruber* shares other features with the halophilic archaea, such as the same environment, the possession of pigment molecules and also the inclusion of retinal proton pumps (molecules that utilise light energy for transmembrane H^+ movement) within the cell membrane [93,204]. It is therefore thought that there could have been a significant amount of gene exchange between the Halobacteriaceae and *S. ruber* [201]. This similarity of *S. ruber* to the halophilic archaea is thought to have arisen via a combination of convergent evolution - perhaps brought about by living within similar environments - as well as by lateral gene transfer from the halophilic archaea that share the same environment [205].

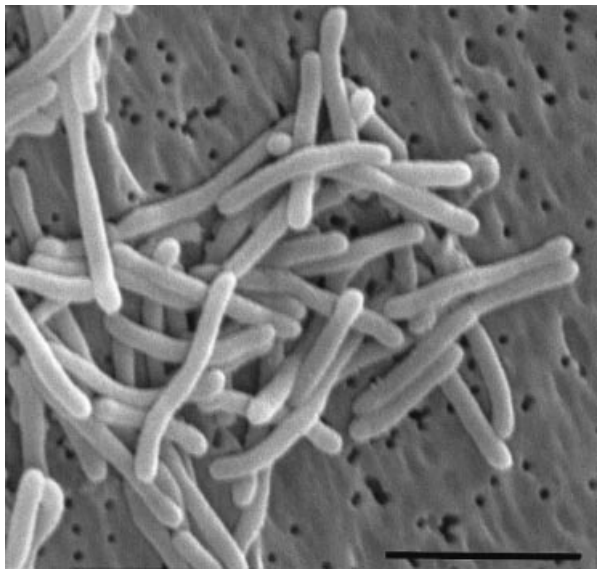

Figure 1.13. Scanning electron micrograph of *S. ruber* strain M31. The scale bar corresponds to 2.5μm. Adapted from Anton *et al*., 2002.

In addition, *S. ruber* cells have been reported to contain a similar level of K^+ as the extremely halophilic archaea [172]. No substantial concentrations of compatible solutes have been found within *S. ruber* cells, which further supports the idea that it adapts to its environment via the salt-in strategy [172].

1.5: Halophilic proteins

As mentioned in section 1.3.2, the proteins from salt-in halophiles - halophilic proteins - show some key adaptations that allow them to remain stable in the presence of molar concentrations of salt [206]. These will be discussed further in the following sections; however, the key adaptation strategy of salt-in halophilic organisms is a general increase in the acidic nature of the whole proteome [170]. Only the salt-in halophiles show these proteome-wide adaptations, which are the main focus of the remainder of this chapter.

1.5.1: Stability in high salt concentrations

1.5.1.1: Adaptation

As mentioned previously, halophilic enzymes contain modified compositions, containing elevated levels of acidic amino acids, lower levels of basic amino acids and also a lower level of the larger hydrophobic amino acids, which are replaced by the smaller hydrophobic amino acids [191]. The elevated level of acidic amino acids are mostly present on the surface of the proteins, which is in contact with the solvent, with the interior of the proteins containing a similar amino acid composition to what is found for non-halophilic proteins, as is shown in Figure 1.14 [88]. Halophilic proteins have also been found to contain less hydrophobic residues on their surfaces [172]. The lower level of hydrophobicity seen in halophilic proteins is thought to be due mostly to a decrease in surface-exposed lysine [34]. For example, the replacement of aspartic acids with lysines on the surface of the glutamate dehydrogenase from the halophilic archaeon *Haloferax mediterranei* resulted in the protein becoming slightly less halotolerant [207]. Halophilic proteins generally contain higher levels of aspartic acid, glutamic acid, valine and threonine and lower levels of lysine, methionine, leucine, isoleucine and cysteine [170].

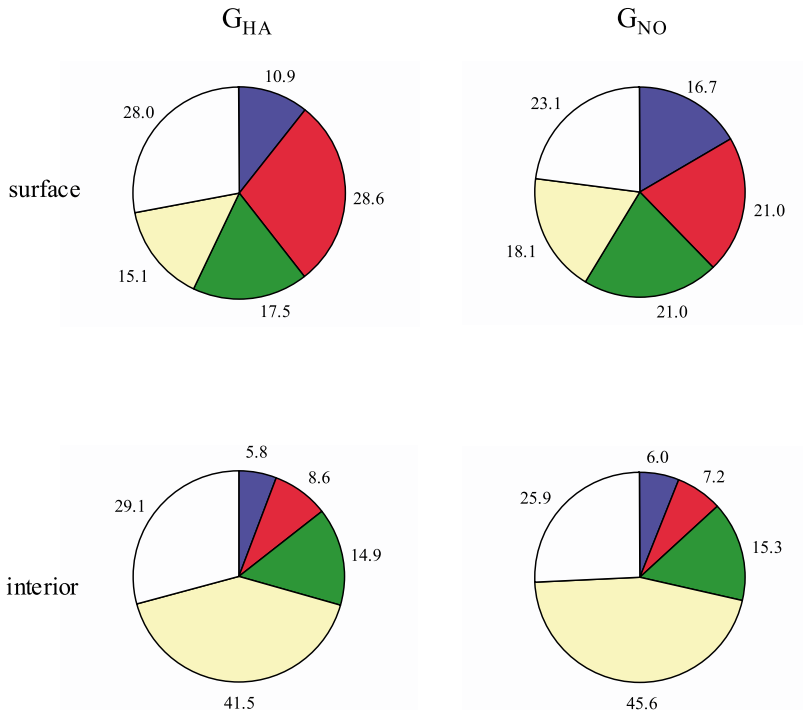

Figure 1.14. General amino acid composition of halophilic as compared to non-halophilic proteins. G_{HA} corresponds to halophilic proteins and G_{NO} corresponds to non-halophilic proteins. Basic amino acids are purple, acidic amino acids are red, polar amino acids are green, apolar amino acids are yellow and other amino acids are white. Adapted from Fukuchi *et al.*, 2003.

In the salt-in adaptation strategy, almost all of the proteins in the cell have to be stable at high internal salt concentrations [95]. It has been suggested that the proteome of an organism may be acidic in proportion to the salinity of its environment – i.e. an organism may have a more acidic proteome if it lives in an environment that is more saline, and as this salinity increases, often the proteome will be more acidic [180].

It has also been found that the formation of salt bridges (hydrogen bonding and ionic bonding between carboxylic acid and amide side chains on amino acids resulting in increased rigidity of the protein) plays a role in the stabilisation of halophilic proteins, especially regarding the maintenance of the association of the subunits of a multi-subunit protein [172,198]. In addition to increased levels of salt bridges, halophilic proteins generally contain intersubunit Cl^- binding sites as well as cation binding sites on the surface of the protein, which aid the stabilisation/association of protein monomers at high salt concentrations [189].

One of the most extensively studied halophilic proteins is the malate dehydrogenase (MDH) from the halophilic archaeon *H. marismortui* [197]. This protein was found to contain more acidic amino acid residues and more salt bridges than a non-halophilic MDH (Dogfish MDH). It was also found, from this particular study, that aspartic acid and glutamic acid (both acidic) made up 20.5% of the amino acid composition of the protein (in comparison to 10.8% for the non-halophilic homologue). In addition to this, and in comparison to the non-halophilic homologue, which has an overall charge of +16, *H. marismortui* MDH (HmMDH) has an overall charge of -156, emphasizing the high degree of negative surface charges on the halophilic enzyme, as is shown below in Figure 1.15. HmMDH has also been found to contain salt bridge clusters between individual subunits (as was mentioned previously), which provide added stability to the enzyme [208]. This enzyme has been reported to require at least 2M NaCl/KCl in order to remain stable – generally halophilic proteins will become inactive when the salt concentration falls below a certain level, which has been reported to be around 2M for HmMDH [189,209].

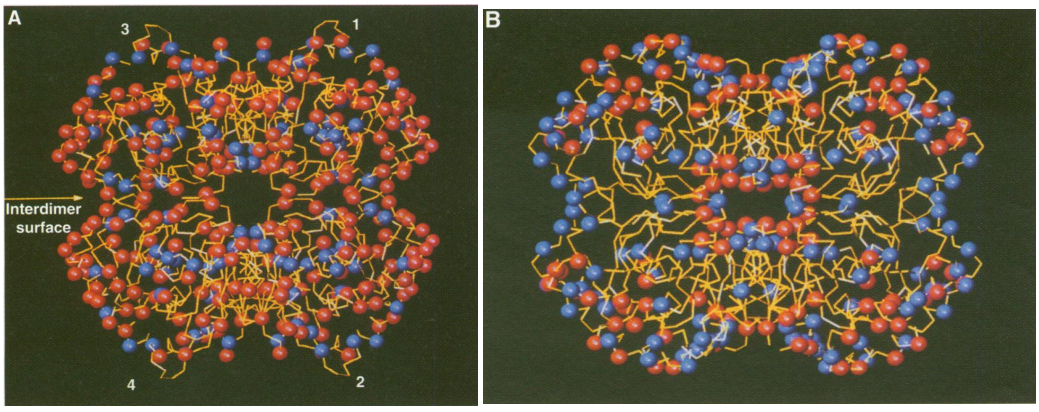

Figure 1.15. HmMDH and DogFish MDH. The images show the 3D structures of HmMDH (A) and DogFish MDH (B). Red balls: acidic amino acids; blue balls: basic amino acids. Figure adapted from Dym *et al.*, 1995.

1.5.1.2: Mechanisms of stability

Regarding non-halophilic proteins, in salt concentrations at 0.1M or lower only very highly charged proteins will be affected by this and hence most organisms can survive within these concentrations with enzyme function intact. However, once the concentration increases above 0.1M, most enzymes within a cell will be affected, since the salt ions will interact with the water surrounding the protein and reduce the amount of water available to it, as well as disrupting the protein structure by direct binding to its surface [206]. At high salt concentrations water availability to a protein is lowered as the water is 'pre-occupied' by forming an ionic lattice around the salt ions [210]. This lowered availability of water can cause the hydrophobic amino acids within a protein to become dehydrated and aggregate, causing the denaturation of the protein. This is thought to be why halophilic proteins generally contain a lower level of hydrophobic amino acids, which assists the protein in competing more effectively with the salt ions for water. Halophilic proteins are generally very unstable at low salt concentrations and will regain activity when placed into solutions containing higher salt concentrations [88]. Enzymes from salt-in halophiles have been found to generally require between 1 – 4M salt in order for the protein to be both active and stable [206].

Halophilic proteins are stabilised by high salt concentrations since their highly charged surfaces can effectively compete with the salt ions for water, in order to properly hydrate the protein [14]. Acidic amino acids (due to their negative charges) tend to become more strongly hydrated (as compared to uncharged residues) and this increased level of acidic amino acids leads to the formation of a so-called 'salt ion network' surrounding the protein, which assists in its solvation and stabilisation at high salt concentrations [88,181,210,211] . This minimum salt concentration required for the stabilisation of halophilic proteins may be due to the interactions of the salt cations with the increased level of negative charges on the protein surface, therefore 'blocking' any repulsive interactions between the acidic amino acid side chains, which could ultimately cause the protein to unfold [206]. In addition, the decreased level of hydrophobic

amino acids also assists the protein to remain stable in the presence of high salt concentrations. This is due to the interactions between hydrophobic residues being increased in the presence of high salt concentrations, which is counterbalanced by the lower level of these residues present on the halophilic protein surface, thus helping to prevent aggregation of the protein [212]. Furthermore, it has also been found that halophilic proteins can be stabilized by decreasing the pH, due to the charge 'screening' effects of H^+ ions on the negatively charged amino acids on the surface of the protein [191].

Halophilic proteins face the problem of having to maintain flexibility (essential for enzyme activity) but also to remain rigid enough so that they do not unfold as a consequence of the high salt concentrations they are exposed to. This flexibility is thought to be partly due to the repulsions between the negative surface charges of the protein [206]. This increase in protein flexibility is further induced by the lower levels of cysteine in these proteins - this amino acid forms disulphide bridges (which increases the rigidity of a protein), therefore lower levels will result in lower rigidity of the protein [170]. A decrease in the level of large hydrophobic amino acids in the centre of a halophilic protein also helps to decrease hydrophobic interactions, hence further increasing the flexibility of the protein [210].

1.6: Ion effects on protein structure

1.6.1: The Hofmeister Effect

Different ions possess different stabilising and destabilising effects on proteins, according to the Hofmeister effect [191]. Franz Hofmeister discovered in 1888, using egg white, that different salts had a different degree of effects when considering the precipitation of the protein [213]. The order that Hofmeister proposed is shown in Figure 1.16.

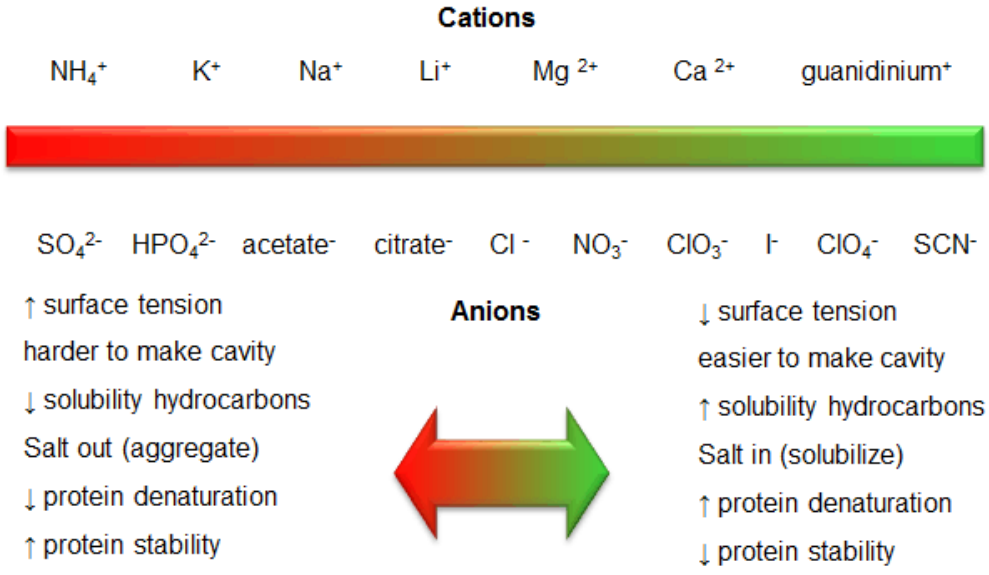

Figure 1.16. The Hofmeister series. Ions in the red are more stabilising towards proteins and those in the green are more denaturing towards proteins. Cations and anions are listed separately in the series. Adapted from Okur *et al.*, 2017.

In the above Hofmeister series, ions are given an order that is dependent on their interactions with water. In other words, dependent on their charge density [215]. This means that smaller ions (higher charge density), such as Li^+, will have a greater affinity for water and will be 'higher' in the Hofmeister series than larger (lower charge density) ions. Regarding cations (the focus of the current study), this means that higher charge density cations are more destabilizing towards protein structures. Due to the fact that anions can interact with both of the positively charged hydrogen atoms of a water molecule, whereas cations are only able to interact with the one negatively charged oxygen atom of the water molecule, the Hofmeister effect is therefore most pronounced for monovalent anions over monovalent cations [215,216].

As is shown in Figure 1.16, ions can also be thought of in terms of salting in and salting out (not to be confused with the 'salt in' and salt out' strategy of halophilic adaptation). This refers to the fact that some ions have been found to lead to protein precipitation, and have hence been used for processes such as crystallization – this is known as salting out. Other ions have been found to increase the solubility and hence lead to the denaturation of a protein – this is

known as salting in [217]. The ability of a salt to 'salt-out' a protein is based on its preferential exclusion from the surface of a protein and hence will lead to the protein becoming more strongly hydrated, whereas salting in is thought to be caused by direct interactions of the salt with the surface of the protein, leading to destabilization [218].

Small ions with a high charge density are usually strongly hydrated, whereas large ions with a low charge density are usually weakly hydrated (Figure 1.17) [219]. Ions can subsequently be split into kosmotropes and chaotropes: kosmotropic ions have high charge density (small) and hence will interact more strongly with water and have more significant effects on its structure, in contrast to the lower charge density of chaotropes (large), which do not have as much of an influence on the structure of water - their interactions with water are in fact weaker than the water-water interactions [220,221]. Kosmotropic anions will compete for the water around a protein and this will then mean that the anion will preferentially interact with the water (as opposed to the protein) and will be excluded from the surface of the protein, hence helping the protein to fold properly (stabilisation), in order to minimize its solvent exposed surface area. The lower affinity for water of chaotropic anions mean that they are more likely to interact with the protein surface and hence cause its destabilization. The situation is different for cations: as ionic size increases, so does the polarisability, therefore larger cations are more likely to be adsorbed to the protein surface than smaller cations, but are less likely to cause precipitation as it is the interaction of smaller (more densely charged) cations with the protein that promotes precipitation by removing water from the protein surface [213]. Therefore, kosmotropic anions and chaotropic cations will have more stabilising effects on proteins, whereas chaotropic anions and kosmotropic cations will have more destabilising effects on proteins, due to their increased binding directly to protein surfaces.

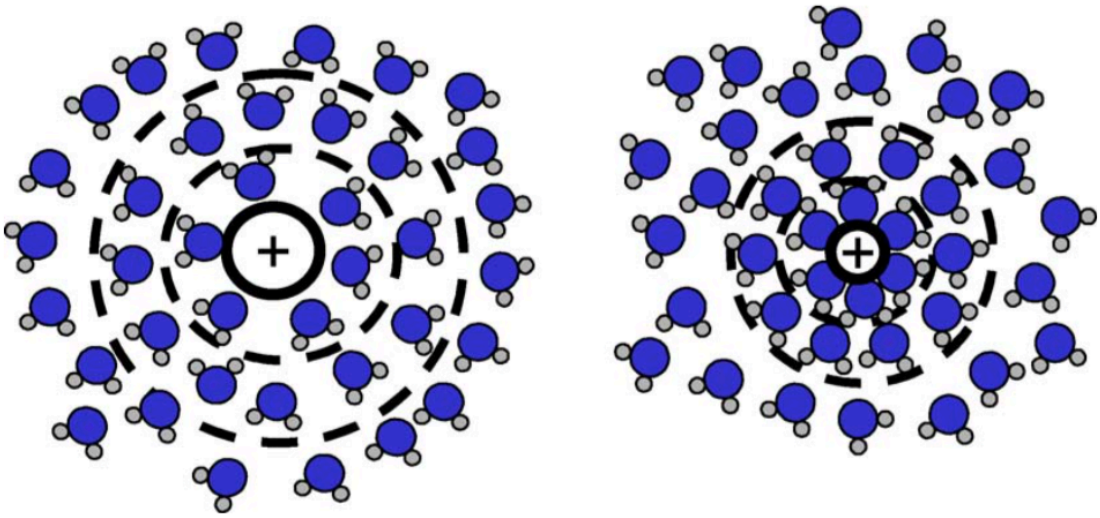

Figure 1.17. Schematic of the hydration shell around a large and small cation. Small ions have a larger charge density and hence water molecules will pack more tightly, whereas larger ions will have a lower charge density and water molecules will be more weakly associated with the ion. Adapted from Tansel *et al*., 2006.

1.6.2: Protein-ion interactions

Proteins are of central importance within a cell, but when considering protein stability it may be surprising to find that proteins are only marginally stable and any minor disruption to their structure may result in destabilization and potential inactivation of the protein [223]. This 'marginal' level of stability is thought to be due to the need to maintain flexibility, i.e. increased 'disorder' – due to the 3D structure of a protein being largely dependent on hydrogen bonding, which is susceptible to alterations due to minor environmental deviations [224]. The fact that proteins, at any one time, are only a few non-covalent interactions away from destabilising explains why protein stabilisation within extremophiles is a topic which has received research attention [14,225].

Na^+ and K^+ have been found to have different effects on amino acids: the higher charge density Na^+ ion can form contact ion pairs with the carboxylic acid side chains on amino acids (meaning that no solvent molecules are between them),

whereas K^+ tends to not interact as strongly with the carboxylic acid side chains within proteins [116]. This is thought to be due to the lower charge density of K^+ ions as compared with Na^+ ions (as can be seen from the Hofmeister series), meaning that K^+ ions have a weaker interaction with the negative side chains of amino acids, and also cannot compete with the water in the first solvation shell surrounding a protein, as effectively as Na^+ [116]. This can be rationalized based on the law of matching water affinity (LMWA), proposed by Collins [226]. This theory states that ions of opposite charge with similar charge densities (i.e. similar affinities for water) tend to associate together, which has major implications for the biological effect of specific ions. This results in higher charge density cations (kosmotropes) having a greater affinity for forming contact-ion pairs with COO^- groups on proteins (which are also kosmotropic), whereas chaotropic cations, such as K^+, do not form contact-ion pairs with the COO^- groups on proteins, as they do not have matching water affinities (i.e. greatly different sizes) and this association would hence be energetically unfavourable. Regarding anions - kosmotropic anions tend to become excluded from the protein surface, since NH_4^+ groups are chaotropic - whereas chaotropic anions can result in protein destabilization due to forming contact ion pairs with these chaotropic NH_4^+ groups on protein surfaces [227,228]. Figure 1.18 shows a schematic of the basis of the theory of LMWA.

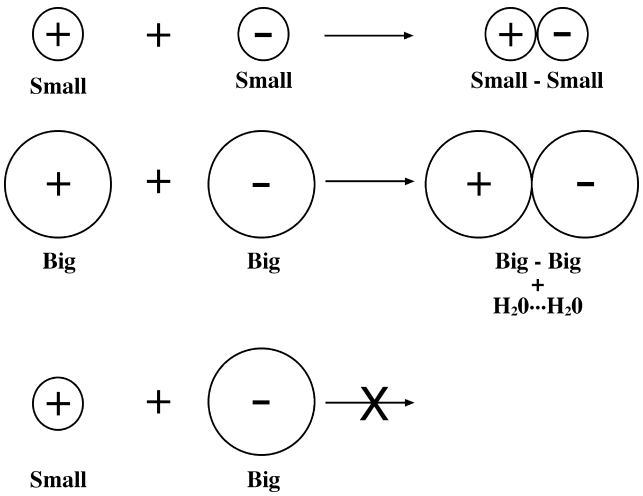

Figure 1.18. The law of matching water affinity. This theory states that two ions of equal water affinities (similar charge density) will spontaneously form ion pairs, whereas those of differing water affinities (large difference in ionic size) will not. Adapted from Collins, 2004.

The peptide Poly-L-Glutamic Acid (PLGA) has been used in various studies as a model protein to understand protein stabilisation/destabilisation, as it can be altered to transition from alpha helix to random coil secondary structure relatively easily [229]. Fedorov *et al*. reported that the addition of K^+ to a solution of PLGA (via molecular dynamics simulations) did not have a significant effect on the polypeptide structure, whereas the addition of Na^+ ions could provoke the transition from random coil to alpha helix conformation, as is shown in Figure 1.19 [116]. This random coil to alpha helix transition generated by the addition of Na^+ is thought to be due to the negative charges between carboxylic acid side chains on the glutamic acid residues being 'screened' (repulsions cause the elongated random conformation), hence allowing tighter packing and hydrogen bonding between side chains [230]. This charge 'screening' is similar as was previously described for the stabilisation of halophilic proteins (section 1.4.1.2).

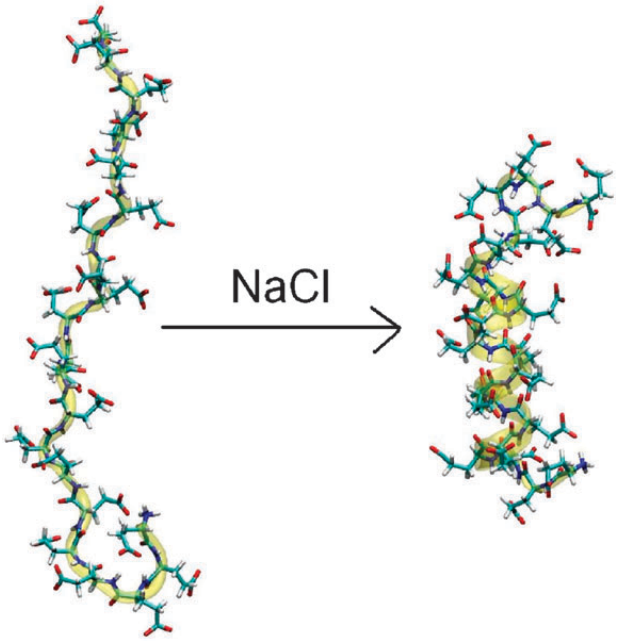

Figure 1.19. The NaCl-induced transition of PLGA from random coil to alpha helix. Poly-L-Glutamic-Acid (PLGA) adopts a random coil (left) conformation when only water is present, but transitions into an alpha helix conformation (right) when moderate (0.3M) concentrations of NaCl are added. Adapted from Fedorov *et al.*, 2009b.

The different effects of specific ions on protein stability and enzyme function has been well studied, and has generally been found to follow the Hofmeister series [232–235]. For example, Na^+ has been found to bind twice as strongly to protein surfaces (carboxylic acid groups) than K^+, with Li^+ binding more strongly than Na^+ to these groups, which obeys the order presented in the Hofmeister series [236,237]. The HIV-1 protease is a particularly well-studied enzyme regarding the effects of specific cations on enzymatic activity. It has been found that the activity of this enzyme follows the Hofmeister series, i.e. activity is lower in the presence of Li^+ than Na^+, and also lower in the presence of Na^+ than K^+, etc. (with the exception of Cs^+), due to the stronger interactions of higher charge density cations with the protein leading to increase destabilisation [238,239].

Moreover, it has been found that specific salts may have different effects with regards to the stabilisation of halophilic proteins [189]. The MDH from *H. marismortui* has been studied within the presence of various salts. It was found from these experiments that adding different salts results in differences in relation to protein/water interactions, which is largely dependent on the nature

of the ionic species present and its charge density [240]. For example, Ebel and Zaccai found that (from the salts they tested) $MgCl_2$ interacted the most strongly with the protein, after which was NaCl, then $NaCH_3CO_2$ and the weakest interaction of the salts tested was $(NH_4)_2SO_4$, which obeys the Hofmeister series. Furthermore, D_2O (deuterium oxide, i.e. 'heavy' water – water with two extra neutrons) has a stronger ability to stabilise this protein than ordinary water, which may be a consequence of its stronger hydrogen bonding with the high level of carboxylic acids on the surface of the protein. Moreover, it was also found that HmMDH unfolds at lower concentrations of NaCl as compared with KCl, suggesting that Na^+ interacts more strongly with the protein surface and maintains its stability more effectively than K^+, as is predicted from the Hofmeister series - making it more efficient for halophilic enzyme stabilisation at lower concentrations.

Ebel *et al.* found that, in terms of the stabilisation of HmMDH, the order of cations was (from high to low): $Li^+ \sim Na^+ > K^+ > Cs^+$, which is the opposite of what was found for the HIV-1 protease, as mentioned previously [191,238]. This seems reasonable when considering the charge density of these ions, as Cs^+ is the largest in terms of ionic radius and Li^+ is the smallest: ions of higher charge density may interact more strongly with the increased level of carboxylic acids on the surface of the halophilic protein (due to LMWA) and will stabilise it more effectively, whereas these cations destabilize non-halophilic proteins [240]. It has been found that the order of the Hofmeister series is preserved to a greater extent for neutral and negatively charged proteins (hence emphasising why it may be so profound for halophilic proteins), whereas the effects may follow a 'reversed' Hofmeister series if the protein has a positive charge – resulting from the stronger adsorption of larger (as opposed to smaller) cations to the protein surface, due to their larger polarisibilities. This effect has also been observed to depend on the pH of the environment – i.e at higher pH the direct Hofmeister series applies (due to deprotonated COO^- groups) whereas at lower pH this 'reversed' Hofmeister series may apply (due to protonated COOH groups) [241].

NaCl and KCl have also been found to have different effects on halophilic proteins from other studies. In a study by Oren and Mana, it was reported that NaCl was

more likely to cause inhibition of the activity of the *S. ruber* G-6-P dehydrogenase at concentrations of up to 2.5M, whereas KCl did not have this effect [203]. Oren and Mana also reported that the enzymes of *S. ruber* have very different tolerances to salts, and this can vary depending on the specific salt present, as well as the individual protein, with some salts more likely to result in increased or decreased activity than others, which also may be dependent on the protein [203]. This adds to the complexity of salt effects on proteins as well as halophilic adaptation.

1.7: Relevance and Applications of Current Study

1.7.1: Insights into protein stabilisation mechanisms

The fact that some compatible solutes isolated from halophiles can assist in protein stability and even in the re-folding of mis-folded proteins makes halophiles a potentially valuable tool for medical research, as these molecules could potentially be used therapeutically for the treatment of various disorders [186]. Compatible solutes have been found to be able to stabilise proteins (via preferential exclusion from the protein surface), and could have a wide array of applications for the general stabilisation of proteins, particularly regarding protein folding disorders [183,242]. Such disorders include Parkinson's disease, Creutzfeldt-Jacob Disease (CJD), Huntington's disease, Alzheimer's disease and retinitis pigmentosa, all of which are progressive disorders for which there are no known cures [243,244]. Moreover, correcting these mis-folded proteins by regaining their native conformations is a promising therapeutic strategy [245]. For example, the compatible solute ectoine, which is found in a range of halophilic organisms (first isolated from *Ectothiorhodospira halochloris*), has been found to help prevent the amyloid aggregation associated with Alzheimer's disease and other neurodegenerative diseases of humans [246,247]. The fact that ectoine may be able to stop proteins from becoming mis-folded and does not interfere with the structures of other proteins within the cell suggests that it is

safe to use in relatively high concentrations, which makes it an excellent candidate for the therapy of these diseases.

Ectoine has many applications and has already been utilised in various products by the German company Bitop [248]. Such current applications include allergy relief, helping with the treatment of dry eye syndrome, a throat spray (for sore throat treatment), a skin cream for allergic disorders, an additive to nebulisers for the treatment of COPD and asthma, and as an additive to skin products such as moisturisers or sun creams [249–255]. The mechanisms of action of ectoine for conditions such as dry eye syndrome and for dry skin disorders are mostly based on the fact that ectoine has been found to be able to protect the cell membrane against damage caused by detergents and other chemicals and (as it interacts strongly with water) is able to prevent water loss from the skin by structuring the water surrounding cells, hence aiding in the retention of water to the surface of the skin, and preventing dry skin, both topically and on the surface of the eye. This preferential exclusion mechanism is also the reason why ectoine has potential for the treatment of protein folding disorders – by becoming excluded from the protein surface and organising the water molecules surrounding the proteins, allowing the effective hydration and folding of these mid-folded proteins. The strong UV absorbing ability of ectoine further supports its inclusion in sun creams. Additionally, ectoine has been found to have anti-inflammatory properties as well as its ability to stabilise the epithelial barrier – i.e. for the treatment of pharyngitis and allergic rhinitis [256]. Many of these ectoine-based protects are available within Europe and one of these products, a nasal spray marketed by Benadryl, is available within the United Kingdom, for the treatment of allergic rhinitis [257]. The source of the ectoine used for these applications is from a halophile which was isolated from the Wadi El Natrun salt lake in Egypt [248].

In addition, the compatible solute betaine and its derivatives have also been shown to have potential therapeutic benefits, including the treatment of alcohol-induced liver disease, atherosclerosis, homocystinuria and as a general anti-convulsant [258–263]. The mechanisms of the anticonvulsant actions are

unknown but its anti-atherosclerosis activities are thought to be due to anti-inflammatory action and it has been shown to lower the levels of homocysteine in plasma by the ability of betaine to methylate homocysteine into methionine [264,265]. Therefore, these halophile-derived molecules have a wide range of applications and show a great deal of promise in the therapy for many conditions. In addition, insights into protein stabilisation/destabilisation could shed light onto protein stability, the conditions required to make a protein stable and to assist in the understanding of pathologies of protein folding.

1.7.2: Industrial applications of halophiles

As mentioned in section 1.6.1, the compatible solute ectoine has a wide array of applications, as do other compatible solutes from halophilic organisms. Ectoine can be produced in large quantities by a process known as 'bacterial milking', whereby these organisms (*H. elongata*) are grown in large quantities and osmotic shock is applied (to stimulate the production of ectoine), after which the salinity of the medium is decreased and the organisms release the ectoine into the medium - as they do not require it anymore - thus making it available to collect and utilise for commercial applications [192]. This technique could be applied to other halophilic/halotolerant organisms for the extraction of large quantities of alternative potentially useful compatible solutes, hence these substances have the capacity to be produced en-masse and at a low cost [266].

Carotenoids (among other pigments) are produced by various halophiles for UV-protection [267]. Carotenoids have various benefits for human health, including the prevention of cancer and eye diseases, such as age related macular degeneration (ARMD), with beta-carotene being the most valuable carotene [268,269]. *H. elongata* has been engineered in order to produce beta-carotene - the main benefit of this particular method of beta-carotene production is that only beta-carotene was produced but other methods involve the production of other carotenoids, and so removal of these has to be performed, which adds both time and expense to the procedure [166]. Also involving beta-carotene production, the halophile *Dunaliella salina* accumulates this pigment in response

to high UV exposure and high salt concentrations withinin its natural environment - this organism has also been used for the production of beta-carotene, as the variety it produces (cis-beta carotene) has been found to be more beneficial to human health than the trans version [270]. Beta-carotene also has non-medicinal uses, including a food colourant and vitamin A precursor (food/vitamin additive) [266]. Halophiles are known to produce a variety of different carotenes and in large quantities, which could be exploited via 'bacterial milking', to utilise these compounds for human health and commercial applications [271,272].

Crop salt tolerance is another potential application of halophilic and halotolerant organisms. Salt-tolerance genes from these organisms could be engineered into crops in order to improve their salt-stress tolerance and hence improve crop yield [201]. This application could have a significant economic impact by transferring salt tolerance to commercially important crops that are blighted by hypersaline soils [266].

Halophiles have also been proposed to be utilised for the treatment of oil-contaminated hypersaline waters, as well as the treatment of hypersaline wastewaters from industry – these processes would take advantage of the natural abilities of these organisms to be able to break down hydrocarbons and pollutants, producing less toxic products [273–275]. Moreover, desalination is another potential application of halophiles. Halophiles as well as marsh plants (halophytes) have been suggested to be able to 'work' together for the desalination of salt water in the Middle East [89]. This method would be considerably cheaper than current alternatives, as well as being renewable. The removal of salt from water using current processes, such as reverse osmosis, is very expensive, so the availability of a cheaper, renewable method may have considerable appeal [276]. The halotolerant bacterium *Staphylococcus xylosus* has been proposed to be used for the treatment of saline wastewater as it was found that this organism was effective at treating wastewater containing as much as 7.2% salt [276]. In addition, a group of UK universities set up a research initiative in 2011 in order to investigate the potential of using halotolerant

Cyanobacteria for the removal of Na^+ and Cl^- from seawater, thus emphasising the recent interest in this potential application of halotolerant species [5,277].

1.7.3: Limits of life and astrobiology

An understanding of halophilic microorganisms may help to gain a better understanding into evolution and the origins of life [278]. Modern life may have evolved from an extremely hot and salty environment (high levels of UV caused water evaporation and hence increased the salt concentration in 'salt pools'), in water at 2 times more concentrated than the seawater of today [279]. In addition, the high salt concentrations were thought to perhaps offer protection to the DNA of the organisms towards the high levels of UV exposure, thus minimizing double strand breaks which could result in mutation or even cell death [280]. The fact that halophilic organisms have been isolated from ancient salt deposits (many millions of years old) could also aid in the study of the evolution of modern day halophiles as well as evolution in a more general sense [281]. The fact that halophiles are widely distributed throughout the tree of life and are not restricted to a few orders shows that halophiles often have close phylogenetic links to non-halophilic organisms. In a review by Ian Dundas he discusses whether life evolved from a hypersaline environment and also asks an interesting question about whether there is an evolutionary connection between halophilic rhodopsins and the rhodopsins found in the human eye [100]. In addition, it has been found that several adaptations found in proteins from halophilic organisms may have also been found in 'prebiotic' proteins (i.e. early proteins), and hence supports the idea that early life may at least have been partially exposed to high salt concentrations [210]. However, since no halophiles have been found close to the roots of the tree of life, this theory remains controversial [94].

The fact that organisms are able to tolerate some of the most extreme environments on the planet may support the idea of life elsewhere in the universe, as well as the theory known as panspermia [11]. This theory was

proposed by various scientists, however, Svante Arrhenius described this theory in the most detail and therefore made this concept more respectable - it states that organisms can travel from one part of the universe to another (via being propelled by radiation pressure) and can grow anywhere that allows them to, as long as the basic conditions that organism requires are met, and most likely travel through space in spore form [282]. This theory has gained an increased level of research attention in recent years [283,284]. Other than the theory of interplanetary transfer, halophiles have relevance to astrobiology due to the fact that it is thought that the soils on Mars contain high concentrations of $MgSO_4$, as well as generally high salt concentrations and high UV levels [285]. However, due to the increased contribution of divalent cations (e.g. Mg^{2+}) towards ionic strength, life within these highly concentrated Mg^{2+} brines may not be similar to terrestrial halophilic life [286]. In addition, brines are thought to have existed in the past on Mars and halite (NaCl) crystals were found in a chondrite that fell in Texas in 1998, as well as halite being found in other chondrites that have fallen to Earth [287–289]. Therefore, life on Mars, whether past or present, is most likely to be halophilic in nature.

There are various sites on the Earth that may be able to act as 'models' for environments elsewhere in the universe [290]. These include glaciers and hot-geysers, which could serve as analogues for life on other planets and their moons. The search for life on other planets continues to gather momentum on the basis that if organisms can survive within extreme environments on Earth then perhaps any environment could contain life, as long as there is a small amount of water and nutrients available [291].

Regarding halophilic 'model' environments for life on other planets, many of these exist, including the Basque lakes in Canada (British Columbia), as this region has been found to have a similar chemical composition to various sites found on Mars [292]. Fendrihan *et al.* exposed the halophile *Halococcus dombrowskii* to UV light similar to that which has been found on the surface of Mars (200nm – 400nm) and it was found that this organism was able to survive this and was still viable after exposure. Since it is possible to simulate such

environments in the laboratory and using these 'analogue' sites, halophiles could offer insight into potential life on Mars without costly space exploration.

Moreover, the BIOPAN mission was launched in 1994, whereby halophilic organisms were sent into space and orbited around the Earth, in order to test for survival within this harsh environment of high radiation exposure. The halophiles were embedded in clay and salt crystals (similar to what has been found on Mars), and it was found that the cells remained partly viable after this journey, more so than non-halophilic organisms [293]. Another mission was subsequently launched, known as the EXPOSE-R mission, whereby two more halophilic organisms were exposed to a space environment, and it was found that the halophiles had increased resistance towards space conditions than non-halophilic organisms, giving further support to the potential of halophilic life elsewhere in the Universe [294] [295].

1.8: Current project

The correlation between specific ion effects and the salt tolerance of bacteria has been poorly studied, with very few studies published on this topic. The only study that has analysed the effects of non-physiological ions in terms of intracellular ion accumulation in a halophilic organism is that of Jensen *et al*., who only used under 1M concentrations and did not compare these data to non-halophiles [10]. There have been no studies carried out in order to compare specific ion effects between a halophile, non-halophile and halotolerant organism. Therefore, the current study is a comparative study of the ion metabolism within a halophile, non-halophile and halotolerant organism, at the interface of biology, physics and chemistry. The aims of this investigation were five-fold:

1. To gain an insight into general ion metabolism in bacteria
2. To understand how ion metabolism within bacteria varies with salt tolerance
3. To gain insight into the mechanisms of bacterial salt tolerance
4. To understand the effects of specific ions at the protein and whole organism level
5. To rationalise specific ion effects on bacteria in terms of physical chemistry

The above were assessed via the analysis of bacterial growth in a variety of different salts, ICP-MS analysis of the cellular contents of these organisms, enzymatic activity assays in the presence of various salts, as well as bioinformatics analysis, to gain additional insight into haloadaptation and bacterial salt tolerance.

Chapter 2: Materials and methods

2.1: Bioinformatics analysis

S. ruber was selected as the halophile to investigate for this study due to the fact it is a bacterium, whereas most salt-in extreme halophiles are Archaea [206], and bacteria are the focus of this particular project. Malate dehydrogenase from *S. ruber* was selected as the enzyme to study due to the fact that the crystal structure of this enzyme is available on the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do): PDB ID 3NEP [296], and also due to the fact that MDH already has an established assay system to detect its activity [296,297].

2.1.1: BLAST search and identification of non-halophilic and halotolerant organisms to study

A schematic showing the steps that led to the selection of the proteins/organisms is shown in Figure 2.1. *Echinicola vietnamensis* was selected due to both the high level of similarity of its MDH to SrMDH as well as the fact that it has had one publication [298], whereas many of the other matches had no publications, so preference was given to the organisms that have already been at least partially characterised. *E. coli* was chosen to use as the non-halophile, as its metabolism has been extensively studied and it is a good model organism for a non-halophile.

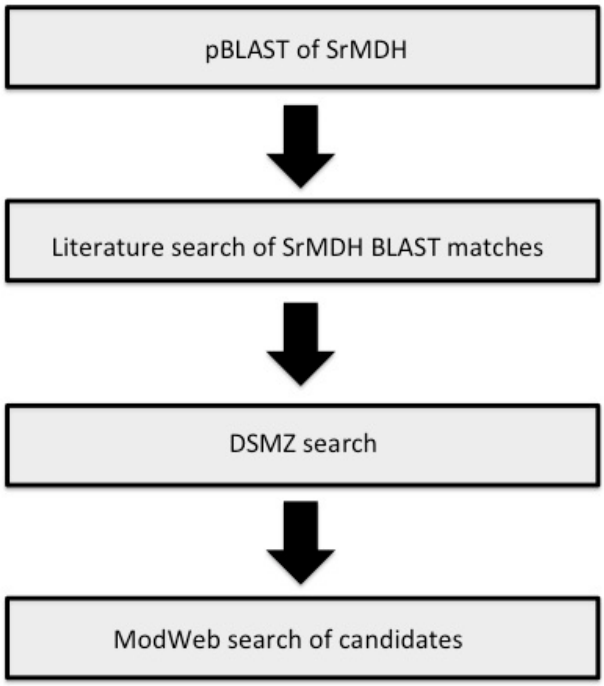

Figure 2.1. Schematic showing the steps that led to the selection of the organisms/proteins to use for the study. A protein BLAST (Basic Local Alignment Search Tool) search was carried out using the resource at https://blast.ncbi.nlm.nih.gov/Blast.cgi [299,300], on the *S. ruber* malate dehydrogenase (SrMDH) in order to find the most similar MDHs to determine which organisms to use alongside *S. ruber* in this study. Only halotolerant organisms were included in the analysis (halotolerant matches were determined by literature searches on the closest matches to SrMDH). This list was further narrowed down by searching the DSMZ (biological resource centre based in Germany) (https://www.dsmz.de/), in order to determine which organisms could be procured. The list of candidates was shortened further by excluding organisms that had more complex growth requirements than could be provided (due to laboratory growth constraints). Finally, the list was narrowed down further by submitting the MDH sequences of all of these organisms to the Modweb protein structure modelling server (https://modbase.compbio.ucsf.edu/modweb/) [301], which was used to find the 3-dimensional sequence similarities of the matches to the template (SrMDH).

2.1.2: Clustal Omega sequence alignments

All protein sequence alignments were carried out using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) [302]. Protein sequences were downloaded in FASTA format from UniProt (http://www.uniprot.org/) [303].

Alignment scores (in terms of sequence identity) were also obtained from Clustal Omega.

2.1.3: Construction of phylogenetic trees

Clustal Omega multiple protein sequence alignments were used to produce a phylogenetic tree using ClustalW2 Simple Phylogeny: http://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/ [304]. The neighbour-joining method was used with distance correction turned on and gaps turned off [305,306]. The ClustalW2 tree file was downloaded and the image was created using SeaView software [307].

2.1.4: *E. vietnamensis* MDH homology modelling

To obtain a 3D structure for *E. vietnamensis* MDH (EvMDH), homology modelling was carried out using the Swiss-model automated homology modelling server (https://swissmodel.expasy.org/) [308] The protein sequence for EvMDH was obtained from UniProt and was submitted to the Swiss-model server. The template used for the homology modelling was SrMDH, as this was the closest match found within the Swiss-Model database. A structural homology model, in PDB format, was created for EvMDH (QMEAN4 score of 0.78).

2.1.5: Structural alignments of the Malate Dehydrogenases

The crystal structures of the MDH from *E. coli* (PDB ID: 3HHP) and *S. ruber* (PDB ID: NEP) were downloaded from the PDB, and the homology model of *E. vietnamensis* (section 2.1.4) was also used. Protein structural alignments were carried out using the UCSF Chimera program, developed by the Resource for Biocomputing, Visualisation and Informatics at the University of California in San Francisco: http://www.rbvi.ucsf.edu/chimera, utilising the MatchMaker tool using the Needleman-Wunsch algorithm [309,310]. This tool performs a

pairwise sequence alignment on the two protein sequences and utilises this 'flat' alignment as well as secondary structural features present in the proteins in order to superimpose the two structures, to create a 3-dimensional structural alignment. EcMDH and EvMDH were broken down into their monomeric forms before alignment (the SrMDH PDB file consisted only of the monomeric form) and individual proteins were coloured separately. In addition, homology modelling was carried out on SrMDH using Swiss-model (see section 2.1.5) in order to create a tetrameric structure, which was also used for the alignments.

2.1.6: Coulombic surface colouring

The PDB files of the three enzymes were imported into the UCSF Chimera program. In order to view the net charges on the surfaces of the proteins, the structures were coloured by the surface electrostatic potential using the 'Coulombic surface colouring' tool, whereby the structures are coloured based on Coulomb's law [310], shown in Equation 2.1.

$$\varphi = \Sigma \left[q_1 q_2 / (\varepsilon d_i) \right]$$

Equation 2.1. Coulomb's law. Where φ is the potential, q are the atomic partial charges, d is the distance between the atoms and ε is the dielectric. This calculation results in the colouring of a protein structure by the sum of Coulomb potentials on the surface of the protein, whereby blue represents positive potentials, red represents negative potentials and white is neutral [311].

2.1.7: Selection of additional organisms to include in analysis

The additional organisms which were included for bioinformatics analysis were selected based on two criteria: to have an MDH sequence available within the UniProt database and to be halophilic, non-halophilic, halotolerant or thermophilic. The organisms were determined to be suitable by extensive literature searches.

2.1.8: Theoretical 2D gels

Theoretical 2D gels were constructed by using the ExPASy pI/mW calculator tool: http://web.expasy.org/compute_pi/ [312]. Whole organism proteomes were downloaded from UniProt and these were used in order to compute the molecular weight and isoelectric point of each protein in the full proteome of the organism (via the pI/mW tool). Data were exported to GraphPad Prism (version 6.0) and were plotted in order to take the form of a theoretical 2-dimensional gel, where the proteins from an entire proteome are displayed as molecular weight vs pI (IEP).

2.1.9: Analysis using the ProtParam and Pepcalc tools

The ExPASy ProtParam tool was used to obtain the theoretical pI as well as negative and positive amino acid residues and amino acid compositions of the proteins used within this study (http://web.expasy.org/protparam/) [313]. The protein sequences were obtained from UniProt and the protein accession number was entered into ProtParam to compute the above-mentioned characteristics of the protein. For the pI, values were plotted using GraphPad Prism software and Tables were produced using Microsoft Excel. Negative and positive amino acid and amino acid composition data were plotted in GraphPad Prism. Amino acid compositions were separated into 5 categories of amino acid properties: basic (Arg and Lys); acidic (Asp and Glu); polar (Asn, Gln, Ser, Thr); nonpolar (Ile, Leu, Met, Phe, Trp, Tyr, Val) and others (Ala, Lys, Gly, His, Pro) – according to the same scheme used by Fukuchi *et al* [88].

Additionally, the resource at pepcalc.com was used to calculate the net charges on the proteins (http://pepcalc.com/).

2.1.10: Analysis of Na^+ and K^+ channels and transporters

The full proteomes of *E. coli* k12, *S. ruber*, and *E. vietnamensis* were searched on UniProt to obtain a list of all potential cation transporters. The searches carried out consisted of the following strings: K^+/H^+; Na^+-dependent; K^+/Na^+; cation transport; cation efflux; efflux; monovalent cation transporter; mechanosensitive channel; K^+ transport; Na^+ transport Li^+ transport; Rb^+ transport; Cs^+ transport; voltage-gated; magnesium transport; Na^+/H^+; K^+-transporting ATPase; Trk system; kup system; kdp system; Nha system. The full proteomes were then analysed manually to acquire any missed proteins and results that were verified to be cation transporters were added to an Excel table (containing the UniProt annotation and the gene name) and comparisons were made between the three organisms.

2.2: Bacterial growth experiments

2.2.1: Bacterial strains and culture conditions

All chemicals used for the preparation of bacterial growth media were purchased from Sigma-Aldrich. All of the media described below were autoclaved after preparing and prior to use.

E. coli DH5α were grown in LB medium: 5g yeast extract; 10g NaCl; 10g tryptone, in 1L of diH_2O. Cells were grown at 37^{o}c in conical flasks, with shaking at 250rpm.

E. vietnamensis is a halotolerant, light-pink pigmented, heterotrophic, Gram-negative bacterium that was isolated from seawater off the coast of Vietnam [298]. *E. vietnamensis* were grown in a modified version of the Marine Broth Medium (Marine Broth medium composition was obtained from the DSMZ website), which was composed based on an initial analysis of growth in different

media. This consisted of (per 1L diH_2O): 5g peptone; 1g yeast extract; 0.10g Fe(III) citrate; 19.45g NaCl; 5.9g $MgCl_2$; 3.24g Na_2SO_4; 1.8g $CaCl_2$; 0.55g KCl; 0.16g $NaHCO_3$; 0.08g KBr. Cells were grown at 30^{o}c in conical flasks with shaking at 220rpm. *E. vietnamensis* bacteria were purchased in lyophilized form from the DSMZ (https://www.dsmz.de).

S. ruber bacteria were grown in a slightly modified version of Salinibacter medium (Salinibacter medium composition was obtained from the DSMZ website), consisting of (per 1L of diH_2O): 195g NaCl; 34.6g $MgCl_2$ x 6H_2O; 49.5g $MgSO_4$ x 7H_2O; 5g KCl; 0.25g $NaHCO_3$; 0.25g KBr; 0.5g yeast extract. Cells were grown at 37^{o}c in conical flasks at 250rpm. *S. ruber* strain M31 was kindly provided by Charles Cockell, at the University of Edinburgh.

All bacterial stocks were stored in 50% glycerol at -80^{o}C.

2.2.2: Formulation of media of varying salt compositions

A novel 'base' medium was formulated that allowed for the growth of all 3 bacteria, which had varying salt requirements. Various media compositions were tested that allowed the growth of all three organisms – this involved growing *E. coli*, *E. vietnamensis* and *S. ruber* in a range of media of various compositions to test whether substantial growth could occur in any of these media (8 variations were tested). Many of these media were not suitable due to the fastidious nature of *S. ruber*, although most could support the growth of *E. coli* and *E. vietnamensis*. Since it has been found that *Salinibacter* species require magnesium salts [314], this was added to the medium, as both *E. coli* and *E. vietnamensis* were able to tolerate this addition. It has been reported in previous studies that *S. ruber* does not tolerate a high concentration of yeast extract in its medium [190]. For this reason, the General Medium only contained a very low concentration of this.

The final composition for the 'base' General Medium (excluding the main salt – see below) consisted of: 0.5g yeast extract; 10g tryptone; 34.6g $MgCl_2$ x 6H_2O;

49.5g $MgSO_4$ x $7H_2O$, per 1L diH_2O. This medium was formulated to allow for the growth of all 3 organisms.

The General Medium was used for the growth experiments (section 2.2.5). This base medium was used to make media of 21 different salts/salt combinations: each of 8 – 12 different concentrations - 218 different media were made in total. The masses of salt added to the medium to make each concentration of a pure salt medium (concentrations between 0M – 5.5M) are shown in Table 2.1 and Table 2.2. In addition, a range (14 different salt combinations) of equimolar salt combination media were composed (containing 2 different salts in a 50:50 ratio) and the masses of each of the seven individual salts used to make these media are shown in Tables 2.3 and 2.4, in which the concentration refers to the total salt concentration in the medium: masses shown refer to the amount of a specific salt added to make equimolar media containing a mixture of two specific salts. Due to the limits of solubility, KCl-containing media contained a maximum concentration of 4.5M. In addition, LiCl media contained a maximum concentration of 4.5M, and RbCl and CsCl media contained a maximum concentration of 3.5M.

Table 2.1. Masses of NaCl, KCl, NaBr and KBr used to make each medium. The table shows the mass (g/L) of salt added to the General Medium in order to make a medium of the appropriate concentration of the specified salt.

Medium salt concentration (M)	NaCl	KCl	NaBr	KBr
0.5	29.22	37.28	51.45	59.50
1	58.44	74.55	102.89	119.00
1.5	87.66	111.83	154.34	178.50
2	116.88	149.10	205.79	238.00
2.5	146.10	186.38	257.24	297.51
3	175.32	223.65	308.68	357.01
3.5	204.54	260.93	360.13	416.51
4	233.76	298.20	411.58	476.01
4.5	262.98	335.48	463.02	535.51
5	292.20	-	514.47	595.01
5.5	321.42	-	565.92	654.51

Table 2.2. Mass of LiCl, RbCl and CsCl used to make each medium. The table shows the mass (g/L) of salt added to the General Medium in order to make a medium of the appropriate concentration of the specified salt.

Medium salt concentration (M)	LiCl	RbCl	CsCl
0.5	21.20	60.46	84.18
1	42.39	120.92	168.36
1.5	63.59	181.38	252.54
2	84.79	241.85	336.72
2.5	105.99	302.31	420.90
3	127.38	362.78	505.08
3.5	148.58	423.24	589.26
4	169.77	-	-
4.5	190.97	-	-

Table 2.3. Masses used to make equimolar media. The table shows the mass (g/L) of each salt added to the General Medium in order to make media with a total salt concentration that is a 50:50 ratio of two salts.

Medium total salt concentration (M)	NaCl	KCl	NaBr	KBr
0.5	14.61	18.64	25.72	29.75
1	29.22	37.28	51.45	59.50
1.5	43.83	55.91	77.17	87.25
2	58.44	74.55	102.89	117.00
2.5	73.05	93.19	128.62	146.75
3	87.66	111.82	154.34	176.50
3.5	102.27	130.46	180.06	206.25
4	116.88	149.10	205.79	236.00
4.5	131.49	167.74	231.51	265.75
5	146.10	-	257.23	294.75
5.5	160.71	-	282.95	324.50

Table 2.4. Masses used to make equimolar media. The table shows the mass (g/L) of each salt added to the General Medium in order to make media with a total salt concentration that is a 50:50 ratio of two salts.

Medium total salt concentration (M)	LiCl	RbCl	CsCl
0.5	10.60	30.23	42.09
1	21.20	60.46	84.18
1.5	31.80	90.69	126.27
2	42.39	120.92	168.36
2.5	52.99	141.15	210.45
3	63.59	171.38	252.54
3.5	74.19	201.61	294.63
4	84.79	-	-
4.5	95.38	-	-

2.2.3: Overnight cultures

Overnight cultures of *E. coli* and *E. vietnamensis* were set up whereby 0.5ml of culture was taken from a continuously growing culture and inoculated into Universal tubes containing 10ml of fresh medium (LB and Marine Broth medium, respectively). Incubation was at 37^{o}c/250rpm and 30^{o}c/220rpm for 16 hours (respectively), before the growth experiments were set up. Due to the slow growth rate of *S. ruber*, these were grown for 7 days in 250ml conical flasks (in Salinibacter medium), until the organisms reached the mid-exponential phase of growth.

2.2.4: Preparation of cells

The bacteria were harvested by centrifugation at 3400g for 10 minutes, in order to remove the cells from the medium and to remove residual medium to ensure an as low level of salt contamination as possible during the experiments. The cells were then washed in potassium phosphate buffer (pH 7.4), centrifuged at 3400g, supernatant removed, and the cell pellets were left to air dry for 60 minutes in a microbiological cell culture hood.

2.2.5: Growth experiment set up

Growth experiments were carried out in 96 well microplates. Each organism was incubated in a separate plate – to avoid cross-contamination. After the cell pellets were air-dried, they were gently re-suspended in the appropriate medium and inoculated into the relevant well of the 96 well plate. Individual experiments were carried out in triplicate: wells containing cells contained 150µl of medium and 50µl of culture (cells re-suspended in medium of the appropriate salt concentration). The blank (control) wells contained 200µl of media, with each blank containing media of the same salt composition/concentration as the sample wells to account for potential differences in the optical density of different salt concentrations/different salts. General medium containing the specified salt/salt combination was prepared prior to experimental set up, as was described in section 2.2.2. Each individual experiment was carried out multiple (3 – 9) times.

2.2.6: Measurement of growth

E. coli and *E. vietnamensis* growth experiments were carried out using a Synergy HT microplate reader (BioTek) with spectra analysed using Gen5 software and were incubated in the instrument incubator for the full duration of the growth experiment, with OD_{600} taken automatically every 30 minutes. *S. ruber* OD_{600} (optical density at 600nm) was measured once a day for 7 – 14 days, due to its slower growth rate, on a Spectramax 190 spectrophotometer (Molecular Devices), with spectra analysed via Softmax Pro software. All plates were sealed with a radiation-sterilised gas-permeable film (Thermo Scientific, Nunc), to avoid wells drying up and to prevent cross-contamination. All organisms were incubated at the same temperature (37^{o}c), with shaking at 250rpm.

2.2.7: Data analysis

OD_{600} data was analysed using GraphPad Prism software (www.graphpad.com), version 6.0. Raw OD_{600} data were initially plotted as OD_{600} against time, which was then ln-transformed using the 'transform' tool: Y = ln(Y). From this the exponential growth phase was identified and isolated and a linear fit was performed on this portion of the transformed growth curve. The gradient of this straight line is equal to the specific growth rate of the bacteria (units in h^{-1}) [315]. Specific growth rates were plotted via GraphPad Prism as a scatter of individual replicates (separate experiments) around the mean, with standard deviations calculated via Prism and shown as error bars. Doubling times (DT) in hours were calculated by Equation 2.2.

$$DT = \ln 2 / k$$

Equation 2.2. Bacterial doubling time calculation. k is the specific growth rate (gradient of the straight line) in units of h^{-1} and DT is the doubling time, in hours [316].

2.3: Inversely Coupled Plasma Mass Spectrometry (ICP-MS) analysis

2.3.1: Bacterial growth

Cultures of *E. coli*, *E. vietnamensis* and *S. ruber* were set up by inoculating 0.5ml of continuously growing culture into sterile Universal tubes containing 10ml fresh medium (LB, MB and Salinibacter medium, respectively), and were incubated at 37°c (30°c for *E. vietnamensis*) with shaking at 250rpm. Cells were harvested after 12 – 24 hours (*E. coli* and *E. vietnamensis*) or 7 days (*S. ruber*), by centrfugation at 3400g, in order to remove media. The organisms were grown in a multitude of different media, as were described in detail section 2.2.1, by re-

suspending cell pellets in 10ml of fresh medium. The different media consisted of 21 different salts/salt combinations and at concentrations from 0M – 5.5M (concentrations depended on the range of growth of that particular organism). *E. coli* and *E. vietnamensis* were incubated for 1 – 3 days, until they reached the mid-exponential phase of growth ($0D_{600}$ of 0.6 – 0.8). *S. ruber* were grown for 7 – 14 days until the OD_{600} reached 0.4 – 0.6.

2.3.2: Sample preparation

After growth, the cells were harvested by centrifugation at 3400g and the medium was removed. The cell pellets were air-dried for several hours in a microbiological cell culture hood.

After drying, the cells were lysed in 100µl cellytic B + benzonase + lysozyme (Sigma-Aldrich), and the cell extracts were removed and cell debris discarded. The lysates and media were diluted in deionised water (diH_2O), in order to prepare them for mass spectrometry analysis. Samples were diluted via serial dilution: samples from organisms grown in salt concentrations from 0M – 1.5M were diluted by 1 in 10 000 (2µl sample added to 20ml diH_2O); samples from 2M – 2.5M were diluted by 1 in 100 000 (a 1 in 10 000 dilution was carried out and 2ml of this was added to 20ml diH_2O); and samples from 3M – 5.5M were diluted 1 in 1000 000 (a 1 in 100 000 dilution was carried out and 2ml of this was added to 20ml diH_2O). This was to account for the sensitivity of the mass spectrometer and to avoid 'overloading' the machine when very high salt concentrations were used, since the ICP-MS instrument is very sensitive. Nitric acid (2%) was added to the sample prior to analysis, in order to stabilise and dissolve the components of the sample [317].

2.3.3: ICP-MS

An Agilent 7700x ICP-MS instrument with liquid argon (supplied by BOC) as the carrier gas was used to analyse the samples. The instrument was tuned (using a

1ppb solution of yttrium (Y), lithium (Li), cobalt (Co) and titanium (TI)) and calibrated (refer to Appendix C for calibration curves) before analysis. Samples were analysed in helium mode and interferences were kept at 0.5%. Method parameters and internal standards were selected using the MassHunter software (Agilent). The ions of interest were: sodium (Na^+); potassium (K^+); magnesium (Mg^{2+}); lithium (Li^+); cesium (Cs^+); rubidium (Rb^+). Crucially, Na^+, K^+, Li^+, Rb^+, Cs^+ and Mg^{2+} all have detection limits of less than 1ppt (part per trillion), whereas Cl^- cannot be detected and Br^- has a detection limit of 1-50ppb (part per billion) [318]. Each sample was measured 3 times and the average value of these was recorded.

2.3.4: Data analysis

2.3.4.1: Data normalisation

After analysis the dilution factor was applied to all of the samples to account for these in the data analysis (refer to section 2.3.2). In order to normalise the data, the OD_{600} measurements for each sample were converted to approximate cell numbers using the Agilent cell culture concentration from OD_{600} calculator (http://www.genomics.agilent.com/biocalculators/calcODBacterial.jsp). This tool gives the approximate number of cells/L. The concentrations obtained from the ICP-MS analysis were converted from µg/L to g/L. The ion concentration (g/L) was divided by the cells/L, to give a concentration of each ion per cell – this was to act as a method of normalising the samples so that they can be directly compared, in terms of estimated cellular concentrations.

2.3.4.2: Data analysis

The values obtained from the normalisation were graphed using Microsoft Excel. Additionally, the data were expressed as the ratio of each cation to potassium inside the cell – [specific ion]:[K^+]. This was achieved by dividing the concentration of the ion of interest (either Na^+/Li^+/Rb^+/Cs^+) by the K^+ concentration.

This resulted in a ratio value – with values less than one showing a greater level of K^+ in the sample than either Na^+, Li^+, Rb^+ or Cs^+ and values greater than one meaning more of Na^+, Li^+, Rb^+ or Cs^+ was present in the sample than K^+. The resultant values were plotted using GraphPad Prism.

2.4: MDH Enzymatic Assays

2.4.1: Preparation of buffer

Potassium phosphate buffer (0.035M) was prepared according to the protocol from Fisher Scientific [319]. 0.35M stocks of KH_2PO_4 (Sigma-Aldrich) and K_2HPO_4 (Sigma-Aldrich) were prepared. In order to prepare buffer with a pH of 7.4, 40.1ml of K_2HPO_4 was added to 9.9ml of KH_2PO_4 and 450ml diH_2O, which was then pH tested and adjusted to pH 7.4 (if required). This was then autoclaved.

2.4.2: Preparation of master mix and stock solutions

Enzyme assay master mixes contained: 50ml pH 7.4 potassium phosphate buffer + 0.4mM NAD^+ (13.2mg per 50ml) (Sigma-Aldrich). Separate master mixes were made for each salt (NaCl, NaBr, KCl, KBr) and each concentration (0, 0.1, 0.2, 0.3, 0.5, 1, 2, 3M), i.e. 32 solutions in total. The salt concentration added to the master mix was added to make the concentration as is shown in Table 2.17. This was to take into account dilution by the addition of substrate when the enzyme assay experiments were carried out (50µl of malate stock added to 150µl master mix). Malate stocks were prepared separately, by adding malate (Sigma-Aldrich) to potassium phosphate buffer (pH 7.4) to make stock solutions according to Table 2.18, to take into account the dilution of malate by addition to the master mix during the experiments. Stock solutions were calculated using the formula given in Equation 2.4.1.

$$C_1V_1 = C_2V_2$$

Equation 2.3. C_1 is the stock concentration, V_1 is the initial volume, C_2 is the final concentration and V_2 is the final volume [320].

Table 2.17. Salt stock concentrations and final concentrations. Separate stock solutions of the enzyme assay master mix were prepared for NaCl, KCl, NaBr or KBr in order to take into account the final assay volume – 150µl of salt-containing master mix was diluted by the addition of 50µl malate. Values refer to the molarity of salt (M) added to the master mix.

Stock concentration (M)	Final concentration in assay (M)
0	0
0.13	0.1
0.27	0.2
0.40	0.3
0.67	0.5
1.33	1
2.70	2
4.00	3

Table 2.18. Malate stock salt concentrations and final concentrations. Stock concentrations of malate were prepared, to give final concentrations that took into account the addition of 50µl malate to 150µl enzyme assay master mix.

Stock concentration (mM)	Final concentration in assay (mM)
120	30
320	80
640	160
2560	640

2.4.3: Cell-extract preparation

Cells were grown in LB medium (*E. coli*), Marine Broth medium (*E. vietnamensis*) and Salinibacter medium (*S. ruber*) in 250ml conical flasks at 37°c and 30°c (*E.*

vietnamensis) with shaking at 250rpm, until the OD_{600} was between 0.6 – 0.8 (or above 0.4 for *S. ruber*, due to its lower cell densities) [203]. The cells were centrifuged at 3000g for 10 minutes and washed in potassium-phosphate buffer (pH 7.4). The concentration of potassium phosphate within the buffer was very low (0.035M) and was removed from the cells prior to lysis, so potassium 'contamination' from this should be minimal. Cells were lysed with cellytic B (Sigma-Aldrich) cell lysis reagent + benzonase (Sigma-Aldrich), + lysozyme (Sigma-Aldrich) and were incubated for 45 minutes with shaking at 30°C. They were then centrifuged for 10 minutes at 16000g, the supernatant obtained (i.e. cell debris were removed) and was stored at -20°C.

2.4.4: Protein concentration measurement

The protein concentrations of the cell lysates were measured via a Bradford assay [321]. BSA concentrations from 0mg/ml – 2mg/ml were prepared in order to construct the standard curve. Cell extracts were diluted by 1:10 in potassium phosphate buffer (pH 7.4). 5µl of each of the BSA standards was added to a well of a 96 well microplate, and 6 x 5µl of diluted sample was added to the following row of a 96 well plate. 250µl of Bradford reagent was added to these and this was mixed and incubated at room temperature for 20 minutes. Absorbance at 595nm was measured using a Spectramax 190 spectrophotometer, with Softmax Pro software. A standard curve was constructed for BSA (absorbance vs protein concentration). The absorbance of the sample was compared with the standard curve in order to calculate the protein concentration of the cellular extracts.

2.4.5: Enzymatic activity assays

The crude cell extracts were used to carry out experiments to analyse the enzymatic activity of the MDHs from the three organisms. These assays took advantage of the fact that the conversion of malate to oxaloacetate involves the conversion of co-enzyme NAD^+ to NADH. NADH absorbs much more strongly than NAD^+ at 340nm and can therefore be used as an indicator of reaction rate,

as NADH will be produced in proportion to the product, oxaloacetate (refer to section 6.1.3) [322].

2.4.5.1: Experimental optimisation

A range of protein concentrations (different volumes of cellular lysate), NAD^+ concentrations and malate concentrations were tested, in order to determine which conditions would be optimal for the assay. In addition, the duration of the enzymatic reaction was assessed, in order to determine the required reaction time and kinetic interval for the experiments.

2.4.5.2: Enzymatic assay

Enzyme assays were carried out in 96 well microplates using a Spectramax 190 spectrophotometer with Softmax pro software, using the Kinetic Read mode. Each experiment consisted of a sample and 2 negative controls: one without protein and one without malate (substrate). Each enzymatic reaction mixture contained the following: 150µl enzyme master mix (described in section 2.4.2); 50µl malate (concentrations of 30mM, 80mM, 160mM or 640mM); 5µl protein (1 – 15mg/ml). For K_m calculations, each protein was assayed in a range (0mM – 640mM) of malate (substrate) concentrations, in the absence of salt, with each reaction carried out multiple (5 – 8) times. Experiments were carried out at 25^{o}C.

Each individual enzyme reaction was carried out separately for a duration of 2 minutes, with readings taken automatically every 4 seconds. The reaction was initiated by the addition of malate (substrate) and the absorption at each time point at 340nm was measured (NADH absorption). Each individual reaction was carried out 3 times.

2.4.6: Data analysis

The raw enzyme assay data was analysed using GraphPad Prism software. Data were initially plotted as absorption at 340nm vs time. The blank was subtracted from the sample data, to give a blank-subtracted curve. The initial velocities (V_0) were calculated by carrying out a linear fit of the initial 4 seconds of the curve - the gradient of this straight line is equal to the initial velocity (V_0).

For K_m calculations, data were plotted as V_0 (obtained as above) against malate concentration. A Michaelis-Menten curve fit (refer to section 6.1.4) was carried out on the data, via GraphPad Prism, which gave the kinetic parameters K_m and V_{max}.

Samples varied with respect to their protein concentrations as the organisms yielded very different amounts of protein: *S. ruber* protein yield was a lot lower than for *E. coli* and *E. vietnamensis*. The specific activity was used in order to express the activities of the enzymes, to take into account the fact that the protein concentrations varied. The specific activity was calculated according to Equation 2.5.

$$\mathbf{SA = [V_0 \times volume\ of\ protein]/mass\ of\ protein}$$

Equation 2.5. Specific activity calculation. Where SA = specific activity and V_0 = initial velocity.

The units of the specific activity calculated are expressed as mM min^{-1} mg^{-1} [233]. The V_0 is in units of mM min^{-1}, which gives the unit of mM min^{-1} mg^{-1} when normalised to protein concentration. The specific activity allows for the direct comparison of the activity of the three proteins, regardless of the protein concentration present within each individual sample.

Chapter 3: Bioinformatics Analysis

3.1: Introduction

3.1.1: Bioinformatics for Proteomics research

The word proteome applies to the study of the entire set of proteins encoded by the genome of an organism [323]. Proteomics is the general area of biology that involves studying the structure, function and properties of proteins [324]. Protein sequence databases, such as UniProt and the Protein Data Bank (PDB), are collections of protein information, such as protein sequences, functional information and 3D structures [325]. Therefore, it is possible to gain extensive knowledge on almost any protein and to gain additional functional, structural and comparative information for that protein, using the wide array of available bioinformatics resources. The PDB is the main resource for obtaining protein structures, attained mainly via nuclear magnetic resonance (NMR) and x-ray crystallography [326]. Figure 3.1 shows how the PDB has developed over the years, in terms of the available structures deposited within this database [327].

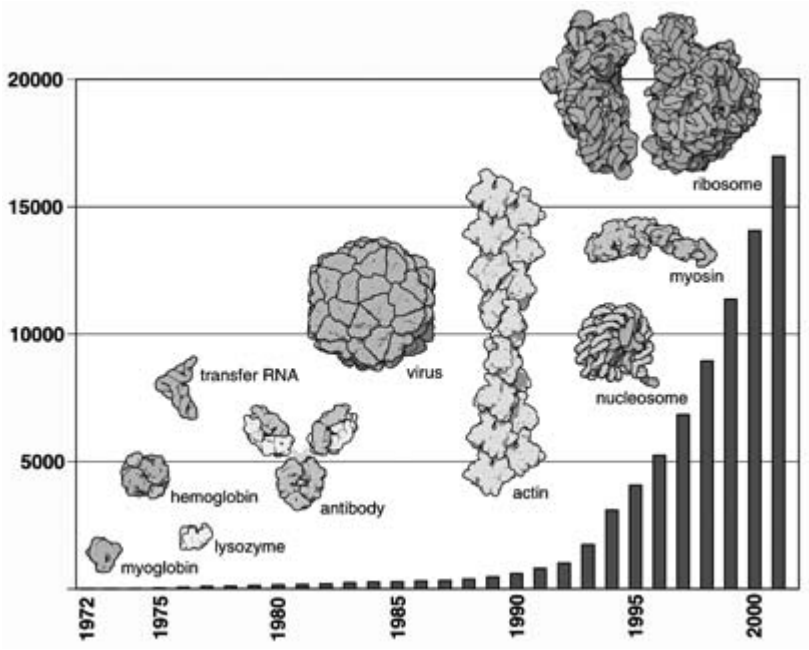

Figure 3.1. How protein structures available on the PDB have increased over the years. The PDB started in 1971 and now contains thousands of structures. The graph charts various 'landmark' structures being solved, such as myoglobin in the 1970s and actin in the 1990s. Image adapted from Chen, 2005.

Structures retrieved from the PDB can be viewed using a variety of programs, including Swiss-model, Pymol and Chimera. The protein structure files stored within the PDB contain the atomic coordinates - i.e. the position of each atom in the protein and where it is in relation to the other atoms within the protein [310,325,328,329]. 3D structures obtained from the PDB can then be utilised for a variety of other analyses, such as 3D structural alignments, calculation of protein surface characteristics, molecular dynamics simulations, among others [326].

In addition to the PDB, there are many other useful bioinformatics resources that can be utilised for proteomics research. UniProt is a protein database that also contains functional information as well as containing the full proteomes from a very large number of organisms: currently the database consists of over 80 million proteins, which is continuously increasing [323,330]. Most of the protein sequences contained on UniProt come from the translation of the nucleotide sequence as opposed to direct protein sequencing [325]. Entries in UniProt are

mostly automatically annotated, which is dependent on knowledge obtained from a smaller number of manually curated entries and from statistical analysis using software, which has a high degree of accuracy [331].

Other than databases that store protein information, there are numerous tools that can carry out complex tasks in order to attain additional information on proteins of interest. pBLAST at the National Centre for Biotechnology Information (NCBI) is the protein version of the Basic Local Alignment Search Tool (BLAST) resource, in which a user inputs a query protein sequence in order to find similar matches to that sequence [332]. This allows for the identification of conserved regions of proteins, as well as obtaining similar sequences for protein comparisons. Matches can be listed based on their similarity to the query sequence, so it is possible to view only the most similar matches, and users can select sequences based on how closely they match the query sequence [325]. Therefore, this tool can be utilised in order to find the most similar proteins to the query, in order to obtain information on potentially similar proteins and to further study the protein of interest, in terms of conserved regions and variable regions [300].

Regarding the comparison between protein sequences, Clustal Omega is a multiple sequence alignment tool, which can align both protein and nucleic acid sequences, in order to determine regions of the protein/nucleic acid which are conserved and those which are variable [302]. Clustal Omega can align up to 2000 sequences and will display the similar and identical regions between the sequences, to give an indication of overall homology and hence evolutionary history of a protein [325]. When sequences are aligned via Clustal Omega, it gives a similarity and identity score: identity specifies how many amino acid positions are identical (i.e the same amino acid at the same position) between the protein sequences, whereas similarity specifies how many amino acid positions are similar - with values above 25% identity suggesting that the proteins are homologous and have similar functions [333]. A high degree of sequence homology within a Clustal alignment may be suggestive that the proteins have similar functions, however, it is also important to consider the 3-dimensional

protein structures in terms of similarities in order to determine the degree of homology between the proteins of interest, to further elucidate whether the proteins share similar functions [334]. Generally, two protein structures which are similar will superimpose relatively easy, which means that structural conservation between proteins can be viewed and interpreted [335]. There are various tools which can perform structural alignments, one of which is Chimera, developed by the University of San Francisco [310]. The MatchMaker tool within the software uses the PDB files to carry out a pairwise alignment and then uses this in order to superimpose the two protein structures onto one another, based on the alignment scores as well as protein secondary structural information [336]. This allows for the observation of which areas of the 3D folded protein are similar and which deviate.

Additionally, sometimes a structure of a protein will be unavailable – in these cases a technique known as homology modelling can be utilised. Homology modelling is based on the relationships between primary protein sequence and tertiary protein sequence [337]. For example, Swiss-Model is an automated homology modelling server that has the ability to compute a 3D structure from a given protein sequence, when no experimentally derived PDB structure is available. It searches the database and uses the closest match as a 'template', from which it bases the 3D model on [328]. Having a 3D structure of a protein is vital in order to better understand its function and potential interactions with other molecules [332].

Furthermore, there are various other useful bioinformatics tools that have applications for proteomics research, and these will be covered in the preceding sections. Before exploring these tools further, however, it is necessary to give some additional background into the topic of halophilic adaptation.

3.1.2: Bioinformatics as a tool to study halophilic adaptation and bacterial salt tolerance

Bioinformatics is a tool that could be well utilised for investigations into the halophilic adaptation of proteins as well as mechanisms of bacterial salt tolerance. The fact that halophilic proteins have generally been found to contain various structural adaptations when compared with non-halophilic proteins (refer to Chapter 1), makes bioinformatics an especially valuable tool for the analysis of these proteins and their comparisons with non-halophilic homologues [338]. A 'homologue' can be defined as a protein with a similar structure (and hence may have a similar function) [339].

In 1995 the crystal structure of the malate dehydrogenase (MDH) from the extreme halophile *H. marismortui* was solved, and it was realised that this protein contained several unique features [197]. It was discovered that this protein had a surface that was covered in acidic amino acid residues and had a highly negative surface charge, as was discussed in section 1.4.1.1. These features show the dramatic adaptation of this protein. Moreover, the availability of bioinformatics resources now make it possible to predict these features on proteins without having to actively crystallise and interpret their structures [332]. A protein structure can be downloaded from the PDB and viewed in a web browser [326]. From this information, various structural features can be interpreted using molecular viewing software, such as the calculation of electrostatic potentials, viewing amino acid compositions, and observing unique structural features, which is especially vital regarding the unique adaptations of halophilic proteins [340].

An analysis of various structural features of proteins from halophiles and non-halophiles using bioinformatics analysis, in order to analyse the proteins in terms of the levels of acidic residues on the surfaces and interior of the proteins has previously been carried out [88]. It was found from this study that although

halophilic and non-halophilic proteins contained similar levels of acidic residues in the interior, the levels on the surface of the proteins were considerably higher in the halophiles than the non-halophiles (see section 1.4.1.1). In addition, the comparisons of full proteomes between salt-tolerant and non-salt-tolerant organisms in terms of their similarities and differences could be significantly aided by bioinformatics analysis, as vast amounts of data can be quickly processed [341]. Therefore, bioinformatics allows for the study of halophilic adaptation at a more holistic level.

When investigating bacterial salt tolerance mechanisms, it is essential to understand how the cell as a whole is affected by salt, as well as considering protein adaptations. Therefore, it is important to look into the membrane ion transporters/channels present within the organisms. The presence or absence of distinct ion transport systems may give insight into how a cell responds to the presence of specific ions. For example, in a study on the halotolerant bacterium *Staphylococcus aureus*, it was found that knocking out the function of one moderate affinity K^+ transporter severely inhibited the salt tolerance of this organism, to such a great extent that it could not compete with wildtype strains *in vivo* [342]. Therefore, it would appear that the function of cation transporters is of vital importance to the survival of an organism, especially within hypersaline conditions. Moreover, via a bioinformatics analysis of ion channels and transporters present in various archaeal halophiles and non-halophiles, Jensen *et al* found that many of these proteins were similar in both groups of organism but there were a few key differences, such as the general finding of more cation transport systems being present in the halophiles as compared with the non-halophiles (as well as halophile-specific proteins such as rhodopsins), suggestive of the increased importance of cation transport within salt-adapted organisms [10]. In addition, other studies that have utilised bioinformatics analysis for the study of halophilic ion transport have found that the up-regulation and an increased level of ion transport systems may be of importance for halophilic organisms, further suggesting the essential nature of ion transport for the salt tolerance of an organism [193,343,344]. Many of the known *E. coli* cation transporters were discussed in section 1.2.2, and nothing is currently

known about cation transport within *E. vietnamensis*. However, a small amount of knowledge exists for cation transporters within *S. ruber*, as it has the Trk K^+ transport system, which has also been found within halophilic archaea, as well as containing a Na-K-Cl co-transporter, within a so-called 'hypersalinity island' [205].

3.1.3: Protein surface charges and halophilic adaptation

As was discussed in section 1.4, halophilic proteins have generally been found to contain an increased level of negative surface charges, as compared to non-halophilic proteins, in addition to lower levels of basic residues [345]. This increased level of negative charges corresponds to an increased number of acidic amino acids and mostly occurs on the surface of these halo-adapted proteins [346]. This adaptation is thought to aid protein stabilisation at high salt concentrations by resulting in a higher level of water binding, as a consequence of the higher level of carboxylic side chains, preventing protein aggregation within a hypersaline cytoplasm [347]. In addition, the decreased level of hydrophobic residues on the surfaces of halophilic proteins is thought to assist their stabilisation at high salt concentrations by reducing the level of hydrophobic interactions, mostly as a reduction in the number of lysine residues - it has been found that many extreme halophilic proteins contain significantly reduced levels of this amino acid [348]. Consequentially, the charges of halophilic proteins will generally be more negative than similar proteins from non-halophiles [197].

It is possible to view protein structures in terms of their distribution of acidic as compared to basic residues. This can be done using the program Chimera, designed at the University of San Francisco, California [310]. This powerful molecular visualisation software allows for the computation of electrostatic potentials on the surfaces of proteins, based on Coulomb's law (see Equation 2.1). This involves the calculation of a sum of Coulomb potentials for the protein surface - resulting in the colouration of the protein surface that is dependent on

the charges of the amino acid residues (i.e. the surface potential). This is especially valuable for the comparisons between halophilic and non-halophilic proteins at a structural level, since these proteins have generally been found to contain highly negative surfaces, due to the high level of glutamic acid and aspartic acid, which contain negatively charged COO^- side chains [206].

3.1.4: 2D gel electrophoresis

The isoelectric point (IEP), or pI, of a protein is the pH at which that protein is neutral, and will depend on the net charge of that protein, i.e. its acidic to basic amino acid composition [349]. Proteins with an overall negative charge will have a pI in the acidic range (due to becoming neutralised with the increased proton concentration at acidic pH), whereas proteins with an overall positive charge will have a pI in the basic range (due to becoming neutralised with the increased hydroxyl concentrations at basic pHs) [350].

Since the halophilic adaptation of proteins involves an increase in the level of negative charges and a decrease in the level of positive charges on the protein surface, it has been found that proteins from salt-in halophilic organisms generally have low pIs [88,170]. For example, the proteome of *H. marismortui* has an average pI of 5.0 [196]. For this reason, the analysis of entire proteomes of these organisms in terms of their charges is extremely useful for understanding how protein charge varies with the salt-tolerance of an organism.

2D gel electrophoresis is an experimental technique where proteins are separated in two dimensions: the 1^{st} dimension involves separation according to their pI (Isoelectric Focusing) and the 2^{nd} dimension is according to their molecular weight (SDS-PAGE) [351,352]. The sample is loaded onto a gel that consists of a pH gradient (IEF gel): where the acidic end of the pH gradient is positioned at the anode (positive electrode) and the basic pH end of the IEF gel is positioned at the cathode (negative electrode). When an electric current is applied, the proteins will migrate to the pH where they are neutral (no overall

charge) and will then stop migrating. This pH is known as the IEP (pI) of that protein. After this the IEF gel is covered in SDS-buffer, which gives all of the proteins on the gel a negative charge. The IEF gel is transferred to on top of an SDS-PAGE gel and an electrical current is applied, so that the proteins will migrate towards the positive electrode, situated at the top of the gel, as is shown in Figure 3.2. Proteins will, as a result, be separated based on their molecular masses, with smaller proteins migrating further through the gel than larger proteins [353].

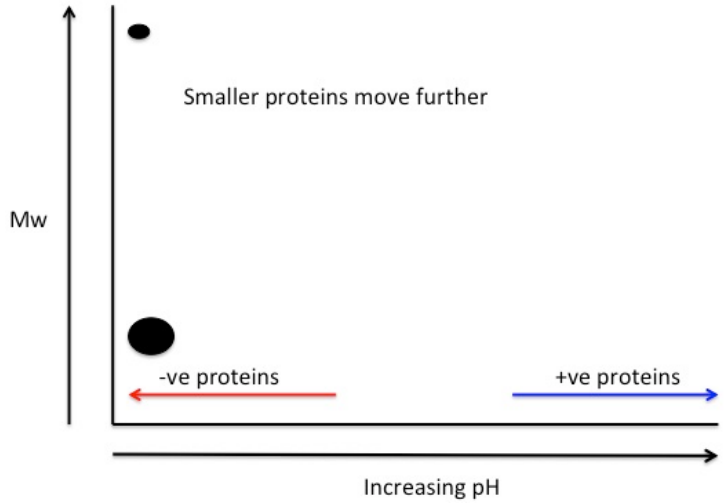

Figure 3.2. Schematic of a 2D-gel. In the 1st dimension (X-axis) proteins are separated based on their isoelectric point (IEP), which is the pH at which the protein will stop moving in an electric field, i.e. it becomes overall neutral. The proteins are then separated based on their molecular weight (Y-axis), with smaller proteins migrating faster and further than larger proteins.

2D gel electrophoresis is largely dependent on the concentration of the different proteins within the sample (i.e. being adequate levels of protein present) as well as the selection of the correct pH range for the isoelectric focusing [354]. Moreover, software exists for the determination of theoretical 2D gels from a pre-loaded list of organisms [355]. However, this is limited by the fact that only organisms added to the software database can be analysed, although this list is quite exhaustive, with around 700 proteomes currently available. The use of theoretical 2D gels has made them effective tools for the study of whole

proteomes, without the expertise and temporal considerations of running them in the laboratory [356–358].

Theoretical 2D gels can be computed based on the amino acid compositions of the proteins from a whole proteome, where both the pI as well as the molecular weight can be determined [359]. Whole proteomes of many organisms are freely available on the UniProt server [303]. The theoretical proteome of an organism is essentially a collection of all of the possible open reading frames (ORFs) from the genome of that organism [360].

The analysis of proteome pIs has been used previously in order to analyse bacterial proteome pI distribution in response to salt tolerance [361,362]. In these particular studies, bioinformatics was utilised to analyse the amino acid compositions as well as the proteome pIs of various halophilic organisms, with the finding that pI may be correlated to the salt-tolerance of the organism, i.e. lower proteome pIs may be found in organisms that can tolerate higher salinites. This further emphasises the benefit of the analysis of proteome pIs for the comparative analysis between various salt-tolerant and non-salt tolerant organisms.

3.1.5: Rationale for Current study

Various bioinformatics resources were utilised in order to initially characterise and to obtain a perspective on similarities and differences between a halophile, non-halophile and halotolerant organism. The first step was to identify organisms that would be used for the experiments and to determine their levels of protein conservation. After which the proteins were compared in terms of structural features, and were also compared with other non-halophiles, halophiles and halophilic organisms. This was performed in order to analyse differences in terms of protein compositions between organisms of different salt tolerances.

S. ruber was selected as the halophile to use for this project, due to the fact it is one of the few salt-in bacterial halophiles currently known. Upon selecting *S. ruber* as the halophile of study, since this project contains a comparative enzyme component, the malate dehydrogenase (MDH) of *S. ruber* was selected as the enzyme of study, since its crystal structure was available on the PDB [296]. Protein BLAST searches (pBLAST) were carried out on *S. ruber* MDH (SrMDH) to find a homologue from a halotolerant organism. The closest match for SrMDH for a protein from a halotolerant organism was the MDH from the organism *E. vietnamensis*. *E. coli* MDH (EcMDH) was selected as the non-halophile/non-halophilic protein to study.

For the comparison of the MDH protein sequences from these three organisms, multiple as well as pairwise sequence alignments were performed. In addition to protein sequence alignments, phylogenetic trees were constructed to visualise the evolutionary relationships between the proteins [363]. The neighbour-joining method was used, which calculates the number of substitutions between two protein sequences (i.e. the number of different positions) and divides this by the length of the protein sequence (excluding gaps) to compute the evolutionary divergence between the sequences [306,364]. This allows for the comparisons of sequences in terms of their evolutionary divergence.

To complement the above, it is important to consider the similarities between proteins in terms of tertiary structures, as structural differences between the proteins has important implications with regards to function [325]. Structural alignments were therefore carried out on the proteins. Comparisons of the MDH from *E. coli*, *E. vietnamensis* and *S. ruber* in terms of their similarity at the protein sequence and structural level, as well as looking at phylogenetic relationships was essential as it is important to determine how homologous the sequences were in order to determine if they were suitable for the comparative enzymatic studies later in the project.

Various analyses were carried out on the three MDHs, as well as a selection of other proteins from *E. coli*, *E. vietnamensis* and *S. ruber*, in addition to the MDHs

from a selection of other halophiles, non-halophiles and halotolerant organisms. This included an analysis of the pIs, net protein charges, electrostatic surface charges and amino acid compositions of the proteins. In addition, theoretical 2D gels were constructed in order to analyse and compare the pI distribution of proteomes from organisms of different salt tolerances.

An analysis of cation transport systems within different organisms can give important insight into the responses of those organisms towards specific cations. Therefore, the current study also aimed to compare cation transport within *E. coli*, *E. vietnamensis* and *S. ruber* in order to better understand how their tolerances towards specific ions relates to ion transport into and out of the cell.

3.2: Results

3.2.1: Sequence Alignments

After the initial selection of the MDHs to study (EcMDH, EvMDH and SrMDH), these proteins were aligned via Clustal Omega. These are shown in Figure 3.3, as well as pairwise alignments in Figures 3.4 – 3.6, and the identity scores for the alignments are shown in Table 3.1. In addition, structural alignments were computed between the three proteins and the data for these are shown in Figures 3.7 and 3.8. EvMDH and SrMDH structures superimposed the most effectively, whereas both EvMDH and SrMDH did not align as closely with EcMDH.

To understand the evolutionary (and hence structural) relationships between the malate dehydrogenases used in this study, phylogenetic trees were constructed to compare the MDHs from *E. coli*, *E. vietnamensis* and *S. ruber*, as well as the MDHs from various other organisms (throughout all three domains), as is shown in Figure 3.9. EvMDH and SrMDH appear to be distantly related to one another, whereas EcMDH is less closely related to the other two proteins.

Figure 3.3. Multiple sequence alignment of EcMDH, EvMDH and SrMDH. The malate dehydrogenase protein sequences from *E. coli*, *E. vietnamensis* and *S. ruber* were aligned using Clustal Omega. The conserved active site histidine residue is highlighted in red. Identical regions (same amino acids) are indicated by '*' and similar regions (amino acids have similar properties) are indicated by ':'.

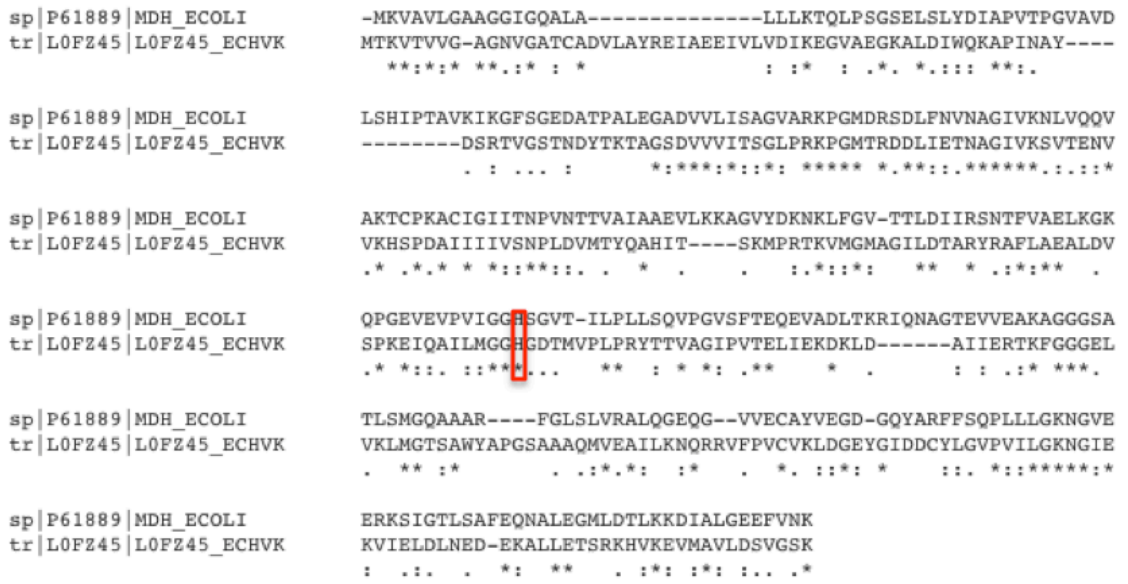

Figure 3.4. Pairwise sequence alignment of EcMDH with EvMDH. The malate dehydrogenase protein sequences from *E. coli* and *E. vietnamensis* were aligned using Clustal Omega. The conserved active site histidine residue is highlighted in red. Identical regins are indicated by '*' and similar regions are indicated by ':'.

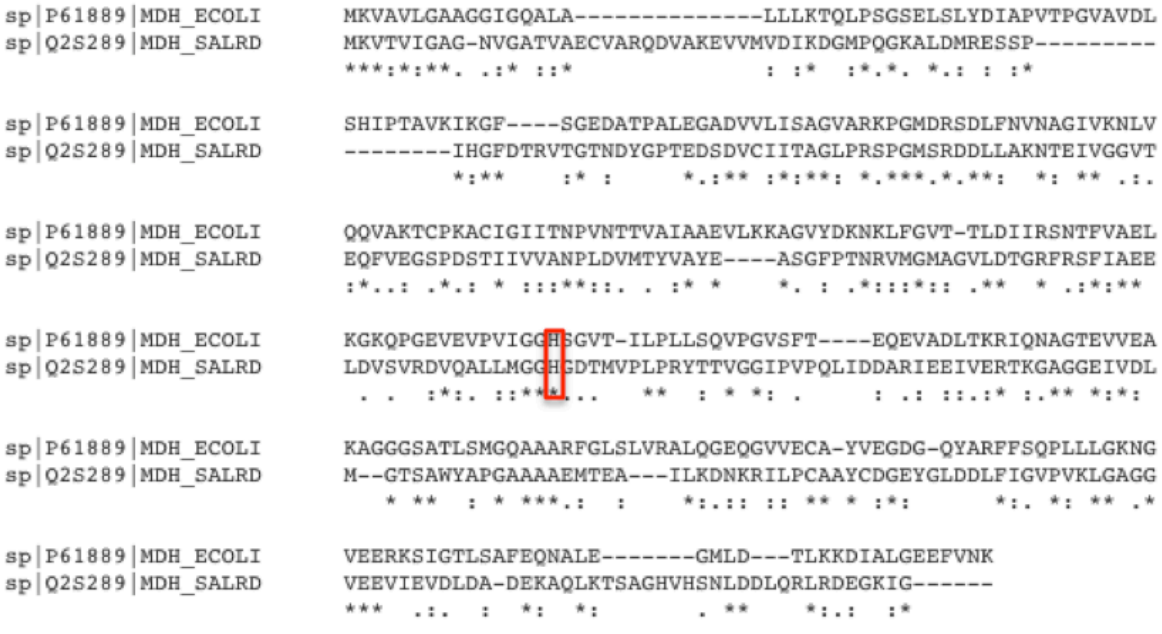

Figure 3.5 Pairwise sequence alignment of SrMDH with EcMDH. The malate dehydrogenase protein sequences from *S. ruber* and *E. coli* were aligned using Clustal Omega. The conserved active site histidine residue is highlighted in red. Identical regins are indicated by '*' and similar regions are indicated by ':'.

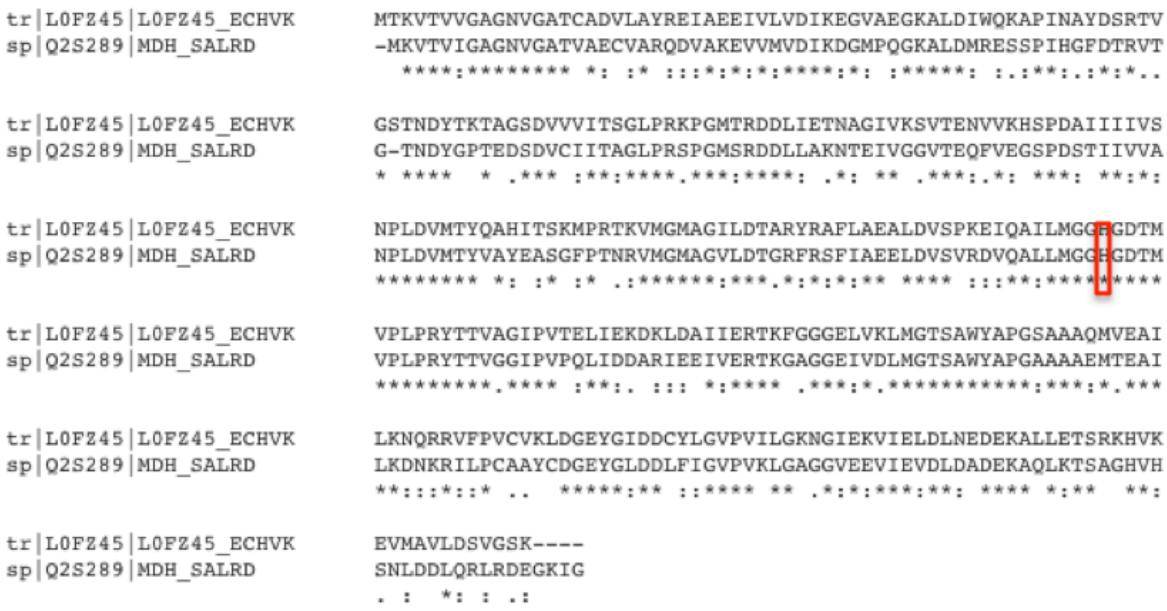

Figure 3.6. Pairwise sequence alignment of SrMDH with EvMDH. The malate dehydrogenase protein sequences from *S. ruber* and *E. vietnamensis* were aligned using Clustal Omega. The conserved active site histidine residue is highlighted in red. Identical regins are indicated by '*' and similar regions are indicated by ':'.

Table 3.1. Alignment scores for the MDH sequence alignments. Multiple sequence alignment between the 3 MDHs (top row), and individual pairwise alignment scores are shown.

Proteins	Identity (%)	Identical positions	Similar positions
3 MDHs	19.13	66	111
Ec and Ev	26.19	88	108
Ec and Sr	25.29	88	110
Ev and Sr	57.60	182	88

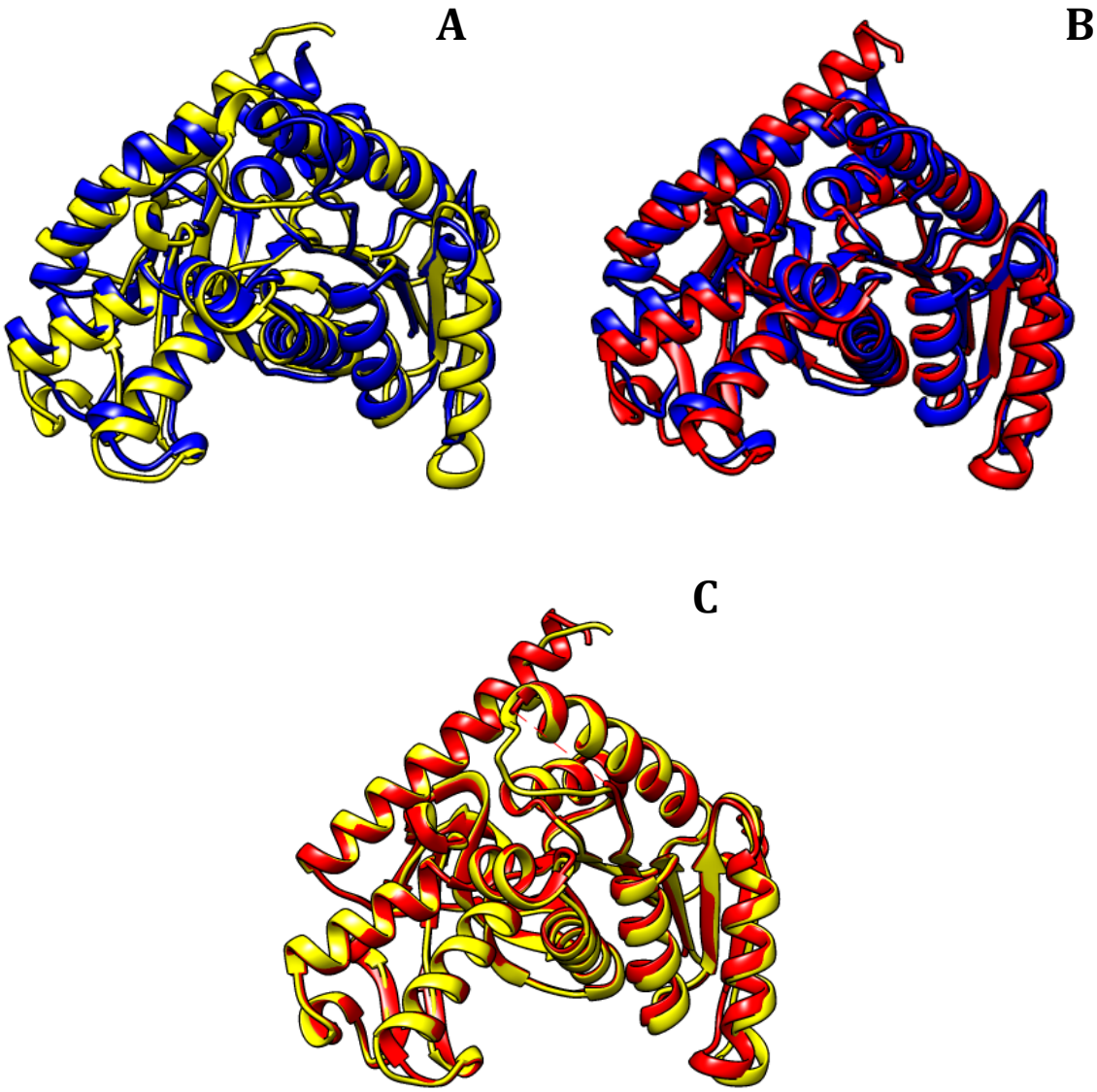

Figure 3.7. 3D structural alignments between monomeric EcMDH, EvMDH and SrMDH. A: EcMDH (blue) and EvMDH (yellow); B: SrMDH (red) and EcMDH (blue); C: SrMDH: (red) and EvMDH (yellow). Alignments were computed on one monomer of the MDHs, via the Chimera MatchMaker tool.

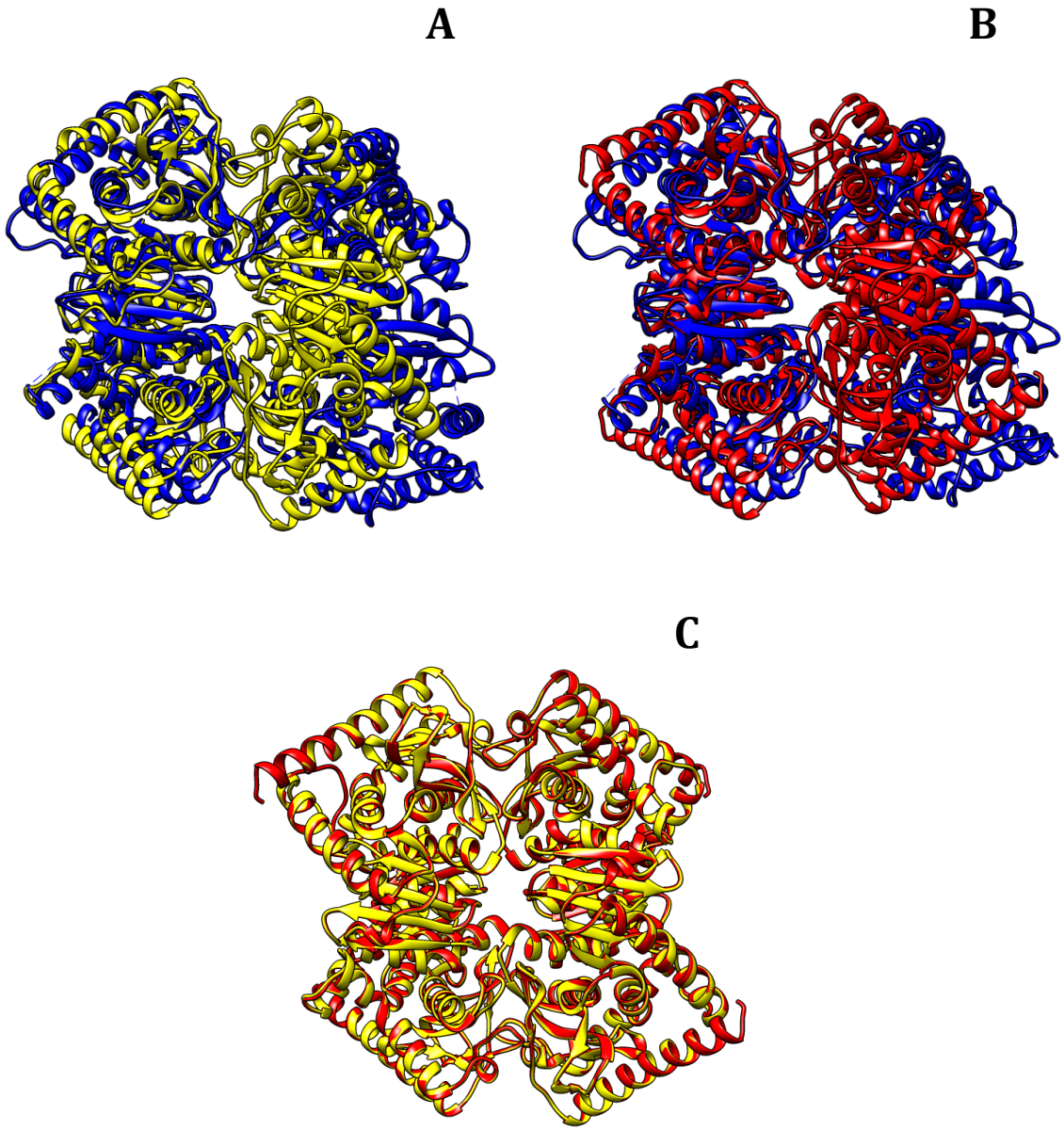

Figure 3.8. 3D structural alignments between tetrameric EcMDH, EvMDH and SrMDH. A: EcMDH (blue) and EvMDH (yellow); B: SrMDH (red) and EcMDH (blue); C: SrMDH: (red) and EvMDH (yellow). RMSD values between protein backbones were: EcMDH:EvMDH: 1.197Å; EcMDH:SrMDH: 1.212Å; EvMDH: SrMDH: 0.177Å. EvMDH and SrMDH tetramers were obtained by homology modelling via the Swiss-model server and alignments were computed using the Chimera MatchMaker tool.

Figure. 3.9. Phylogenetic tree of various MDHs. The tree was constructed from the alignment file of 19 proteins using the Clustal Phylogeny tool using the neighbour-joining method, and was viewed using SeaView. Scale bar refers to the amount of amino acid substitutions. MDHs were analysed from: *Thermus thermophilus*; *Halorhodospira halophila*; *Bordetella pertussis*; Human (cytoplasmic); *Taenia solium* (cytoplasmic); *Echinococcus granulosus* (cytoplasmic); *Pelagibacter ubique*; *Rhodothermus marinus*; *Salinibacter ruber*; *Echinicola vietnamensis*; *Haloarcula*

marismortui; *Haloferax volcanii*; *Halobacterium salinarum*; *Vibrio chloerae*; *Salmonella typhimurium*; *Escherchia coli*; *Haemophilus influenzae; Candida albicans* (cytoplasmic); *Lactobacillus plantarum*.

3.2.2: Protein charges

The pIs of the MDHs from several halophiles, non-halophiles and halotolerant organisms, as well as the pIs from 10 additional proteins from *E. coli*, *E. vietnamensis* and *S. ruber*, were calculated. These are shown in Figure 3.10 and Table 3.2. In addition, the net charges of these additional ten proteins (plus MDH) from *E. coli*, *E. vietnamensis* and *S. ruber* were calculated, which are shown in Table 3.3. The salt-in halophiles, *H. marismortui*, *S. ruber*, *H. volcanii* and *H. salinarum*, have an MDH with a significantly lower pI than the non-halophiles. The only exception to this is the halotolerant organism *L. plantarum*, which has an MDH with a pI of 4.75. Regarding the protein net charges, there is a significant difference between *S. ruber* and the non-halophiles (*E. coli* and *E. vietnamensis*), in terms of the charge of its proteins. The average value for the *S. ruber* proteins is -31.99, the average for the *E. coli* proteins is -10.57 and the average for the *E. vietnamensis* proteins is -11.35.

The levels of positively and negatively charged amino acids withinin the MDHs were also calculated. This was plotted as a ratio chart and this is shown in Figure 3.11. With the exception of *H. halophila*, the MDHs from the halophilic organisms generally show a much lower level of positively charged (basic) amino acids and higher levels of negatively charged (acidic) amino acids. The amino acid compositions of the MDHs from *E. coli*, *E. vietnamensis* and *S. ruber* were plotted in terms of their properties (i.e. basic, acidic, polar and apolar) and are shown in Figure 3.12 – which shows the amino acid composition of the full protein, i.e. the interior as well as the surface. There is a general increase in acidic and decrease in basic residues in SrMDH, which may also be the case for EvMDH (but to a lesser extent).

To compliment this, EcMDH, EvMDH and SrMDH protein structures were coloured in terms of surface charge, as is shown in Figures 3.13 and 3.14. Negative potentials are coloured red, positive potentials are coloured blue and neutral areas of the protein surface are coloured white. There is a clear discrepancy between the proteins in terms of the electrostatic surface potentials - SrMDH contains the most negatively charged surface, EcMDH contains the most neutral surface, whereas EvMDH is between these two 'extremes'.

Protein pI may be related to level of salt tolerance of the organism

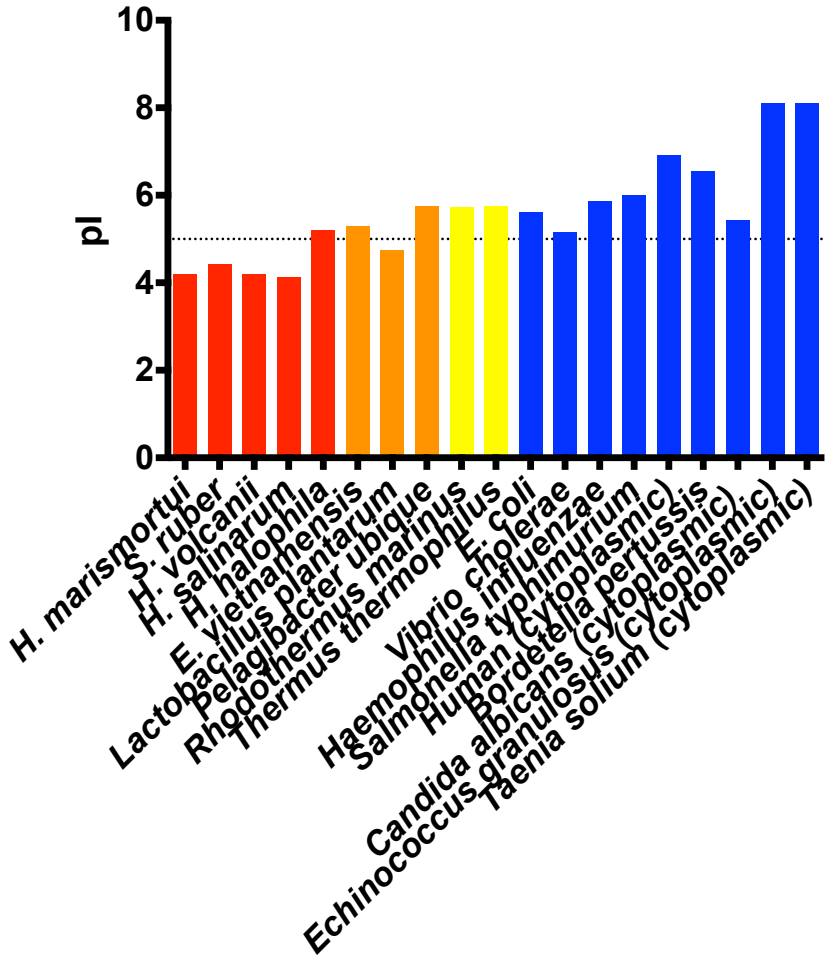

Figure 3.10. pI values of the malate dehydrogenases from a range of organisms. The pI of the MDH from each organism was computed using ProtParam. Halophiles: red; halotolerant: orange; thermophiles: yellow; non-halophiles: blue.

S. ruber proteins have lower pIs and are much more negatively charged than *E. coli* and *E. vietnamensis* proteins

Table 3.2. The pI of 10 proteins from *E. coli*, *E. vietnamensis* and *S. ruber* .The pI of 10 proteins from each organism was calculated using ProtParam.

Protein	*E. coli*	*E. vietnamensis*	*S. ruber*
CTP Synthase	5.6	6.2	4.8
Ketol-acid reductoisomerase (NADP(+))	5.2	-	4.3
Enolase	5.3	4.8	4.3
S-adenosylmethionine synthase	5.1	5.1	5.1
Adenylosuccinate synthetase	5.3	5.3	4.6
Serine--tRNA ligase	5.3	-	4.8
Serine hydroxymethyltransferase	6.0	5.9	4.9
Glutamyl-tRNA reductase	5.4	5.4	4.7
Adenylate kinase	5.5	5.7	4.3
DNA ligase	5.3	5.6	4.6

Table 3.3. Protein net charges of 11 proteins from *E. coli*, *E. vietnamensis* and *S. ruber*. The protein sequence of 11 proteins from *E. coli*, *E. vietnamensis* and *S. ruber* were submitted to the pepcalc tool at http://pepcalc.com/ in order to obtain the net charge of each of the proteins.

Protein	*E. coli*	*E. vietnamensis*	*S. ruber*
CTP Synthase	-12.1	-5.0	-36.7
Ketol-acid reductoisomerase (NADP(+))	-13.7	-	-43.8
Enolase	-10.3	-21.6	-41.0
S-adenosylmethionine synthase	-14.4	-14.2	-19.8
Adenylosuccinate synthetase	-9.6	-9.8	-32.6
Serine-tRNA ligase	-13.3	-11.2	-26.5
Serine hydroxymethyltransferase	-5.9	-8.0	-24.9
Glutamyl-tRNA reductase	-10.3	-11.2	-35.8
Adenylate kinase	-3.8	-3.6	-11.2
DNA ligase	-19.9	-22.1	-55.8
Malate dehydrogenase	-3.0	-6.8	-23.8

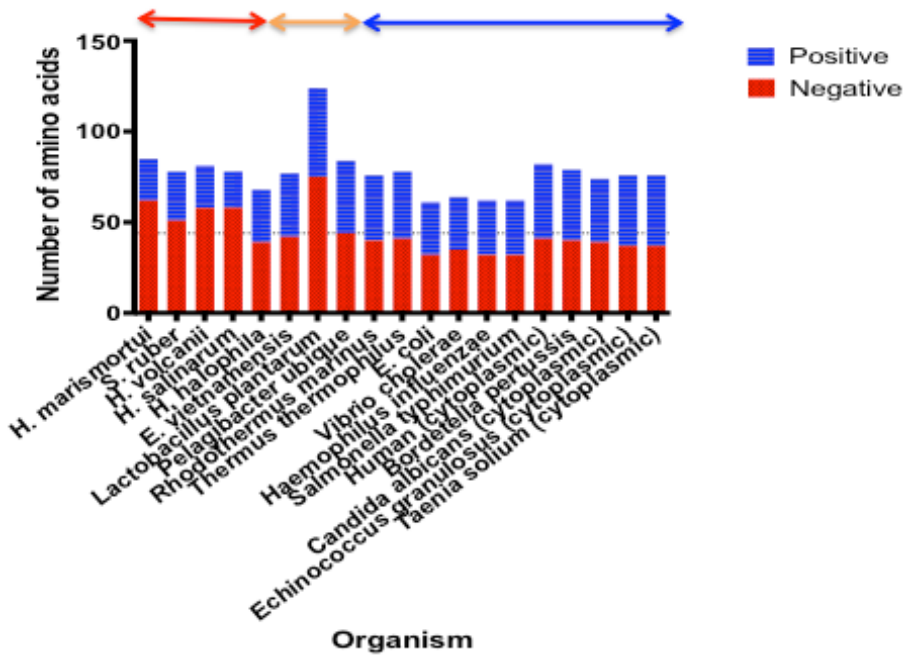

Figure 3.11. Ratio of positive and negative amino acids of MDHs from a range of organisms. Number of acidic (red - dotted) or basic (blue - stripes) amino acids are displayed (Y axis), against the organism (X axis). The dotted line refers to the average acidic amino acid content between the 19 proteins: those above the line have more acidic amino acids than the average. Red arrow: halophiles; orange arrow: halotolerant; blue arrow: non-halophiles.

Level of acidic amino acids in a protein may increase with the salt tolerance of the organism

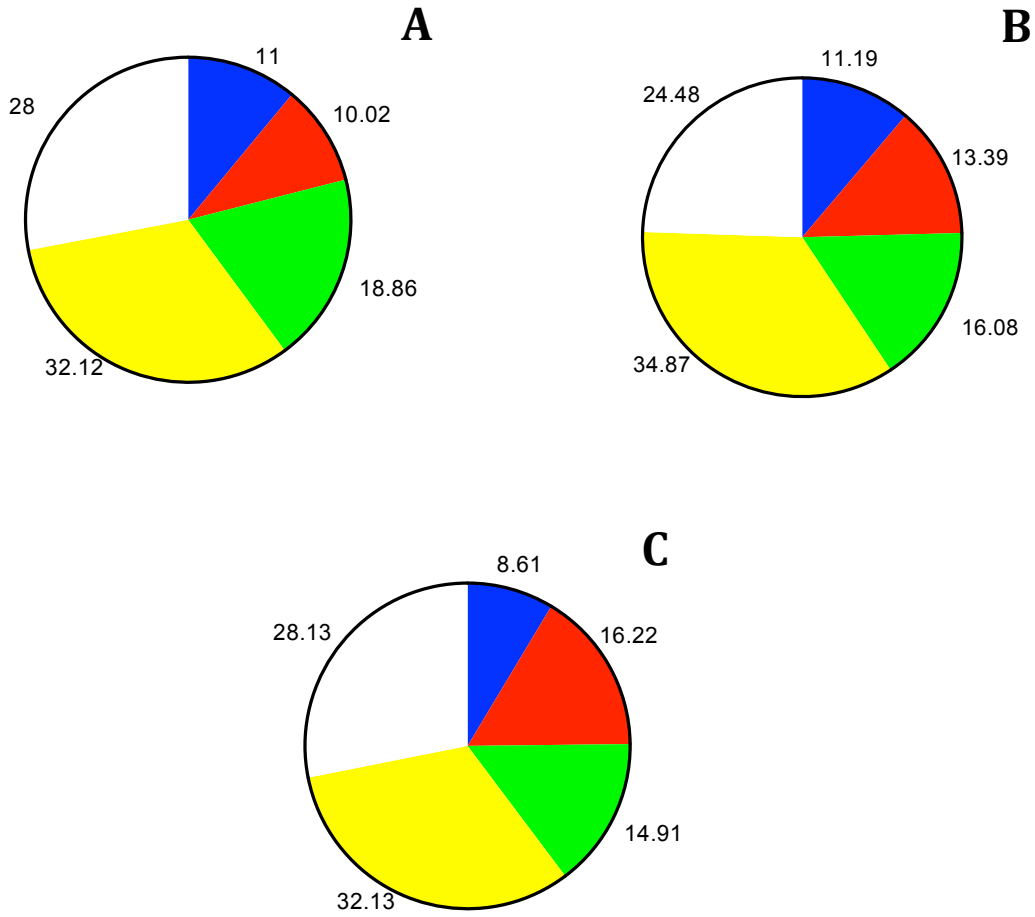

Figure 3.12. Amino acid compositions of EcMDH, EvMDH and SrMDH. Amino acid compositions were calculated via ProtParam, and were plotted in terms of their properties. A: EcMDH; B: EvMDH; C: SrMDH. Blue = basic amino acids; red = acidic amino acids; green = polar amino acids; yellow = apolar amino acids; white = other amino acids.

SrMDH contains a highly negative surface charge

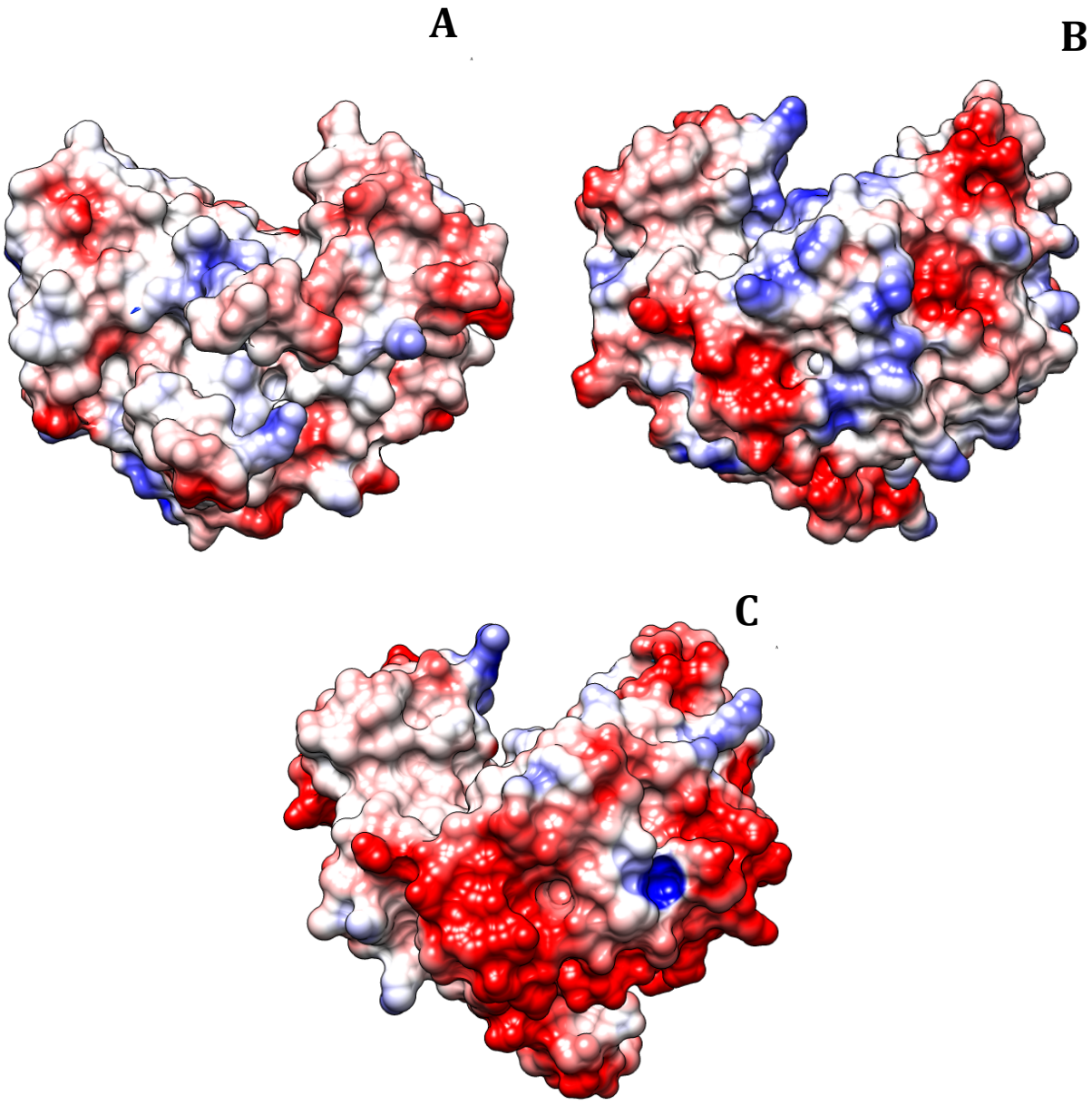

Figure 3.13. Electrostatic potential of the surface of the monomeric MDH from *E. coli*, *E. vietnamensis* and *S. ruber*. A: EcMDH; B: EvMDH; C: SrMDH. Structures are coloured using the Coulombic surface colouring tool in Chimera, according to the surface electrostatic potential: red = negative; blue = positive; white = neutral.

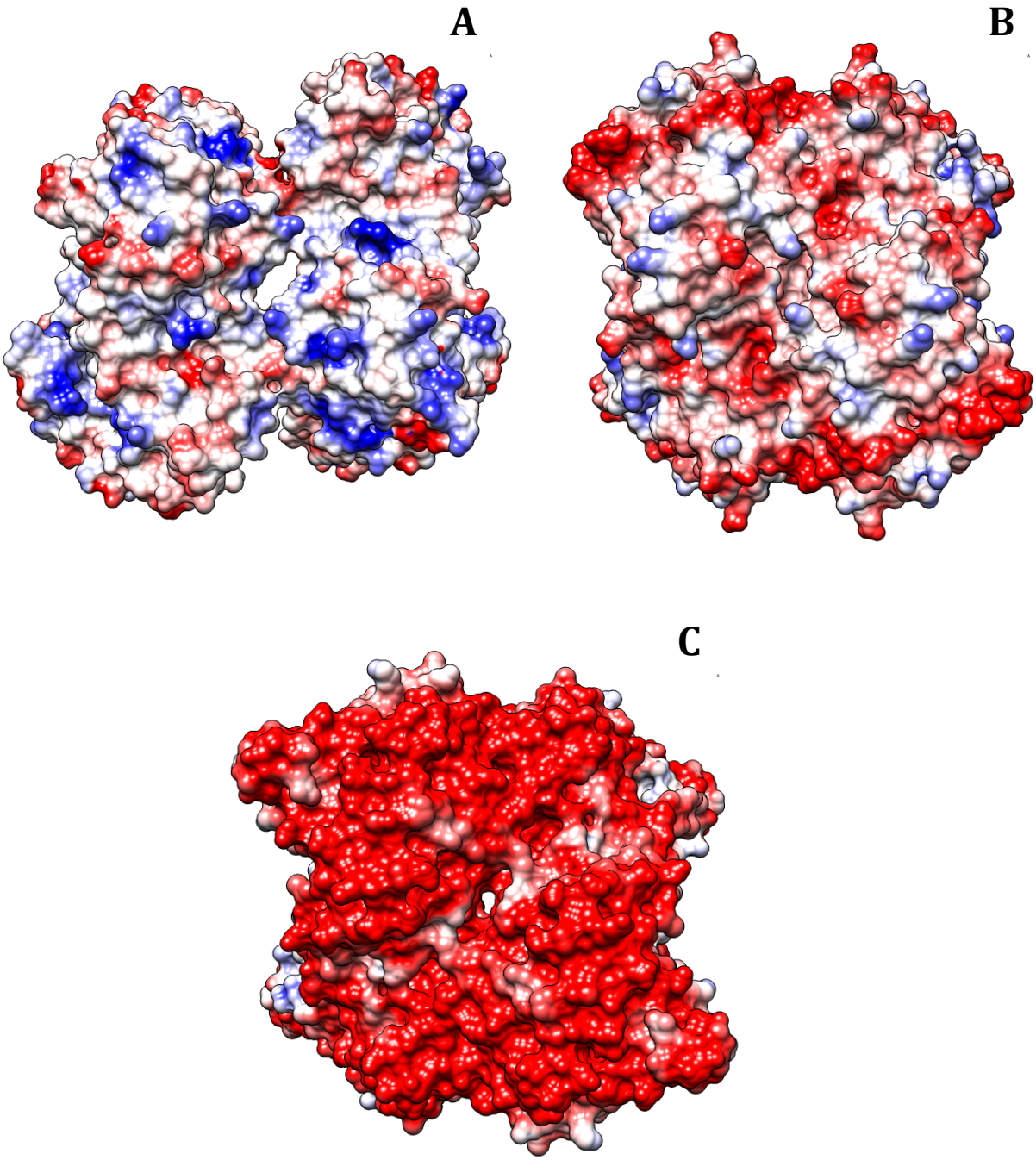

Figure 3.14. Electrostatic potential of the surface of the tetrameric MDH from *E. coli*, *E. vietnamensis* and *S. ruber*. A: EcMDH; B: EvMDH; C: SrMDH. Structures are coloured using the Coulombic surface colouring tool in Chimera, according to the surface electrostatic potential: red = negative; blue = positive; white = neutral. SrMDH and EvMDH tetrameric MDH structures were produced by homology modelling via the Swiss-model server.

3.2.3: Theoretical 2D gels

The full proteomes of the salt-in halophiles (*S. ruber*, *H. marismortui*, *H. volcanii* and *H. salinarum*); salt-out halophiles (*H. halophila*, *H. elongata* and *Halobacillus halophilus*); non-halophiles (*E. coli*, *Helicobacter pylori*, *S. typhimurum*, *H. influenzae*, *V. chlorerae*); and halotolerant organisms (*E. vietnamensis*; *S. aureus*; *Pediococcus acidilactici*; *P. ubique*; *L. plantarum*) were used to construct theoretical 2D gels, which are shown in Figures 3.15 – 3.18 and Tables 3.4 – 3.7. For comparative purposes, a summary of these results is shown in Figure 3.19.

There is a skew towards acidic pIs for the salt-in halophiles, with the majority of proteins falling between a pI of 4 and 5. The proteomes of *H. marismortui*, *H. volcanii* and *H. salinarum* have a slightly more obvious acidic pI skew than that of *S. ruber*. However, the *S. ruber* proteome is clearly acidic, and is comparable to that of the archaeal halophiles. Furthermore, there is also a clear acidic bias of the proteomes of the salt-out halophiles, with the majority of proteins falling within the pI range of 4-6. The mean pI of these organisms are between 6.2 – 6.4, higher than that of the salt-in halophiles.

Non-halophiles and halotolerant organisms have a relatively equal distribution across the pI range: no obvious skews are detected, like was found for the proteomes of the halophilic organisms, although *E. vietnamensis* does have a lower pI than that of the other halotolerant organisms.

Halophilic proteomes are acidic

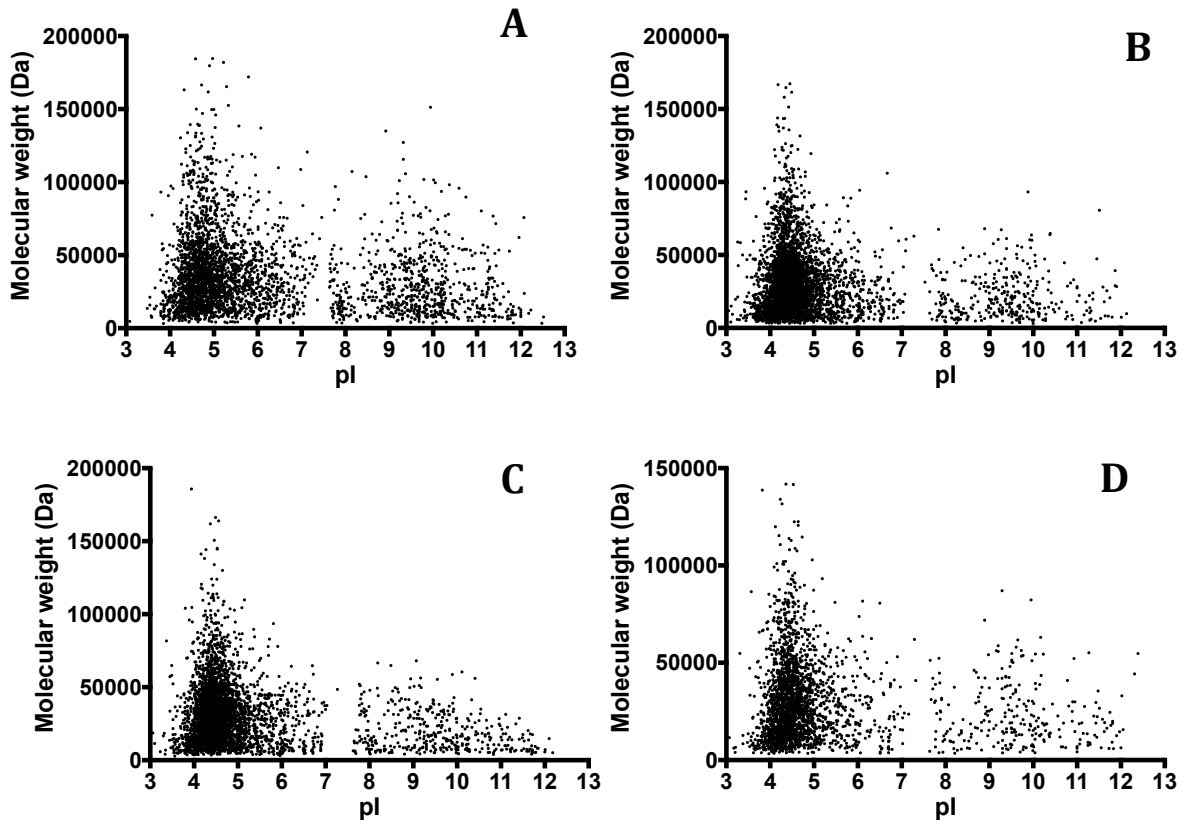

Figure 3.15 Theoretical 2D gels from salt-in halophiles. The entire proteome of the organism is plotted as the molecular weight (Y axis) against the pI (X axis). A: *S. ruber*; B: *H. marismortui*; C: *H. volcanii*; D: *H. salinarum*. Each point refers to an individual protein.

Table 3.4. Statistics of the proteome pIs from 4 salt-in halophiles. The mean, median, maximum and minimum pIs were calculated.

Statistics	*S. ruber*	*H. marismortui*	*H. volcanii*	*H. salinarum*
Mean	6.07	4.95	5.09	5.14
Minimum	3.08	2.93	2.79	3.07
Maximum	12.52	12.13	12.18	12.39
Median	5.09	4.47	4.53	4.52

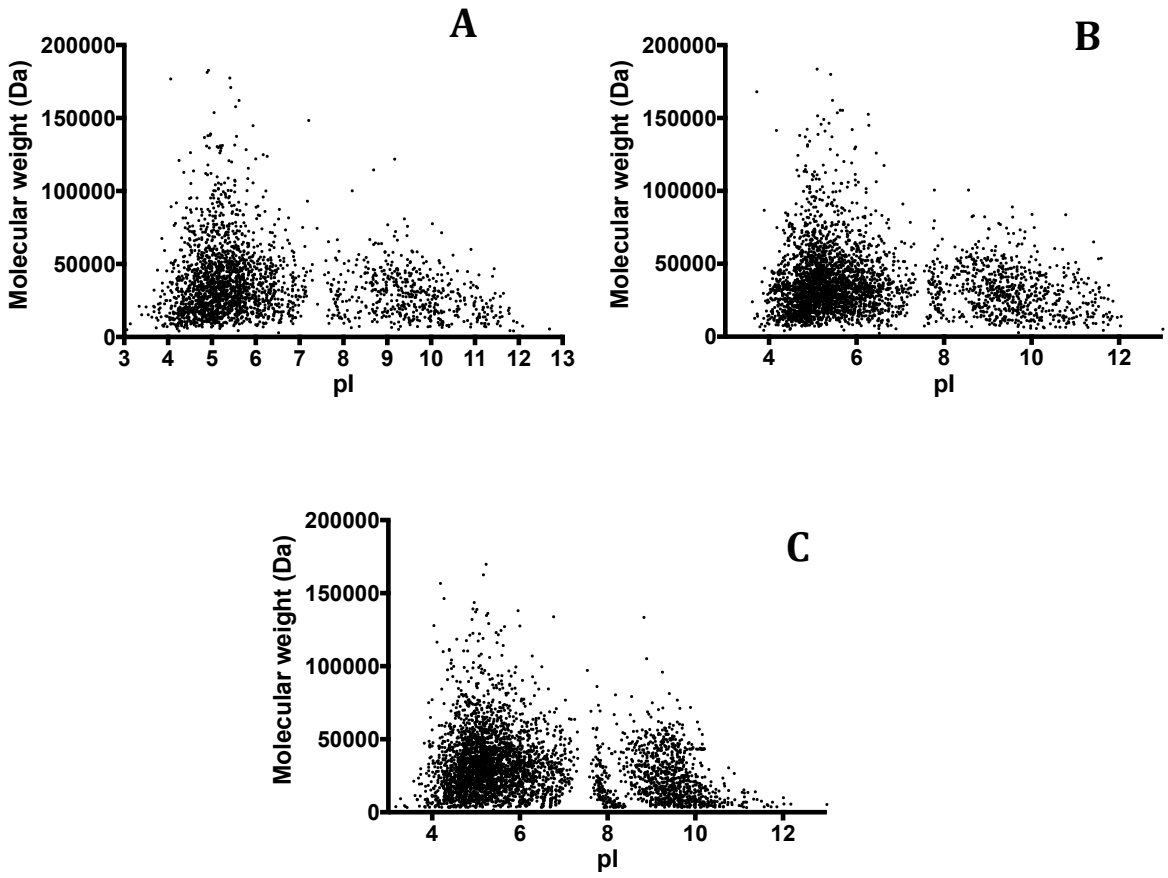

Figure 3.16. Theoretical 2D gels from salt-out halophiles. The entire proteome of the organism is plotted as the molecular weight (Y axis) against the pI (X axis). A: *H. halophila*; B: *H. elongata*; C: *H. halophilus*. Each point refers to an individual protein.

Table 3.5. Statistics of the proteome pIs from 3 salt-out halophiles. The mean, median, maximum and minimum pIs were calculated.

Statistics	*H. halophila*	*H. elongata*	*H. halophilus*
Mean	6.20	6.24	6.35
Minimum	2.63	2.54	2.95
Maximum	12.70	13.00	13.00
Median	5.47	5.50	5.57

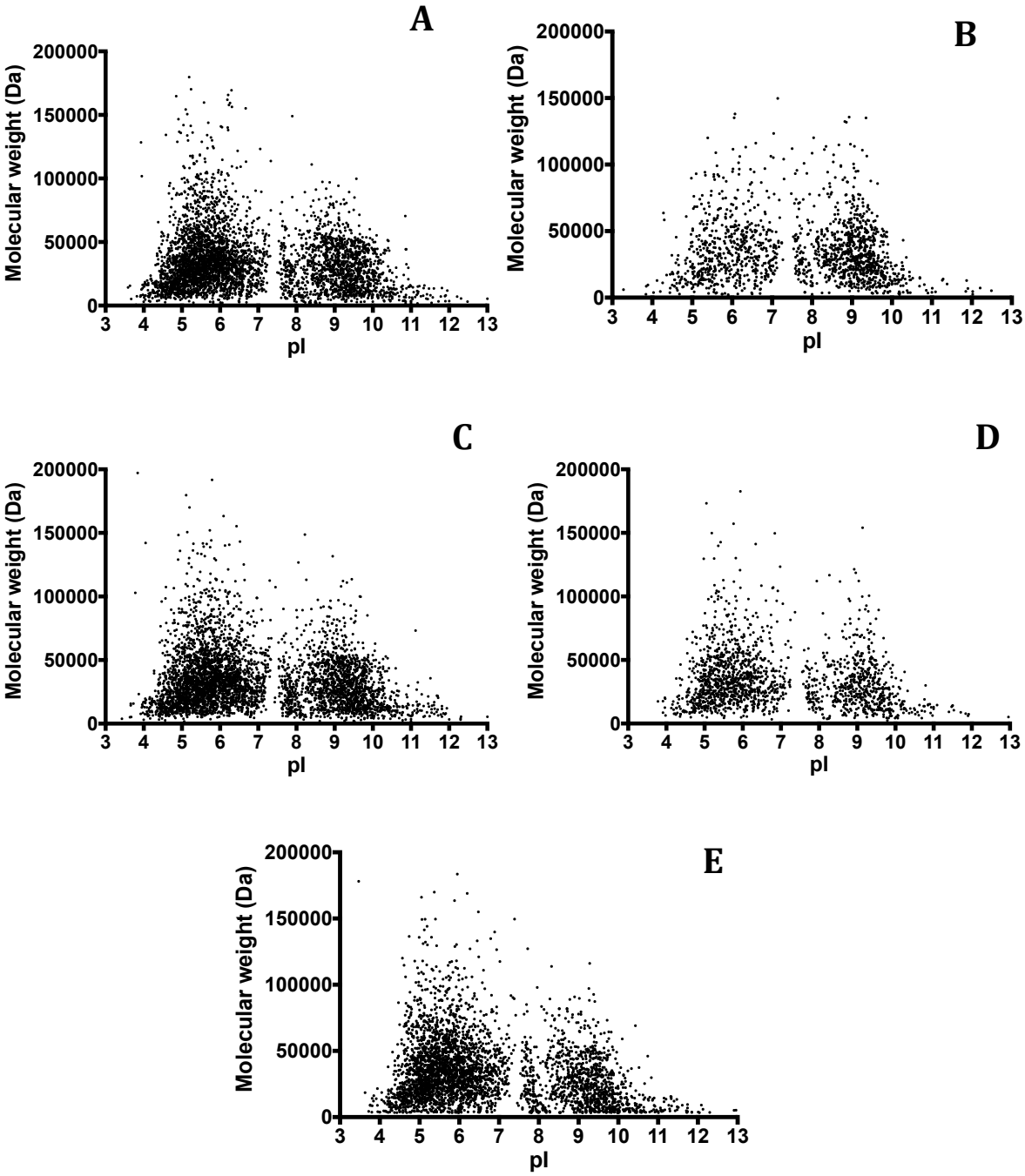

Figure 3.17. Theoretical 2D gels of various non-halophiles. The entire proteome of each organism is plotted as the molecular weight (Y axis) against the pI (X axis). A: *E. coli k12*; B: *H. pylori*; C: *S. typhimurum*; D: *H. influenzae*; E: *V. cholerae*. Each point refers to an individual protein.

Table 3.6. Statistics of the proteome pIs from 5 non-halophiles. The mean, median, maximum and minimum pIs were calculated.

Statistics	*E. coli*	*H. pylori*	*S. typhimurum*	*H. influenzae*	*V. cholerae*
Mean	6.85	7.76	6.97	6.97	6.74
Minimum	3.59	3.28	3.43	3.78	3.47
Maximum	13.00	12.49	13.00	12.96	12.96
Median	6.12	8.33	6.27	6.30	6.05

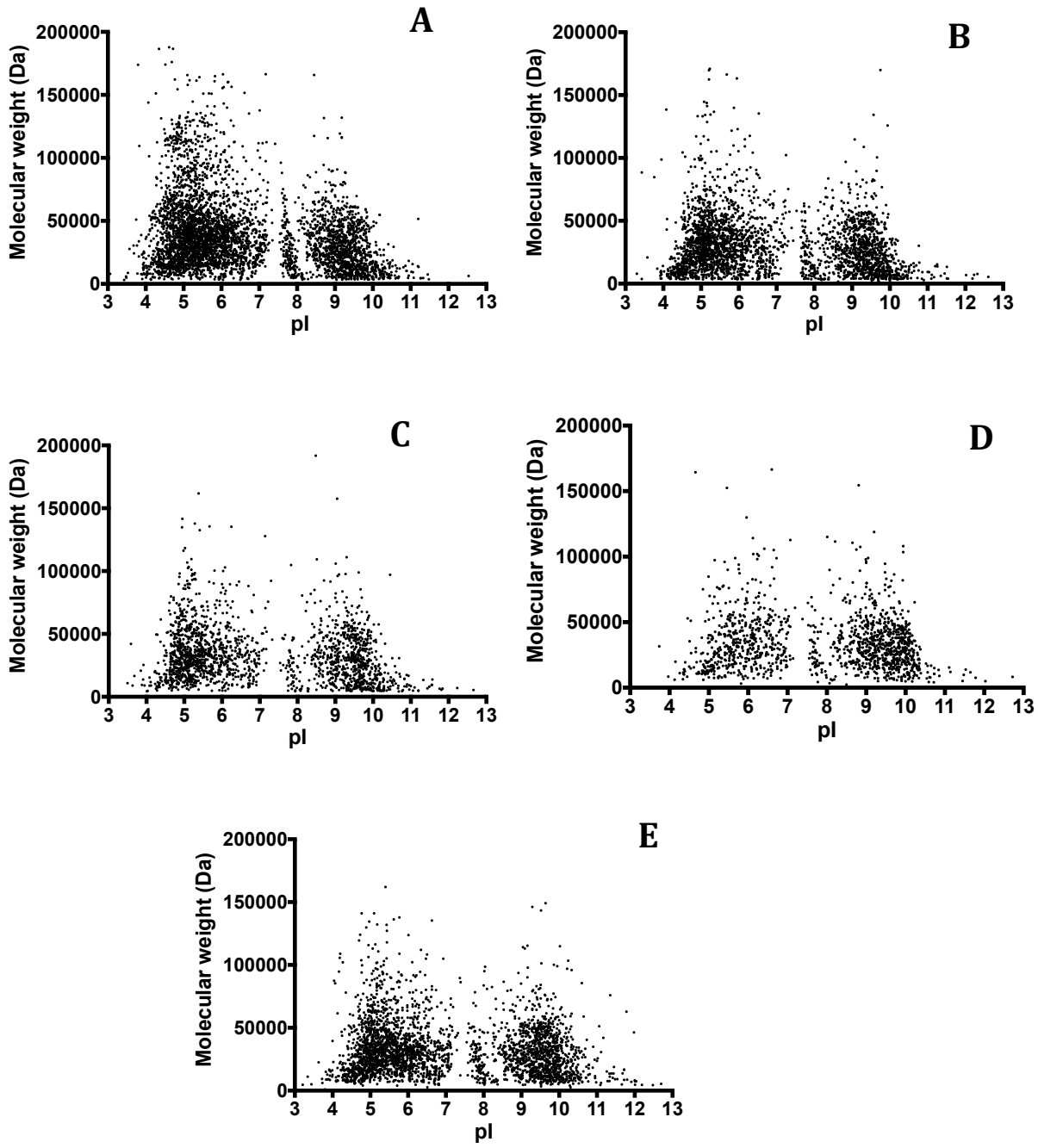

Figure 3.18. Theoretical 2D gels of various halotolerant organisms. The entire proteome of each organism is plotted as the molecular weight (Y axis) against the pI (X axis). A: *E. vietnamensis*; B: *S. aureus*; C: *P. acidilactici*; D: *P. ubique*; E: *L. plantarum*. Each point refers to an individual protein.

Table 3.7. Statistics of the proteome pIs from 5 halotolerant organisms. The mean, median, maximum and minimum pIs were calculated.

Statistics	*E. vietnamensis*	*S. aureus*	*P. acidilactici*	*P. ubique*	*L. plantarum*
Mean	6.60	6.95	7.13	7.97	7.13
Minimum	3.06	3.30	3.50	3.56	2.76
Maximum	12.53	12.61	12.66	12.72	12.70
Median	5.89	6.15	6.35	8.71	6.38

Summary

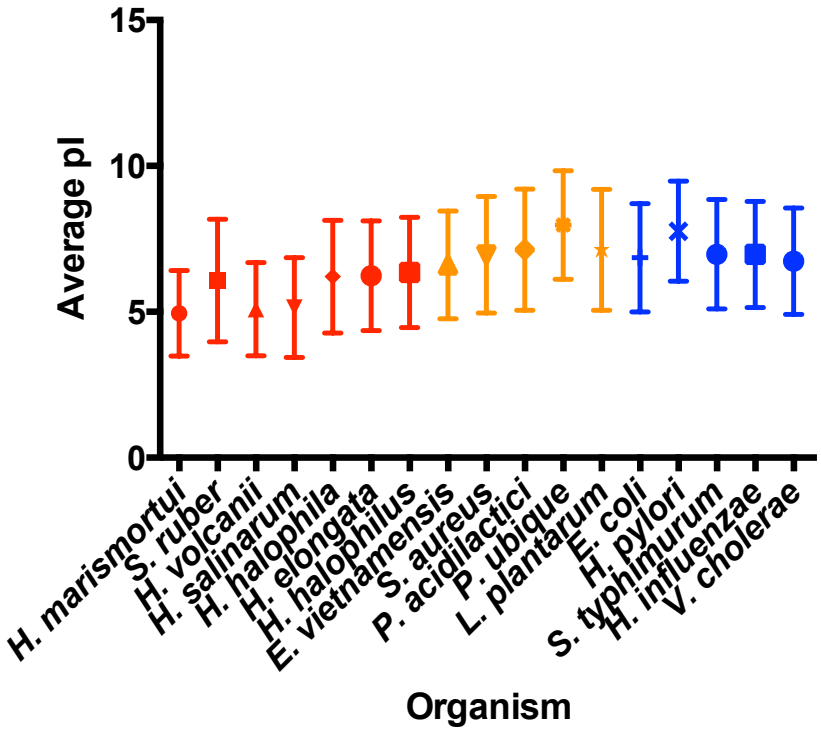

Figure 3.19. Average proteome pI for a selection of halophiles, non-halophiles and halotolerant organisms. The mean pI of the proteins from each organism is plotted, along with the standard deviation of this mean. Red: halophiles; orange: halotolerant; blue: non-halophiles.

3.2.4: Cation transport

An extensive search on the UniProt database was undertaken in order to find all of the main cation transporters and channels present in *E. coli*, *E. vietnamensis* and *S. ruber*. The results from this are shown below, in Table 3.8.

Table 3.8. Cation transport systems present in *E. coli*, *E. vietnamensis* and *S. ruber*. An extensive search on UniProt was performed in order to find the cation transporters present in the three organisms: *E.coli* (EC), *E. vietnamensis* (EV) and *S. ruber* (SR). The 'Annotation' refers to what the protein is known as on UniProt and the 'gene name' lists the gene associated with the function of the protein.

Annotation	Gene name	*EC*	*EV*	*SR*
Efflux transporters				
Cation				
Cation efflux system protein	-	Yes	Yes	Yes
Cation efflux system protein CusA (czcA)	CusA (czcA)	Yes	No	Yes
Cation efflux system protein CusB (czcB)	CusB (czcB)	Yes	No	Yes
Cation efflux system protein CusC	CusC	Yes	No	No
Cation efflux system protein CusF	CusF	Yes	No	No
Cation/multidrug efflux pump	-	No	Yes	Yes
Potassium				
pH adaptation K^+ efflux system phaF	phaF	No	No	Yes
Efflux transporter, outer membrane factor lipoprotein, NodT family	NodT	No	Yes	No
Efflux transporter	-	No	Yes	Yes
K^+ efflux system protein	-	No	No	Yes
K+-efflux system protein	-	No	No	Yes
Glutathione-regulated K^+-efflux system protein KefB	KefB	Yes	No	No
Glutathione-regulated K^+-efflux system protein KefC	KefC	Yes	No	No
Glutathione-regulated K^+-efflux system ancillary protein KefF	KefF	Yes	No	No
Glutathione-regulated K^+-efflux	KefG	Yes	No	No

system ancillary protein KefG				
Kef-type K^+ transport system	kef	Yes	Yes	No
Sodium				
Na^+-driven multidrug efflux pump	-	No	Yes	No
ABC-type Na^+ efflux pump	-	No	Yes	Yes
Magnesium				
Mg^{2+} and cobalt efflux protein CorC	CorC	Yes	No	No
Mg^{2+} efflux protein	-	Yes	No	No
General cation transporters				
Cation symporters	-	Yes	No	No
Cation-transporting ATPase pacS	pacS	No	No	Yes
Cation diffusion facilitator family transporter	-	No	Yes	No
Cation transport regulator ChaB	ChaB	Yes	No	No
Cation transport ATPase	-	No	Yes	No
Magnesium and divalent cation transporters	-			
Divalent-cation transporters	-	Yes	No	No
Divalent metal cation transporter MntH	MntH	Yes	No	No
Mg^{2+} transport protein	-	Yes	Yes	Yes
Mg^{2+} transporter YhiD	YhiD	Yes	No	No
CorA Mg^{2+} transport protein	CorA	Yes	Yes	Yes
MgtA Mg^{2+} transporting ATPase	MgtA	Yes	No	Yes
MgtE Mg^{2+} transport protein	MgtE	No	Yes	Yes
K^+ transporters				
K^+-transporting ATPase	-	Yes	No	No
Trk K^+ transport system	Trk	Yes	Yes	Yes
Trk system K^+ uptake protein TrkA	TrkA	Yes	No	Yes
Trk system K^+ uptake protein TrkG	TrkG	Yes	No	No
Trk system K^+ uptake protein TrkH	TrkH	Yes	No	Yes
K^+ uptake system	-	No	No	Yes
kup (TrkD) low affinity K^+ transport system	kup (TrkD)	Yes	No	No
kdp K^+ transporting ATPase	kdp	Yes	No	No
K^+-transporting ATPase K^+-binding subunit, kdpA	kdpA	Yes	No	No
K^+-transporting ATPase ATP-binding subunit, kdpB	kdpB	Yes	No	No
Na^+ transporters				
Na^+-dependent transporter	-	No	Yes	Yes

SSS Na^+ solute transporter	SSS	No	Yes	Yes
K^+-stimulated pyrophosphate-energized Na^+ pump, hppA	hppA	No	Yes	Yes
Voltage-gated channels				
Voltage-gated potassium channel Kch	Kch	Yes	No	No
Mechanosensitive channels				
Large-conductance mechanosensitive channel (gene = mscL)	mcsL	Yes	Yes	No
Miniconductance mechanosensitive channel YbdG	YbdG	Yes	No	No
Mechanosensitive channel MscK	MscK	Yes	No	No
Moderate conductance mechanosensitive channel YbiO	YbiO	Yes	No	No
Miniconductance mechanosensitive channel MscM	MscM	Yes	No	No
Low conductance mechanosensitive channel YnaI	YnaI	Yes	No	No
Small-conductance mechanosensitive channel, mscS family protein	MscS	Yes	No	Yes
Small-conductance mechanosensitive channel	-	No	Yes	No
Antiporters				
General				
$Na^+(K^+)/H^+$ antiporter	-	No	Yes	No
$Na^+(K^+)/H^+$ antiporter, ChaA	ChaA	Yes	No	No
Cation/H^+ antiporter YbaL	YbaL	Yes	No	No
Transporter, monovalent cation:proton antiporter-2 (CPA2) family	CPA2	No	No	Yes
Monovalent cation/proton antiporter, MnhG/PhaG subunit subfamily	MnhG/PhaG	No	No	Yes
K^+/H^+				
K^+/H^+ antiporter	-	Yes	No	No
$K(^+)/H(^+)$ antiporter NhaP	NhaP	Yes	Yes	No
Na^+/H^+	-			
Na^+/H^+ antiporter	-	Yes	Yes	Yes
Na^+ antiporter	-	No	Yes	No
NhaA Na^+/H^+ antiporter	NhaA	Yes	Yes	No
NhaB Na^+/H^+ antiporter	NhaB	Yes	No	No
Na^+/H^+ antiporter NhaC	NhaC	No	Yes	No
Na^+/H^+ antiporter NhaD	NhaD	No	Yes	No
MnhB subunit of Na^+/H^+ antiporter superfamily	MnhB	No	No	Yes

Na^+/H^+ antiporter family	-	No	No	Yes
Symporters				
General				
Cation/acetate symporter ActP	ActP	Yes	No	No
H^+/glutamate (aspartate) symporter (gene = gltP)	gltP	Yes	No	Yes
Glycoside/cation symporter YagG	YagG	Yes	No	No
Sodium				
Na^+: solute symporter	-	No	Yes	Yes
Na^+:dicarboxylate symporter	-	No	No	Yes
Na^+/proline symporter (gene = putP)	putP	Yes	No	No
Na^+/glutamate symporter (gene = gltS)	gltS	Yes	No	No
Na^+/pantothenate symporter (gene = panF)	panF	Yes	No	No
Na^+/phosphate symporter	-	No	Yes	No
Na^+/H^+ dicarboxylate symporter	-	No	Yes	No
Na^+/alanine symporter	-	No	No	Yes
Transporter, Na^+/sulfate symporter family	-	No	No	Yes
Na^+/proline symporter	-	Yes	Yes	Yes
Co-transporters				
Na^+/glucose cotransporter	-	No	No	Yes
Na-K-Cl cotransporter	-	No	No	Yes
Exchangers				
H^+/Cl^- exchange transporter	-	Yes	No	No
K^+ dependent Na^+ exchange related protein	-	No	Yes	No
Na^+/Ca^{2+}-exchanging protein (nce)	nce	No	No	Yes
Na^+/H^+ exchanger YjcE	YjcE	Yes	No	No
K^+- dependent Na^+/Ca^+ exchanger	-	No	No	Yes
K^+ - dependent Na^+ exchanger	-	No	Yes	No
Others				
K^+-stimulated pyrophosphate-energised Na^+ pump	-	No	Yes	Yes
Na^+-translocating NADH-quinone reductase	-	No	Yes	Yes
Na^+-and Cl^--dependent transporter	-	No	No	Yes

3.3 Discussion

The 3 MDHs contain homology to one another

When the three proteins were compared together, there was just under 20% sequence similarity between all three - this is known as the twilight zone of protein homology [365]. This region indicates when the sequence identity cannot act as a reliable indicator for protein structural homology [366]. However, the fact that the pairwise alignments were all above 25%, which has been defined as the region where proteins can potentially be considered homologous, suggests that the sequences are related through divergent evolution [367].

Protein sequence alignments can give insight into the 3D structural alignments: two proteins containing >50% sequence identity have been found to have peptide backbones that do not vary by any more than around 1Å rms (root mean square) and those that have 20-25% identity will generally differ by around 2Å rms [368]. From the structural alignments, this may be the case, as SrMDH and EvMDH are much more closely superimposed than EcMDH and SrMDH or EcMDH and EvMDH, further supported by their respective rms values (Figures 3.7 and 3.8). However, as stated above, the proteins contain an adequate level of similarity to be considered homologous - or in fact orthologous [369] - with SrMDH and EvMDH having the most similarity, in terms of both sequence and 3D structure.

Regarding evolutionary relatedness, the phylogenetic tree shows that EvMDH and SrMDH are more closely related than either is to EcMDH. Phylogenetically, *S. ruber* has been found to be located within the phylum Bacteriodetes, and the family *Rhodothermaceae*, in the same lineage as the thermophile *R. marinus* [93,370,371]. *E. vietnamensis* is also located within the phylum Bacteriodetes,

and is of the family *Cyclobacteriaceae* [298]. Therefore, these organisms are relatively closely related to one another, whereas *E. coli* is placed at a greater distance, in the Proteobacteria phylum [372].

SrMDH contains a more negative surface charge than the non-halophilic proteins

It is clear that there is a difference in the overall charges of the proteins from the halophilic organisms when compared with the non-halophiles. The halophilic proteins predominately have lower pIs than their non-halophilic counterparts. In addition, the overall charges on *S. ruber* proteins (Table 3.3) are more much negative than those from *E. coli* and *E. vietnamensis*, as well as the MDH from *S. ruber* having a significantly more negative overall surface charge, in addition to a general increased level of acidic and decrease in the level of basic amino acids in the protein as a whole, in comparison to that of *E. coli*, which may also be the case for *E. vietnamensis*, but to a much lesser degree (Figures 3.12, 3.13 and 3.14). Madern and Zaccai (2004) stated that SrMDH is not acidic [209], which is questionable from the data presented in the current study, as both the pI as well as the surface charge show that this is not the case and the protein is in fact highly acidic.

Additionally, it would appear, from the data presented in the current study, that the surface charges of the 3 MDHs are directly proportional to the degree of salt tolerance of the organism. SrMDH contains the most highly negative (red) surface charge, EcMDH contains the least charged surface (more neutral) and the surface charge of EvMDH sits in between these two extremes. In addition, it has previously been found that the acidic amino acids on the surfaces of halophilic proteins may 'cluster together' [34]. This is what is seen in the present study for SrMDH, as there are various large clusters of acidic residues (red) on the surface of the protein (Figure 3.13). Coulombic interactions at the surfaces of proteins brought about by an increased level of charged amino acids are more pronounced when these amino acids are found in clusters [14], which may lead to an increased stabilisation of halophilic proteins in hypersaline conditions. This

increased negative charge featured on the surfaces of halophilic proteins is thought to aid their stabilisation by providing increased water binding, leading to a 'hydrated salt ion network' around the protein, hence deterring protein aggregation at higher salt concentrations [88,197]. In addition to increased ion binding, the elevated levels of acidic amino acids also makes halophilic proteins more flexible, which is another mechanism to help prevent the protein from aggregating at higher salt concentrations [206]. Therefore, SrMDH is clearly an example of a halo-adapted protein - containing a highly negatively charged surface.

The proteomes of halophiles are acidic – regardless of their adaptation strategy

From the data presented in section 3.2.3, it seems reasonable to state that there is a clear halophile-specific acidic-pH-skewed adaptation of the proteomes of the halophiles, and this is more than likely due to an adaptation for survival within hypersaline environments. This also appeared to be the case for the so-called salt-out halophiles, which accumulate compatible solutes (see section 1.3.1). The majority of the proteins from these organisms have an acidic pI, although less so than for the salt-in halophiles. It has been previously reported that acidic proteomes have been found in several halophiles that accumulate compatible solutes, as well as in marine bacteria [180]. Deole *et al* (2013) found that the organism *H. halophila*, even though it accumulates compatible solutes, also contains an acidic proteome and is also able to accumulate high concentrations of inorganic ions when grown at higher salt concentrations: a so-called 'hybrid' adaptation strategy [190]. This is suggestive that an acidic proteome may be a general adaptation to saline stress, regardless of the adaptation strategy. The finding, from a previous study that organisms growing at a relatively low level of salinity (9% salt) contained acidic-skewed proteomes changed the traditional view that only the extreme salt-in halophiles could have acidic proteomes [373]. It has previously been suggested that, generally, as the salt tolerance of an organism increases, so might the acidic nature of its proteome [180], although

this may not be the case for the majority of halotolerant organisms, given the data in the present study.

The non-halophilic and halotolerant organisms analysed in the current study appeared to have a relatively equal pI distribution within their proteomes, with the exception of *E. vietnamensis*, which contained a slightly acidic proteome – perhaps indicative of the extent of salt tolerance of this organism (which will be explored further in Chapters 4 and 5). Non-halophilic organisms have previously been described as having a 'bi-modal' pI distribution of their proteomes, i.e. a peak in the acidic range and a peak in the basic range, equating to relatively equal levels of acidic and basic amino acids [190,374].

To conclude, the data presented here give increased insight into protein adaptations with regards to different levels of salt tolerance – with the salt-out halophiles having a low level of adaptation (slightly acidic proteomes) and the salt-in halophiles having a higher level of adaptation (more highly acidic proteomes). These findings suggest that halophilic adaptation needs to be re-evaluated, and may be considered to be more of a 'spectrum' rather than two independent strategies.

The presence of specific membrane transport proteins could be essential for bacterial salt tolerance

All three organisms possess the Trk K^+ transport system. However, only *E. coli* contains both the kdp K^+ transport system as well as the kup (TrkD) K^+ transport system. Most archaeal organisms do not contain a kdp transport system, which includes most of the extreme halophiles [8]. *S. ruber* and *E. vietnamensis* also do not contain this transporter, so this could potentially be a factor that is absent within salt-tolerant organisms. Additionally, Mongodin *et al* (2005) found that the *S. ruber* genome contains a 'hypersalinity island' (see section 3.1), which is composed of various genes involved in K^+ transport, efflux and general cation transport, including the Trk system [205]. Therefore it was no surprise that the

current study found these proteins to be present in *S. ruber* - TrkA and TrkH - thus showing the essential nature of this system for halophiles and non-halophiles alike.

Both *E. vietnamensis* and *S. ruber* lack the glutathione -regulated K^+ efflux pumps KefB, KefC, KefF and KefG, which are present within *E. coli* (kef-efflux transporters). However, a protein annotated as 'Kef K^+ transport system' was found within *E. vietnamensis*, although it contained none of the aforementioned Kef proteins. K^+ efflux through the kef system has previously been characterised in *E. coli* and is regulated by the thiol glutathione (kefB and kefC transporters), with the levels of glutathione generally increasing during osmotic stress [375,376]. This is suggestive of a variation in the adaptation of the three organisms, namely that *E. coli* has a larger requirement to remove K^+ than either *E. vietnamensis* and *S. ruber*, or perhaps due to the potential osmotic balance/protection again stress provided by glutathione [377].

Regarding sodium transport, *E. vietnamensis* and *S. ruber* contain the SSS sodium solute transporter and hppA K^+ stimulated Na^+ transporter, whereas *E. coli* does not possess these. This suggests that Na^+ transport could perhaps be correlated to salt tolerance and that the utilisation of sodium gradients and for solute transport may be less important for non-halophilic organisms. It has been suggested previously that the utilisation of Na^+ as opposed to H^+ for the generation of electrochemical energy could be more important for organisms living in highly saline environments [114]. When the Na^+ concentration inside *E. coli* reaches a threshold level (thought to be around 40mM), the cells increase their expression of NhaA, hence suggesting the importance of this protein for the removal of Na^+ from the cell [114]. The Na^+/H^+ Nha family transporters are completely lacking in S*. rube*r. This may be consistent with the halophilic lifestyle, since *S. ruber* may have a requirement to maintain a cytoplasm with a high internal Na^+ concentration and may not generally exchange Na^+ for H^+. On the other hand, the halophilic archaea *H. marismortui*, *H. volcanii* and *Halobacterium spp.* have all been described to contain the NhaA transporter [10]. However, the fact that this is absent in *S. ruber* may suggest that osmotic

adaptation in *S. ruber* may be different from that of the archaeal extreme halophiles, suggesting that *S. ruber* may not exclude Na^+ to the same extent as these organisms. Conversely, *E. vietnamensis* contains more variants of this transporter family (NhaA, NhaC and NhaD) than *E. coli* does (NhaA and NhaB). This could perhaps be explained by the halotolerant lifestyle of *E. vietnamensis*, as it should be able to grow in the presence of moderately high NaCl concentrations, which would indicate a greater need for more Na^+/H^+ antiporters than a non-halophilie like *E. coli*, or a salt-in halophile such as *S. ruber* (does not need to remove Na^+ from its cytoplasm, due to adapted proteins), due to a larger requirement to remove Na^+ from its cytoplasm. NhaD has been reported to have only have been found within halophilic and halotolerant organisms and it is thought that this protein may in fact have a role in Na^+ import into the cell [378], as opposed to Na^+ export – which may be crucial for the osmotic adaptation of these organisms. Therefore, the greater level of Nha genes present in *E. vietnamensis* suggests that this could be a consequence of its halotolerant lifestyle (especially considering the role of NhaD).

Furthermore, the Mnh cation/proton antiporter system (MnhB and MnhG) was only found to be present in *S. ruber*. The importance of this transporter for halo-adaptation is emphasised by the fact that Jensen *et al.* also found this protein to be present in several archaeal halophiles but mostly absent in non-halophiles [10]. It has previously been found from several studies that the disruption/knockout of the function of the Mnh gene (or of its homologues Mrp/Pha) results in an increased sodium sensitivity and growth hindrance within hypersaline conditions [379,380]. This protein has been found to be a Na^+/H^+ transporter, which may function in a similar way to the Nha-transporters [142]. Additionally, it has previously been stated to confer Na^+ tolerance to *Bacillus subtilis* (paralogue to Mnh) and *S. aureus* [142,379–381], so this may indeed be a factor that is essential for a salt tolerant lifestyle. This system therefore merits further research as a mechanism that may be fundamental to bacterial salt tolerance. Additionally, *S. ruber* also contains the PhaF and CPA2

monovalent cation/proton antiporters, which were not found in either *E. coli* or *E. vietnamensis* – both of which are $Na^+(K^+)/H^+$ antiporters [382]

Regarding magnesium transport, *E. coli* may contain more efflux pumps than *E. vietnamensis* and *S. ruber* due to its lower salt concentration growth range, as magnesium has been found to be required intracellularly in higher concentrations when the external salt concentration is high, in order to compete with other cations for ribosome binding sites [383]. However, the fact that *E. coli* also contains more transporters for Mg^{2+} uptake within its membrane suggests the importance of this cation for general bacterial growth. Note that CorA and MgtE have been found to be the two main Mg^{2+} transporters within bacteria and are thought to be found throughout most species: all three organisms in the current study contain CorA [384]. However, only *E. vietnamensis* and *S. ruber* were found to contain MgtE – this is the only Mg^{2+} transport protein not found in *E. coli*. This protein has been previously found to perhaps have a role in the thermotolerance of *B. subtilis* [385], so there is a possibility that it could perhaps have a similar role regarding salt tolerance (i.e for general stress).

Additional findings to note are the fact that voltage-gated and mechanosensitive channels seem to be much more important for *E. coli* than the other two organisms, as E. coli contains many of these which were found to be absent in *E. vietnamensis* and *S. ruber* (Table 3.8). This may be due to the fact that mechanosensitive channels operate based on detecting membrane stretch changes (mechanical pressure stimulus) [386], which may be more vital for non-salt tolerant organisms such as *E. coli* for proper osmotic control. It has previously been found that *E. coli* lacking MscS and MscL cannot survive osmotic shock [386,387]. These channels are known to be vital for cell survival during hypoosmotic shock (i.e. when the cell turgor increases in response to a decreased solute concentration in the environment) by removing excess osmolytes from the cell [388]. The finding in the current study of a much greater level of mechanosensitive channels being present within *E. coli* as compared to *E. vietnamensis* and *S. ruber* therefore suggests that these channels are more important for non-salt-tolerant organisms, most likely due to the lower levels of

solutes in their natural environments, with *E. vietnamensis* and *S. ruber* having a lesser need for these. Finally, a decreased level of general cation efflux pumps was found in the *E. vietnamensis* proteome. This could be postulated to be due to the lifestyle of this organism: it encounters a wider range of cations within its environment (seawater, where many trace ions are present) than *S. ruber* and so does not require to remove as many cations from its cytoplasm as it is able to utilise and withstand a variety of them [389,390].

Next steps

In order to understand the nature of salt tolerance and mechanisms, it is vital to relate the protein adaptations and specific cation transport systems found in the current chapter to the study of the organisms of different salt tolerances (*E. coli*, *E. vietnamensis* and *S. ruber*) at a range of salt concentrations. This is essential to gain insight into their general osmotic response and how this differs for each organism. Moreover, to fully understand cation effects on bacteria it is of crucial importance to determine the effects of specific cations on the growth of these organisms – i.e. to determine if osmotic or specific ion effects are more decisive regarding bacterial growth. This should give essential insight into specific cation effects at the whole organism level, as well as giving insight into halo-adaptation and bacterial salt tolerance.

Additionally, future research should further investigate protein adaptations of bacteria of varying salt tolerances, including the characterisation of proteomes from organisms not studied in the present study. Investigations into cation transport between bacteria of varying should tolerances should be extended to include more organisms – especially regarding the presence of specific proteins which could be essential for the possession of salt tolerance.

Chapter 4: Effect of salt on bacterial growth

4.1: Introduction

4.1.1: Bacterial salt response

It has been known for many years that bacterial growth is adversely affected by the presence of high salt concentrations [391]. As stated previously (section 1.2), with regards to salt-stress, bacteria have developed different strategies in order to cope with this increased external ionic strength, in order to protect themselves at both a cellular as well as at a protein level [178]. The response of different bacterial species may vary somewhat when faced with high ionic strength in the environment. However, most bacteria cannot grow at salt concentrations greater than 10% (equivalent to 1.7M NaCl or 1.3M KCl) [89].

When an organism is exposed to an environment that has a very high level of dissolved solutes, the loss of cellular water to the environment and the influx of solutes into the cell can cause physiological changes that will be detrimental to that organism [392]. It is for this reason that cells have adapted various mechanisms to cope with such situations, to keep their internal water and solute concentrations constant [393]. The responses of organisms to fluctuations in the osmotic pressure of their environment are based upon whether the environment is hypotonic (lower concentration of solutes in the environment than inside the cell) or hypertonic (higher concentration of solutes in the environment than inside the cell) with their cytoplasm [394]. Without the accumulation of compatible solutes (or inorganic ions, see section 1.3.1 for details), there are three possible outcomes that could occur when the cell water content abruptly changes as a consequence of fluctuations in external ionic strength. A schematic showing the effects of osmotic changes on a bacterial cell is shown in Figure 4.1.

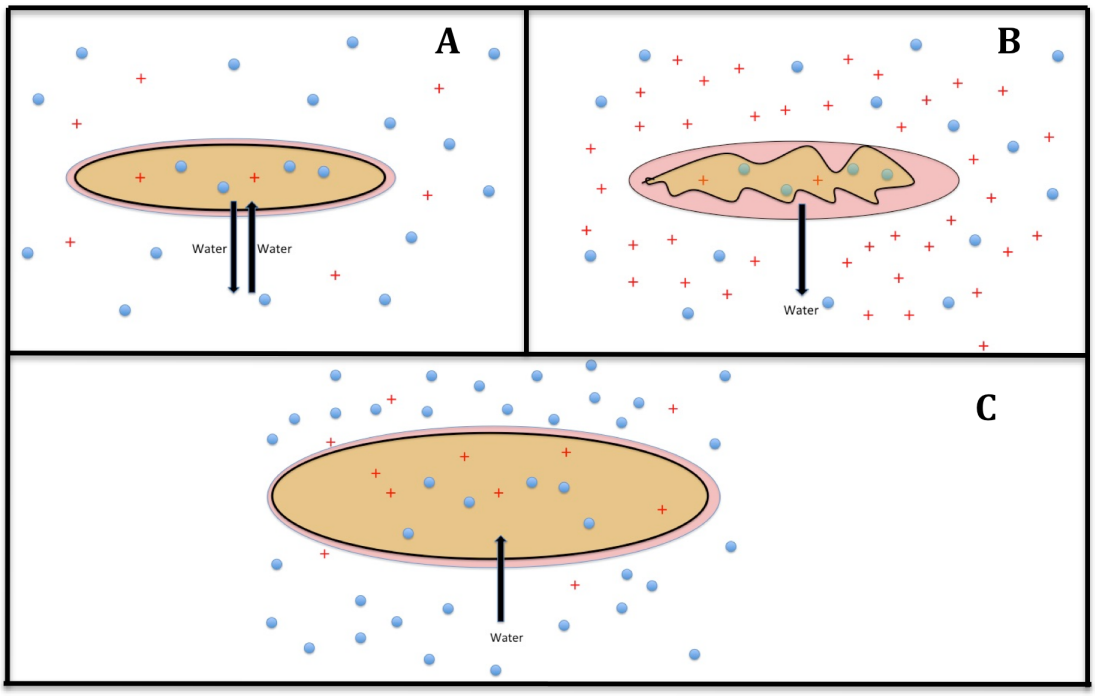

Figure 4.1. Schematic of the effect different solute concentrations on a bacterial cell. When a bacterium is exposed to an environment that contains an equal solute concentration to that inside the cell (isotonic), the net water flow into the cell will equal that leaving the cell (A) [110]. However, if the solute concentration in the environment is greater than that inside the cell (hypertonic), then water will leave the cell by osmosis, flowing to the region of lower water concentration, i.e. the environment (B). This will cause the bacterial cell to shrivel up, meaning the plasma membrane will come away from the cell wall as it shrinks, known as plasmolysis, which will lead to a drop in cell turgor and a decrease in growth rate [104,395]. The inverse to the situation in B, is that of the environment containing a lower solute concentration from that within the cell (hypotonic), which will result in water flowing from the high water concentration (the environment) to the lower water concentration (inside the cell) (C), meaning that the cell will swell up and potentially lyse (burst) [393]. Situation B involves an increase in external osmotic pressure, whereas situation C involves a decrease in external osmotic pressure.

For non-halophilic bacteria, salt-stress can inhibit cellular respiration. However, it has been found that the addition of compatible solutes (solutes used for osmotic balance that do not adversely affect the cell at high concentrations – see section 1.3.1) to the medium of salt-stressed *E. coli* can recover the growth rate [396]. A bacterium will have to expend a large amount of energy to pump out salts from its cytoplasm as well as to accumulate/synthesise compatible solutes,

hence various cellular processes will be inhibited at higher salt concentrations, due to this increased energetic demand [397]. Such processes include cell division and cellular growth [398]. In addition, a drop in cell turgor, caused by a hyperosmotic environment, results in a decreased rate of growth, and in extreme cases, plasmolysis of the bacterial membrane can cause cell death [106].

The growth rate of *E. coli* has been shown to decrease at a linear rate with increasing NaCl concentration, which is typical of a non-halophilic and non-salt tolerant organism [399]. In contrast, halotolerant organisms can often tolerate NaCl concentrations anywhere from 6% up to NaCl saturation, but do not require its presence for growth [86]. Generally, most halotolerant species will not be able to grow at the higher NaCl concentrations. Ramadoss *et al* (2013) reported that only 25% of the halotolerant species they surveyed were able to grow at NaCl concentrations of 3.4M or above [400]. However, halophiles are known to be able to grow in NaCl concentrations up to saturation (over 5M) [84]. Figure 4.2 shows a schematic of the general trend of a non-halophile, halophile and halotolerant organism in terms of the effect of an increasing salt concentration in the environment on their rates of growth.

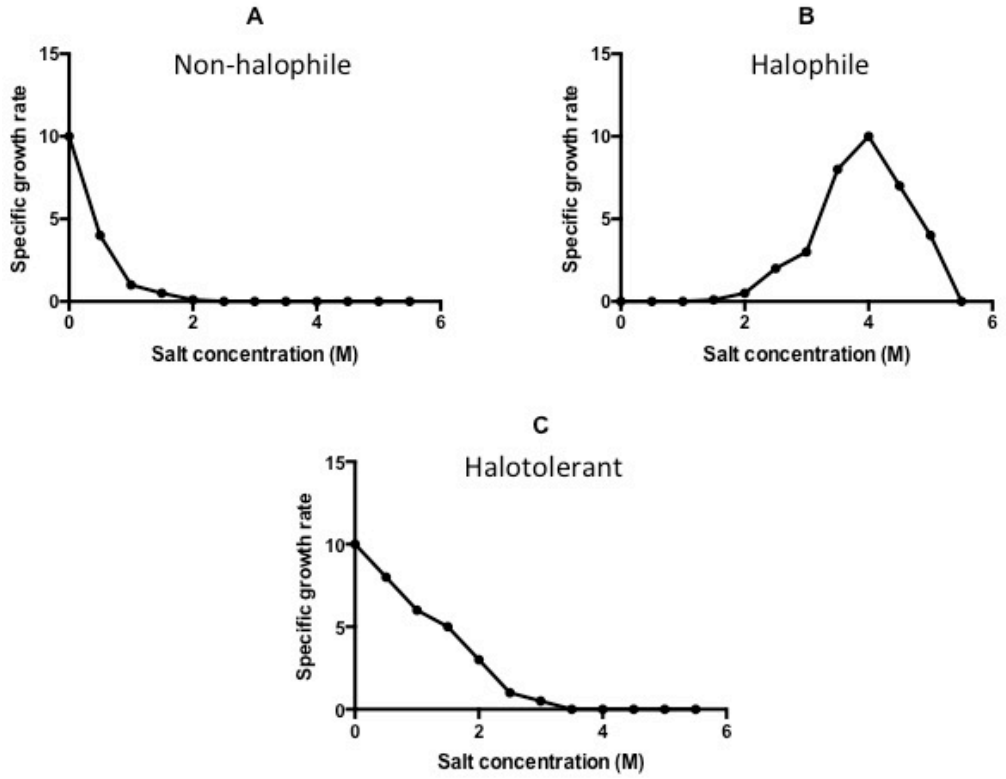

Figure 4.2. General growth rate vs salt concentration of a non-halophile, halophile and halotolerant organism. Figure is a schematic representation to depict a general trend and growth rates do not correspond to the actual specific growth rates of these organisms. Figures are plotted as salt concentration (x axis) against specific growth rate (y axis). The growth rate of the non-halophile will decrease sharply with increasing salt concentration (A), whereas the growth rate of the halophile will gradually increase with increasing salt concentration (B). For the halotolerant organism, its maximal rate of growth will be in the absence of salt but it is able to grow at low and moderate salt concentrations (C).

4.1.2: Effects of different cations on bacterial growth

It has previously been reported that the non-halophilic osmotic-stress response will be the same, regardless of the specific salt encountered [401]. However bacteria have been found to be affected, in terms of their growth rates, by different salts to varying extents [402]. Specifically, it has been known for many years that sodium is generally more toxic to cells than potassium (especially at higher concentrations) and that the effects on growth of a particular organism will vary depending on the specific solute present, hence meaning the particular

salt encountered may determine the severity of growth effects on a specific bacterium [403,404].

Regarding the cations most similar to sodium and potassium (the group one alkali cations), these can be separated into two groups, with sodium and lithium together in one group, and potassium, rubidium and cesium in the other group, due to their chemical behaviour [405,406]. Francium makes up the remainder of this group, but since it is highly radioactive [407] it will not be mentioned further. Potassium, rubidium and cesium are all relatively weakly hydrated (have a single shell of water around them when in solution), whereas lithium is strongly hydrated, with both rubidium and cesium having a very weak interaction with the surrounding water structure and hence cannot organise a water network effectively [408]. In addition, it should be noted that the presence of a second hydration layer around sodium has been disputed [409,410].

Given that high charge density cations are known to be more detrimental towards biomolecule structure, it would be expected that higher charge density cations may also be more detrimental towards bacterial growth, as compared to lower charge density cations [221]. Figure 4.3 shows some kosmotropic and chaotropic cations and anions as well as their relative sizes. As mentioned in section 1.5, small ions are kosmotropic and large ions are chaotropic – determined by their degree of water structuring ability. Table 4.1 displays some of the main physical properties of the alkali cations. Lithium is the smallest of the alkali cations and has the largest charge density, whereas cesium is the largest and has the lowest charge density.

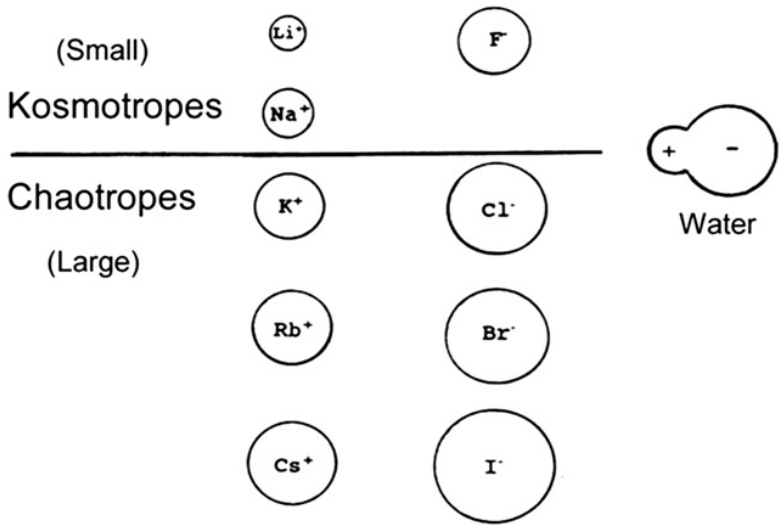

Figure 4.3. Kosmotropes and chaotropes. Kosmotropes are small and interact strongly with water, whereas chaotropes are large and interact weaker with water than it does with itself. Image adapted from Collins, 2007.

Table 4.1. The group 1 alkali cations and some of their properties. Various physical properties of the group 1 alkali cations are shown: electronegativity, electron affinity, ionisation energy, ionic radius and approximate hydrated radius. Ionic radii were obtained from [213], hydrated radii were obtained from [411] and the other properties were obtained from [407].

	Li^+	Na^+	K^+	Rb^+	Cs^+
Electronegativity	0.98	0.93	0.82	0.82	0.79
Electron affinity (KJ/mol)	59.60	52.80	48.40	46.90	45.50
Ionisation energy (KJ/mol)	520.20	495.80	418.80	403.00	375.70
Ionic radius (Å)	0.42	0.67	1.06	1.23	1.62
Approximate hydrated radius (Å)	3.35	2.65	2.20	-	-

As can be seen from Table 4.1, on progressing down the periodic table from Na^+ to K^+ the level of hydration decreases (i.e. smaller hydrated radius), with K^+ being more weakly hydrated than Na^+. This has various implications for cellular functions, as organisms generally have been found to prefer K^+ over the more disruptive Na^+ [412]. The larger cations, which have lower charge densities (chaotropes) will have a different water affinity to the kosmotropic COO^- groups present on intracellular proteins, whereas larger (kosmotropic) cations will have more similar water affinities to these groups (COO^- groups have been found to have a similar water affinity as Na^+, and are hence classed as kosmotropes),

which may have implications for the biological effects of that particular cation (refer to section 1.5) [222,413].

Sodium and potassium are ubiquitous in living systems and so it seems reasonable to assume that most organisms will be able to tolerate a certain level of these cations, as they are essential to life, especially regarding the generation of membrane electrochemical gradients for energetic purposes [406,414]. However, as was discussed in section 1.5, cations have differential effects on biomolecules and cells, with some being stimulatory towards growth (usually at lower concentrations), whereas others are toxic [403]. The nature of K^+ accumulation inside cells is highlighted by a study which found that for some fungi, the effects on growth of NaCl, LiCl, RbCl and CsCl were more adverse than as compared to KCl – with KCl producing the lowest level of growth inhibition, which suggests that KCl may be a less disruptive salt than the others [415]. In addition to K^+ being utilised for initial osmotic balance during osmotic stress, K^+ also has a range of important cellular functions [163]. These functions include protein stabilisation and the maintenance of an adequate membrane potential, required for energy generation, as is discussed in more detail in Chapter 1 [403].

The literature on the topic of cation specificity of halophilic organisms is somewhat lacking - most studies have grown these organisms in the presence of NaCl as the primary salt - although halophile media almost always contains some KCl, albeit at lower concentrations than NaCl [416]. Regarding the small number of studies that have investigated the effects of different cations on the growth of halotolerant or halophilic bacteria (Jensen et al., 2015; Nostro et al., 2005), the comparison between a halophile, non-halophile and halotolerant organism in terms of their cation preferences and utilisation of alternative cations has never been carried out. The effects of different alkali cations upon the growth of non-halophilic microorganisms has been an ongoing area of interest within the field of microbiology: as far back as 1928 investigators had a basic understanding that distinct cations produced different growth effects and this varied between species [417]. For example, more recently, the effects of LiCl have been examined on the growth of the yeast *Saccharomyces cervisiae*, which can be toxic to this

organism [418,419]. Moreover, the halotolerant organism *S. aureus* has also been found to have its growth inhibited by the presence of LiCl - the mechanism of the growth inhibition was thought to be due to Li^+-specific toxicity rather than merely osmotic reasons [420].

Regarding alternative alkali cations to sodium and potassium, the high charge density of the lithium ion (as mentioned above) means that its biological effects have been found to be similar to that of the divalent cation magnesium (Mg^{2+}), and it is because of this that many of its biochemical effects are thought to arise, as it may interfere with cellular processes requiring Mg^{2+} [411]. Li^+ is generally found in trace levels inside most cell types and is not essential to life, or required for any cellular processes [421]. Lithium is known to be toxic towards some organisms and yet other organisms are able to grow in its presence, i.e. this sensitivity towards lithium appears to vary between different species [422,423]. Moreover, LiCl has been investigated for anti-bacterial activities in the past, and it has been found that at lower concentrations it may not actively inhibit bacterial growth but may interfere with certain aspects of their metabolism, particularly regarding aspects related to pathogenesis [424]. However higher concentrations have been reported to lead to a lower level of overall growth for various bacterial species [425]. Interestingly, lithium has also been linked to the inhibition of viral replication, suggesting that its detrimental ramifications go beyond basic osmotic effects [426].

With respect to the other group 1 cations, rubidium and cesium have been reported to show similarities to that of potassium (Table 4.1) [427]. Cesium has been found to be more toxic to certain bacterial species than others, and rubidium has been reported to be a more beneficial potassium replacement than cesium, with less toxic effects reported [428,429]. Therefore, it would appear that even small changes in the chemistry of an ion could result in dramatic differences on the effects it has on an organism.

4.1.3: Rationale for current study

Weakly and strongly hydrated cations and anions have been found to differently affect the growth of different bacteria [9]. Nostro *et al* found that the effects of specific anions on the growth of various bacteria followed the Hofmeister series. Additionally, Jensen *et al.* investigated the effects of low concentrations of LiCl, RbCl and KCl on the growth of the haloarchaeon *H. marismortui* and found that only RbCl was an effective substitute for KCl, and even this resulted in a lower than normal generation time [10]. However, they did not study the effects of NaCl or higher salt concentrations, nor did they compare the halophilic growth in these different salts to non-halophilic growth.

The current study aimed to investigate the effects of specific cations and anions on the growth of a halophile, non-halophile and halotolerant organism. Since *E. coli* is a particularly well characterised bacterial species, it is ideal for use as a model for a typical Gram-negative non-halophilic bacterium, to compare with a halophile and non-halophile [430]. In addition, it is well known to not be able to tolerate high salt concentrations [431].

S. ruber, *E. coli*, and *E. vietnamensis* were grown in various salts at a range of concentrations (0M – 5.5M). The salts chosen for these experiments were alkali cation salts: NaCl, KCl, NaBr, KBr, LiCl, RbCl and CsCl. These salts were also used in various combinations, in a 50:50 ratio (equimolar), in order to further elucidate the differences between osmotic and specific ion effects on the organisms.

4.2: Results

4.2.1: Baseline growth of organisms

Figures 4.4 - 4.6 show the specific growth rates and growth curves of *E. coli*, *E. vietnamensis* and *S. ruber* grown in LB, Marine Broth (MB) and Salinibacter Medium, respectively. Table 4.2 shows the doubling times. In addition, the

organisms were also grown in the General Medium with no added salts, and the growth curves from these are shown in Figure 4.7 with the specific growth rates in Table 4.3.

E. coli had a relatively fast doubling time in LB medium but growth in the General Medium was significantly lower. The doubling time of *E. vietnamensis* in MB medium was just over 8 hours and again growth in the general medium is not as rapid as in MB medium. *S. ruber* has a very slow rate of growth (doubling time of over 100 hours in Salinibacter medium), and did not grow in the general medium with no added salts.

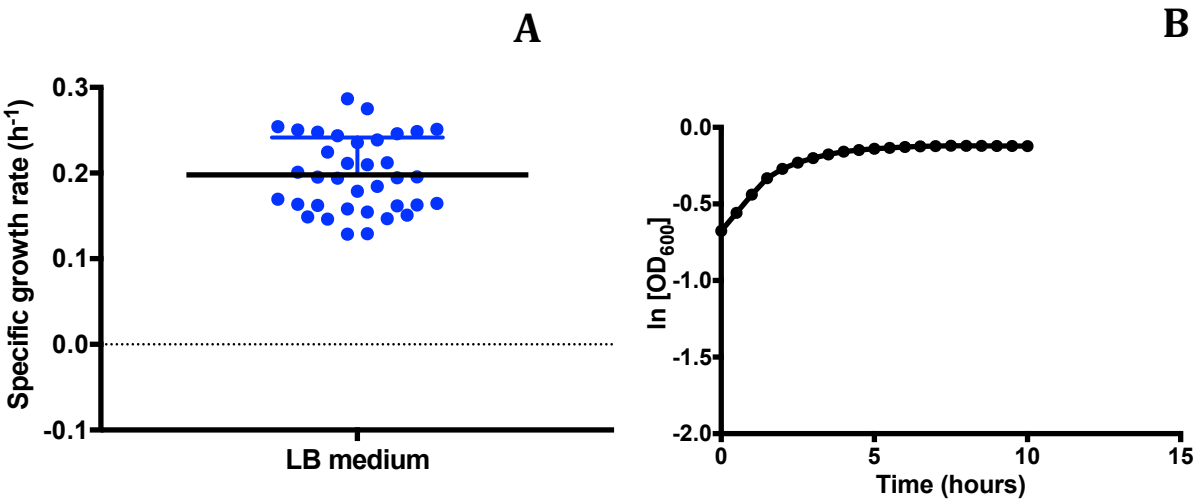

Figure 4.4 *E. coli* growth in LB medium. A: scatter distribution about the mean of the specific growth rates of individual replicates (36). B: averaged ln-transformed growth curve of *E. coli* grown in LB medium. Error bars refer to the standard deviation between individual experiments.

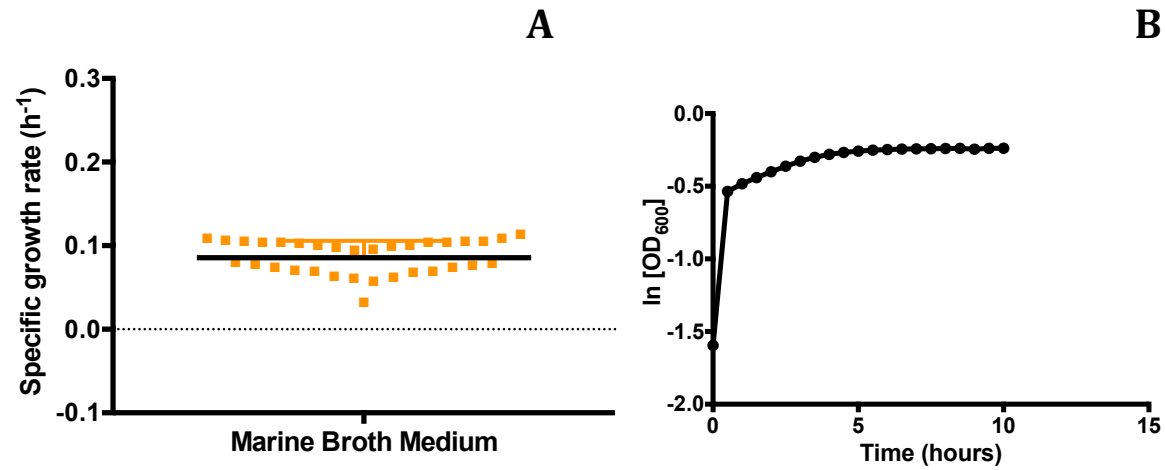

Figure 4.5. *E. vietnamensis* growth in Marine Broth Medium. A: scatter distribution about the mean of the specific growth rates of individual replicates (33). B: averaged ln-transformed growth curve of *E. vietnamensis* grown in Marine Broth medium. Error bars refer to the standard deviation between individual experiments.

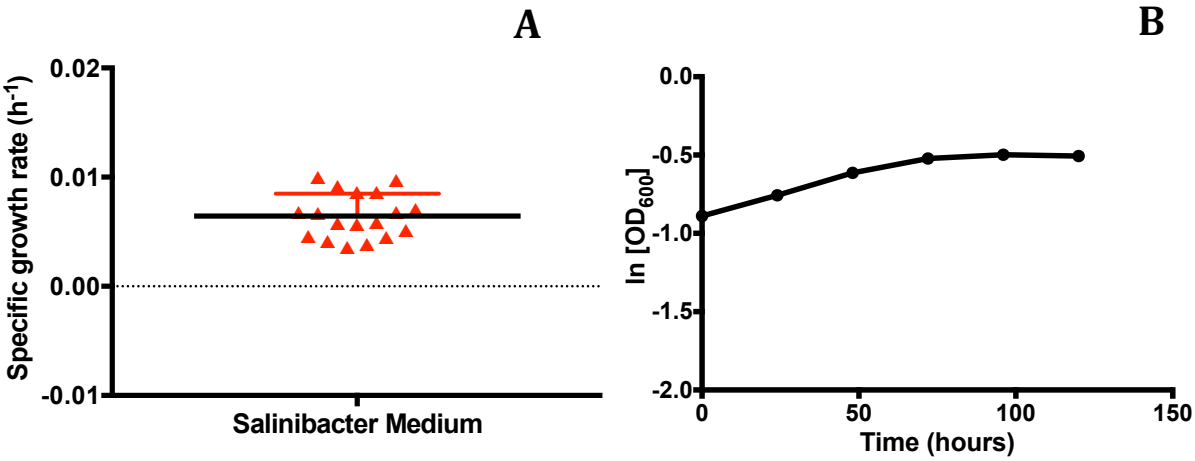

Figure 4.6. *S. ruber* growth in Salinibacter Medium. A: scatter distribution about the mean of the specific growth rates of individual replicates (18). B: averaged ln-transformed growth curve of *S. ruber* grown in Salinibacter Medium. Error bars refer to the standard deviation between individual experiments.

Table 4.2. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in their optimal media. Standard error of mean (SEM) are shown for the average doubling time values.

Organism	Doubling time (hours)
E. coli	3.50 ± 0.14
E. vietnamensis	8.09 ± 0.54
S. ruber	107.82 ± 9.16

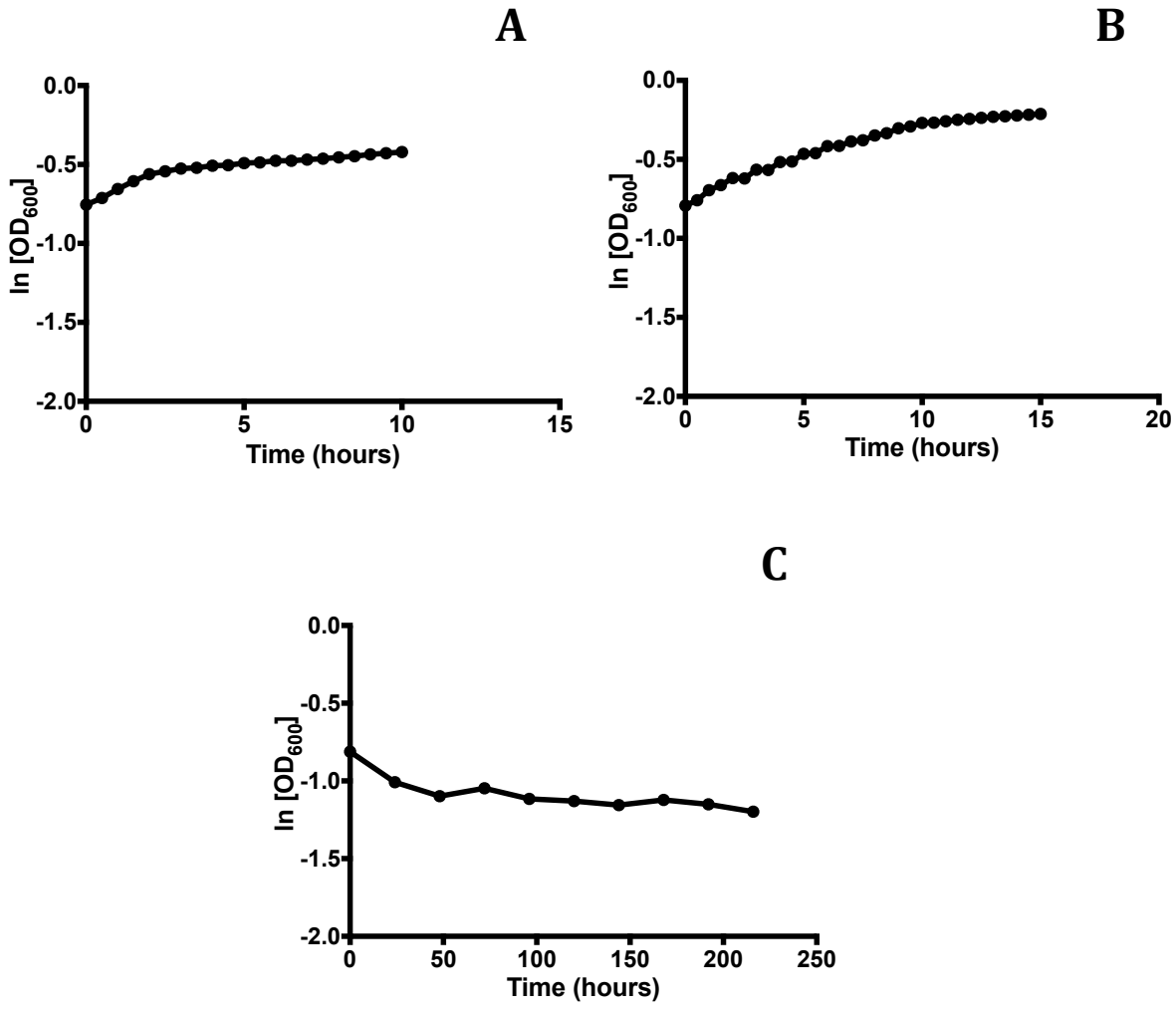

Figure 4.7. *E. coli*, *E. vietnamensis* and *S. ruber* growth curves in 0M salts. *E. coli* (A), *E. vietnamensis* (B) and *S. ruber* (C) were grown in the General Medium containing no salts. The data are plotted as time (hours) (x axis) against ln[OD_{600}] (y axis). The data was averaged from multiple (102 (Ec/Ev) and 125 (Sr)) experiments.

Table 4.3. *E. coli*, *E. vietnamensis* and *S. ruber* specific growth rate (h^{-1}) in the general medium with no added salts (0M). The SEM of the average growth rates is shown.

Organism	Specific growth rate (h^{-1})
E. coli	0.13 ± 0.01
E. vietnamensis	0.08 ± 0.01
S. ruber	0.00

4.2.2: Growth in the presence of sodium

Growth experiments were conducted on *E. coli*, *E. vietnamensis* and *S. ruber* in media containing NaCl, NaBr and equimolar NaCl:NaBr, at concentrations between 0M – 5.5M. These are shown in Figures 4.8 – 4.10 (specific growth rates) and Tables 4.4 – 4.6 (doubling times).

E. coli can tolerate higher concentrations (1M) of equimolar NaCl:NaBr than in either salt alone, but grows better in NaCl than in NaBr. Growth is a lot slower in the presence of sodium salts than in zero salts. *E. vietnamensis* was able to grow in up to 2.5M NaCl but only to 1.5M in NaBr and equimolar NaCl:NaBr – with lower growth in the equimolar combination than in pure NaBr. *S. ruber* requires at least 1M NaCl and 1.5M NaBr for growth, and requires a minimum of 2M equimolar NaCl:NaBr for growth.

E. vietnamensis is extremely halotolerant in terms of its growth in NaCl

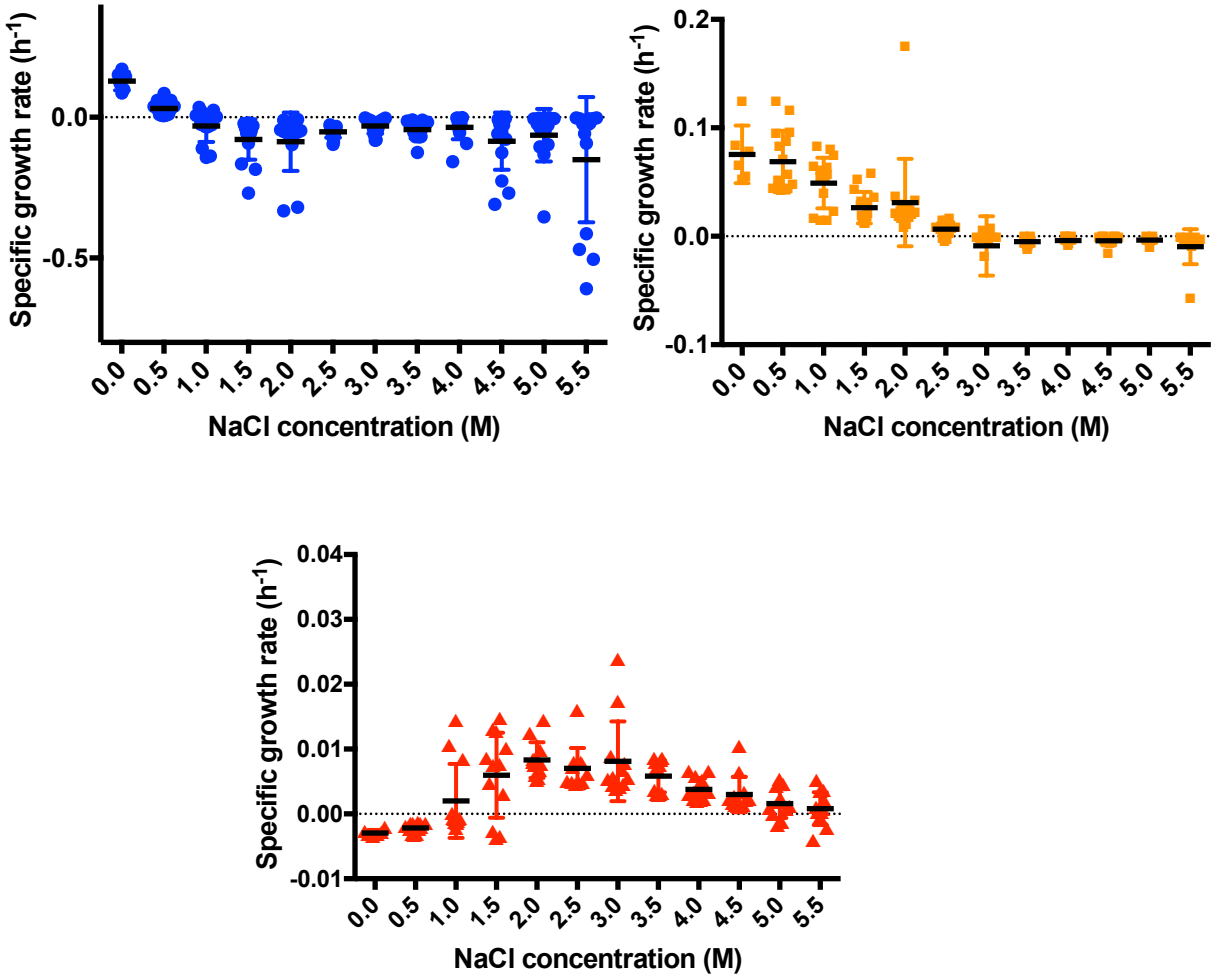

Figure 4.8. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of NaCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (top left – blue circles), *E. vietnamensis* (top right – orange squares) and S*. ruber* (bottom – red triangles) grown in media containing a range of NaCl concentrations (x axis). Error bars indicate the standard deviation between individual experiments (15 replicates). Variance between organisms analysed via ANOVA: F (2, 421) = 142.2, $p < 0.0001$.

Table 4.4. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of NaCl concentrations. Values relate to the average doubling time (hours) of the organism at a given NaCl concentration, with the SEM of this average value shown (15 replicates per condition). Blank cells indicate that no growth occurred.

NaCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.69 ± 0.63	9.18 ± 1.21	-
0.5	20.85 ± 4.67	11.64 ± 1.10	-
1	-	13.43 ± 1.78	345.90 ± 9.78
1.5	-	29.62 ± 3.63	101.87 ± 20.93
2	-	28.53 ± 3.10	90.54 ± 7.56
2.5	-	86.06 ± 10.73	93.32 ± 9.80
3	-	-	84.56 ± 14.63
3.5	-	-	102.58 ± 13.37
4	-	-	163.29 ± 18.28
4.5	-	-	252.67 ± 44.01
5	-	-	439.67 ± 100.92
5.5	-	-	-

Bromide may be less stimulatory/more inhibitory towards growth than chloride

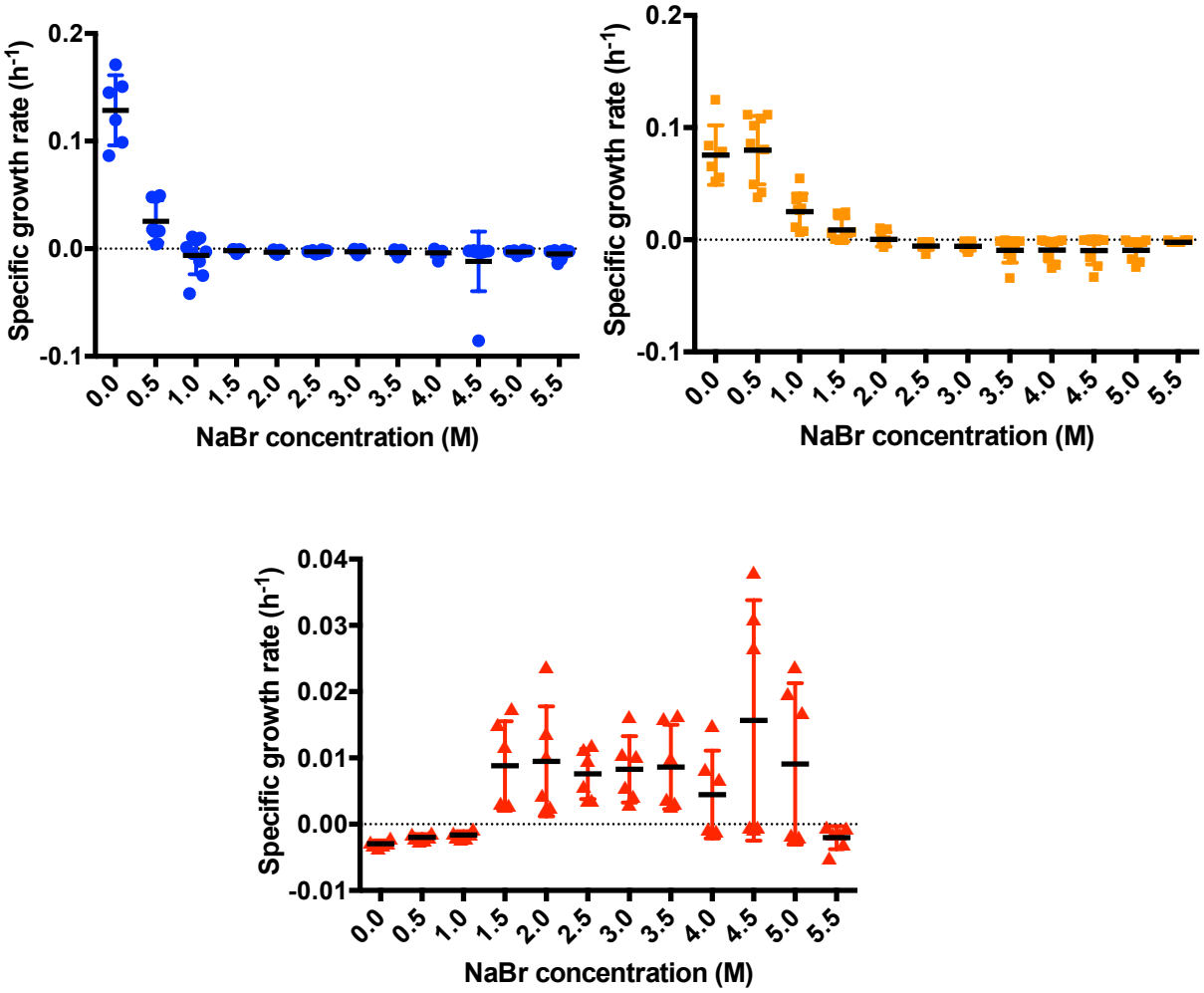

Figure 4.9. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of NaBr concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (top left – blue circles), *E. vietnamensis* (top right – orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of NaBr concentrations (x axis). Error bars indicate the standard deviation between individual experiments (9 replicates). Variance between organisms analysed via ANOVA: F (2, 223) = 46.97, $p < 0.0001$.

Table 4.5. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of NaBr concentrations. Values refer to the doubling time (hours) of the organism at a given NaBr concentration, with the SEM of this average value shown (9 replicates per condition). Blank cells indicate that no growth occurred.

NaBr (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	27.61 ± 5.99	10.27 ± 1.65	-
1	-	21.69 ± 2.28	-
1.5	-	71.86 ± 23.56	78.68 ± 5.79
2	-	-	73.06 ± 27.96
2.5	-	-	91.27 ± 26.00
3	-	-	83.74 ± 17.43
3.5	-	-	80.23 ± 32.99
4	-	-	154.90 ± 16.33
4.5	-	-	44.24 ± 1.80
5	-	-	76.19 ± 3.06
5.5	-	-	-

Bromide and chloride together may lower growth rates

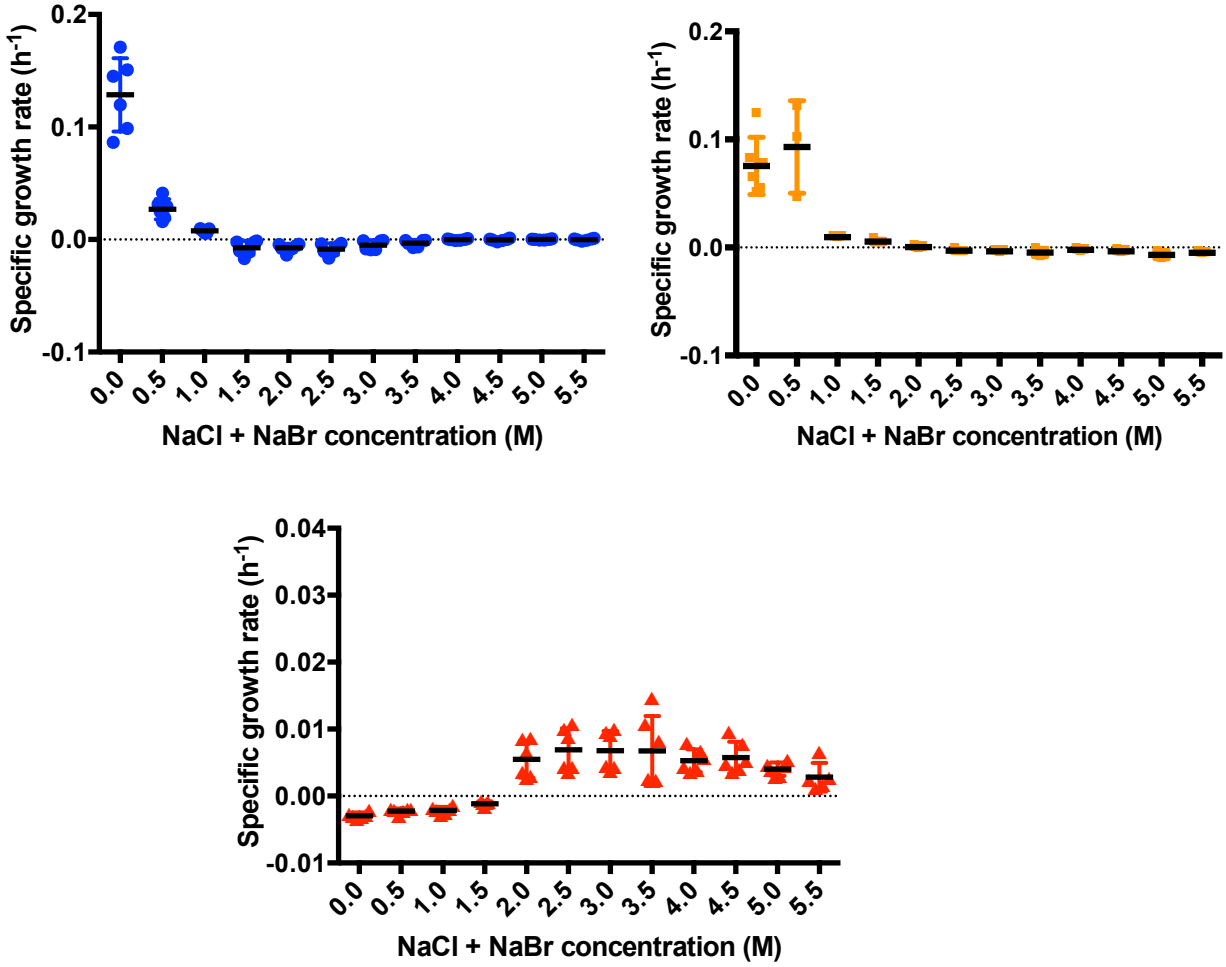

Figure 4.10. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar NaCl:NaBr concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (top left – blue circles), *E. vietnamensis* (top right – orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of NaCl:NaBr concentrations (x axis). Error bars indicate the Standard Deviation between individual experiments (3 – 6 replicates). Variance between organisms analysed via ANOVA: F (2, 129) = 91.72, $p < 0.0001$.

Table 4.6. Doubling times of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar NaCl:NaBr concentrations. Values refer to the doubling time (hours) of the organism at a given NaCl:NaBr concentration, with the SEM of this average value shown (3 (Ev) and 6 (Ec and Sr) replicates per condition). Blank cells indicate that no growth occurred.

NaCl:NaBr (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	28.24 ± 3.97	8.97 ± 2.97	-
1	90.08 ± 11.06	72.70 ± 2.27	-
1.5	-	136.88 ± 24.88	-
2	-	-	114.59 ± 26.39
2.5	-	-	93.32 ± 22.44
3	-	-	107.97 ± 21.32
3.5	-	-	98.57 ± 10.97
4	-	-	130.95 ± 16.83
4.5	-	-	123.43 ± 18.42
5	-	-	152.99 ± 10.37
5.5	-	-	220.28 ± 58.11

4.2.3: Growth in the presence of potassium

E. coli, *E. vietnamensis* and *S. ruber* were grown in media containing a range (0M – 4.5M) of concentrations of KCl, KBr and equimolar KCl:KBr, shown in Figures 4.11 – 4.13 (specific growth rates) and Tables 4.7 – 4.9 (doubling times). The molar solubility of KCl meant that concentrations above 4.5M could not be used for KCl containing media (in comparison, NaCl concentrations of up to 5.5M were used).

E. coli was able to grow in as high as 1.5M KCl, although growth is significantly affected by increasing KCl concentration, but considerably less so than in NaCl. Growth is lower in the presence of KBr and it cannot tolerate as high concentrations of equimolar KCl:KBr as either of the salts individually. KCl proves stimulatory towards *E. vietnamensis* as its growth rate at 0.5 – 1.5M KCl is higher than at 0M. KBr is more growth inhibitory towards *E. vietnamensis* than

KCl, and the equimolar combination proves the most inhibitory. *S. ruber* could not grow in the presence of K^+ containing media.

S. ruber growth is inhibited in KCl

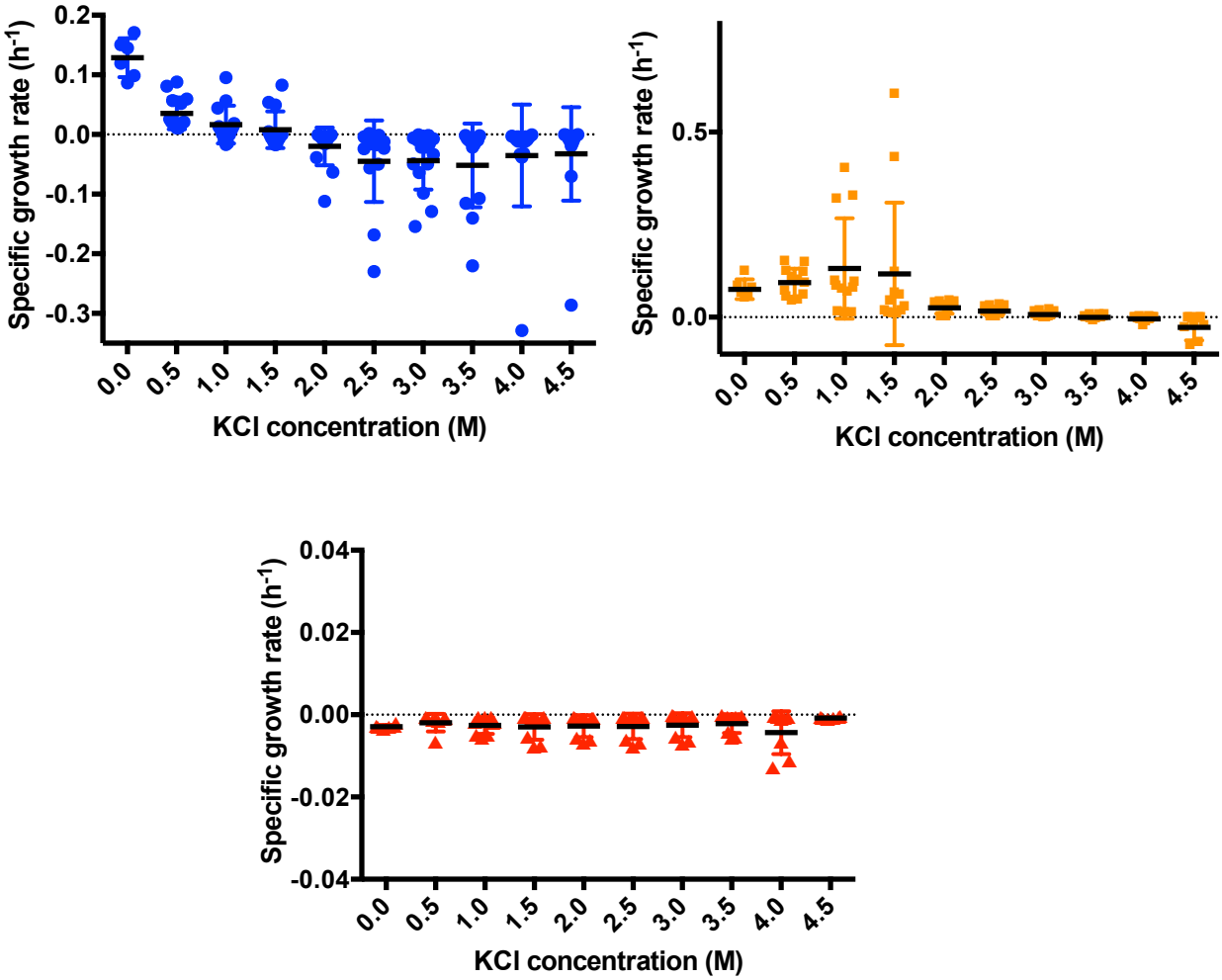

Figure 4.11. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of KCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (top left – blue circles), *E. vietnamensis* (top right – orange squares) and S. *ruber* (bottom – red triangles) grown in media containing a range of KCl concentrations (x axis). Error bars indicate the standard deviation between individual experiments (12 replicates). Variance between organisms analysed via ANOVA: F (2, 280) = 22.72, $p < 0.0001$.

Table 4.7. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of KCl concentrations. Values refer to the doubling time (hours) of the organism at a given KCl concentration, with the SEM of this average value shown (12 replicates per condition). Blank cells indicate that no growth occurred.

KCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	8.85 ± 1.06	7.51 ± 0.89	-
1	41.71 ± 12.05	5.84 ± 1.15	-
1.5	86.54 ± 1.72	6.20 ± 2.22	-
2	-	21.66 ± 2.01	-
2.5	-	30.77 ± 4.39	-
3	-	60.91 ± 11.42	-
3.5	-	-	-
4	-	-	-
4.5	-	-	-

Bromide is more growth-inhibitory than chloride

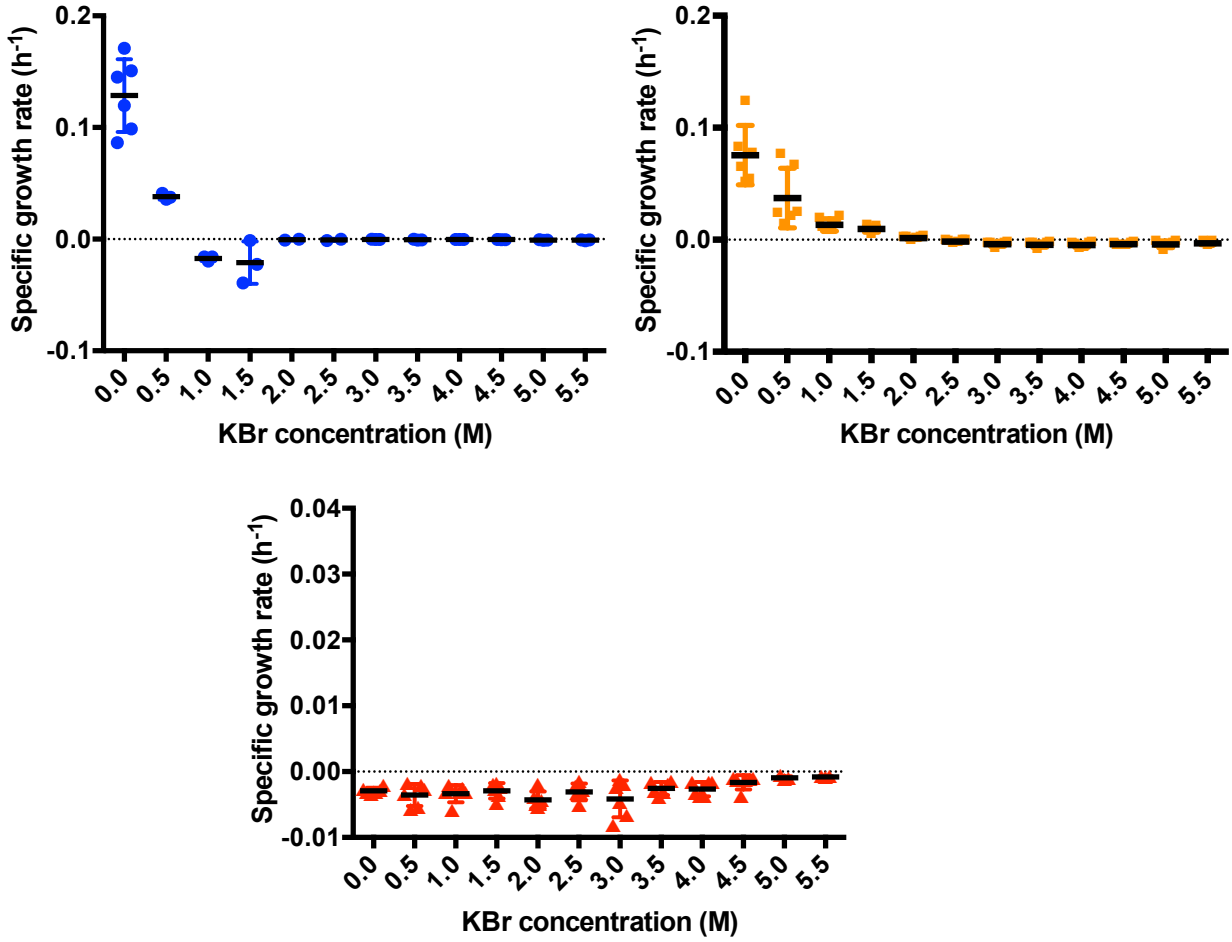

Figure 4.12. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of KBr concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (top left – blue circles), *E. vietnamensis* (top right – orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of KBr concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3 – 6 replicates). Variance between organisms analysed via ANOVA: F (2, 122) = 15.73, $p < 0.0001$.

Table 4.8. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of KBr concentrations. Values refer to the doubling time (hours) of the organism at a given KBr concentration, with the SEM of this average value shown (3 (Ec) and 6 (Ev/Sr) replicates per condition). Blank cells indicate that no growth occurred.

KBr (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	18.19 ± 0.73	19.39 ± 5.56	-
1	-	54.82 ± 9.90	-
1.5	-	64.59 ± 4.50	-
2	-	-	-
2.5	-	-	-
3	-	-	-
3.5	-	-	-
4	-	-	-
4.5	-	-	-
5	-	-	-
5.5	-	-	-

The presence of chloride and bromide together inhibits bacterial growth

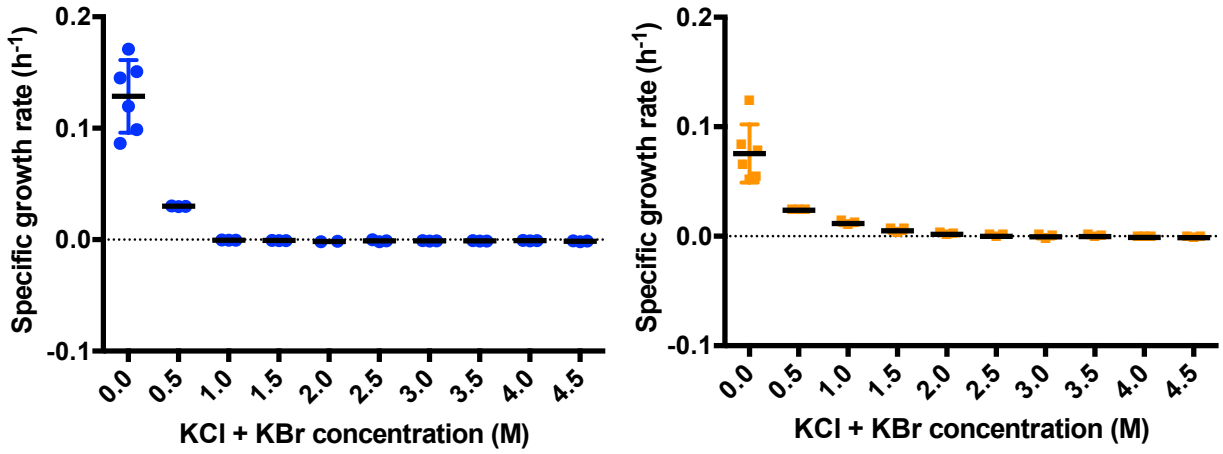

Figure 4.13. *E. coli* and *E. vietnamensis* growth in media containing a range of equimolar KCl:KBr concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles) and *E. vietnamensis* (right - orange squares) grown in media containing a range of KCl:KBr concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3 replicates). Variance between organisms analysed via ANOVA: F (1, 23) = 215.6, $p < 0.0001$.

Table 4.9. Doubling times (hours) of *E. coli* and *E. vietnamensis* when grown in media containing a range of equimolar KCl:KBr concentrations. Values refer to the doubling time (hours) of the organism at a given KCl:KBr concentration, with the SEM of this average value shown (3 replicates per condition). Blank cells indicate that no growth occurred.

KCl:KBr (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	23.04 ± 0.15	23.13 ± 0.21	-
1	-	59.95 ± 5.02	-
1.5	-	163.90 ± 48.79	-
2	-	367.09 ± 125.07	-
2.5	-	-	-
3	-	-	-
3.5	-	-	-
4	-	-	-
4.5	-	-	-

4.2.4: Growth in the presence of sodium and potassium

E. coli, *E. vietnamensis* and *S. ruber* were grown in a range of media containing equimolar combinations of NaCl, NaBr, KCl and KBr, which are shown in Figures 4.14 – 4.17 (specific growth rates) and Tables 4.10 – 4.13 (doubling times).

E. coli was able to tolerate up to 1M equimolar NaCl:KCl and NaBr:KBr, with its lowest growth rates determined to be in the presence of equimolar KCl:NaBr. *E. vietnamensis* has superior growth in equimolar NaCl:KCl than in 0M, but has low growth in both equimolar NaCl:KBr and KCl:NaBr, and growth is better in NaBr:KBr than in both of these.

S. ruber is able to grow when both sodium and potassium are present, but growth is greater when only sodium is present in the medium. However, it could not grow in equimolar NaBr:KBr and growth is particularly low in equimolar NaCl:KBr and KCl:NaBr.

K^+ presence may reduce *S. ruber* growth rate

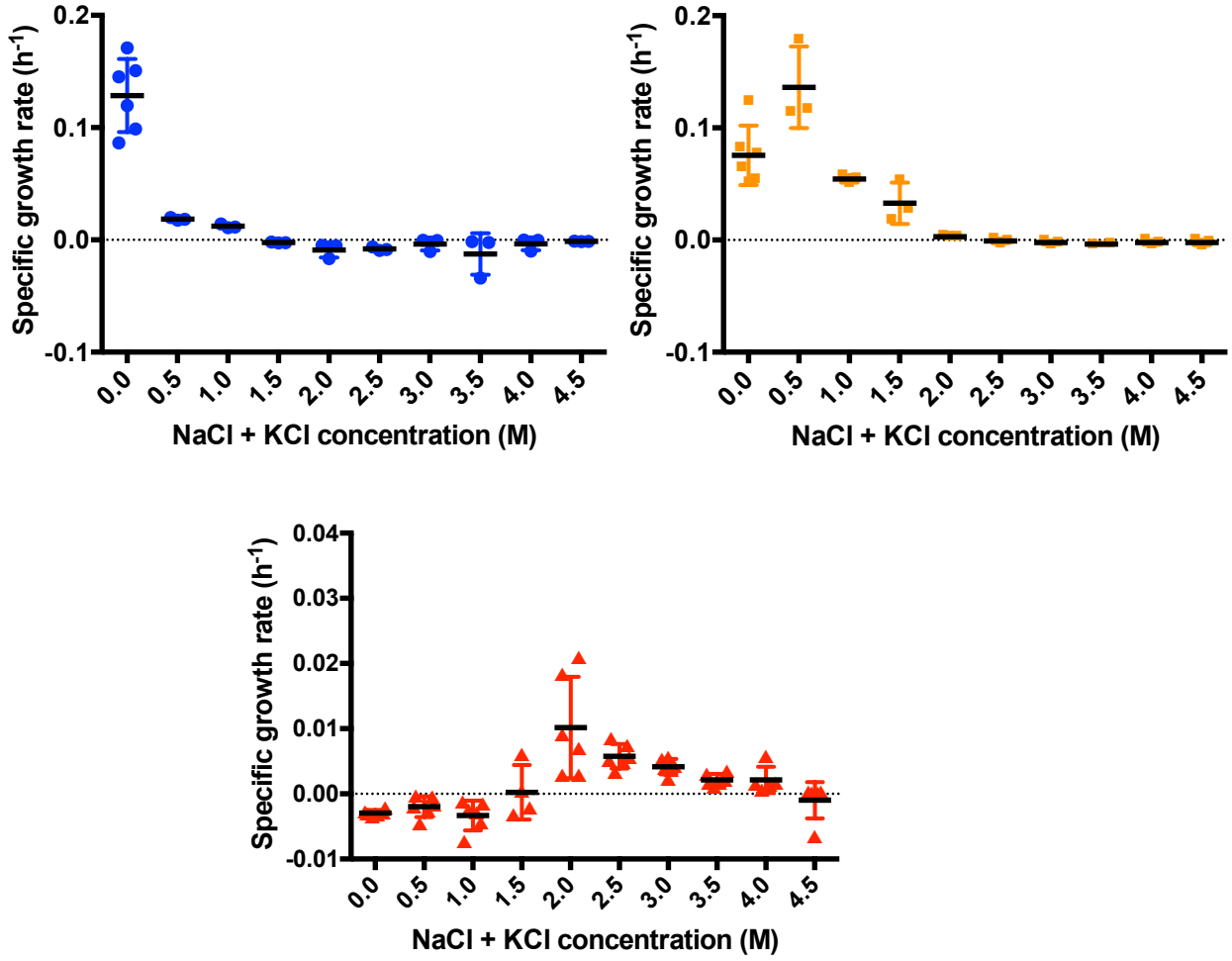

Figure 4.14. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar NaCl:KCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (top left – blue circles), *E. vietnamensis* (top right – orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of NaCl:KCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3-6 replicates). Variance between organisms analysed via ANOVA: F (2, 77) = 11.99, $p < 0.0001$.

Table 4.10. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of NaCl:KCl concentrations. Values refer to the doubling time (hours) of the organism at a given NaCl:KCl concentration, with the SEM of this average value shown (3 (Ec/Ev) and 6 (Sr) replicates per condition). Blank cells indicate that no growth occurred.

NaCl:KCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	37.28 ± 1.41	5.28 ± 0.71	-
1	57.27 ± 4.42	12.73 ± 0.45	-
1.5	-	25.75 ± 7.52	-
2	-	237.13 ± 16.52	61.17 ± 15.72
2.5	-	-	108.23 ± 12.37
3	-	-	156.25 ± 12.59
3.5	-	-	320.28 ± 47.26
4	-	-	337.67 ± 108.89
4.5	-	-	-

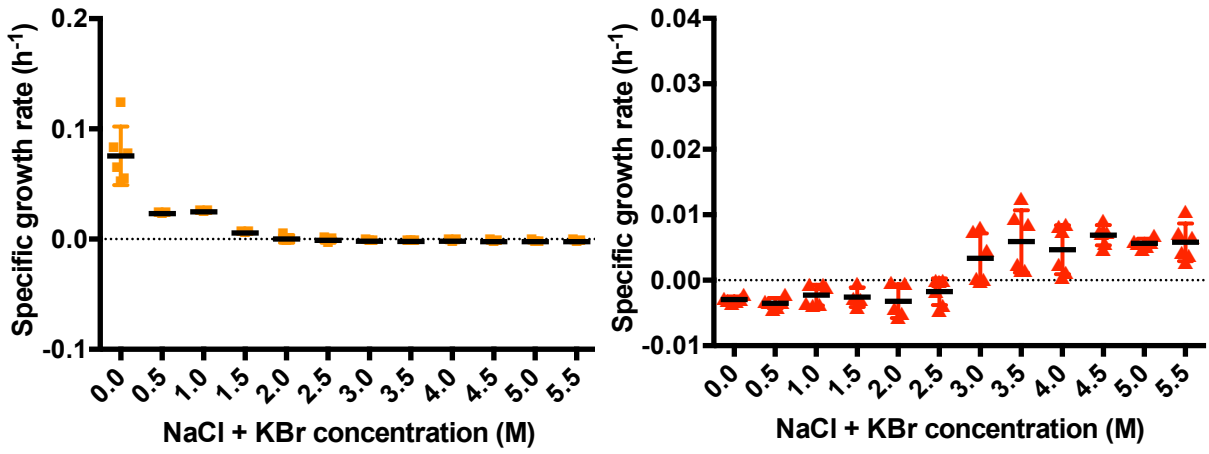

Figure 4.15. *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar NaCl:KBr concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. vietnamensis* (left - orange squares) and *S. ruber* (right – red triangles) grown in media containing a range of NaCl:KBr concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3-6 replicates). Variance between organisms analysed via ANOVA: $F(1, 74) = 31.31$, $p < 0.0001$.

Table 4.11. Doubling times (hours) of *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar NaCl:KBr concentrations. Values refer to the doubling time (hours) of the organism at a given NaCl:KBr concentration, with the SEM of this average value shown (3 (Ev) and 6 (Sr) replicates per condition). Blank cells indicate that no growth occurred.

NaCl:KBr (M)	*E. vietnamensis*	*S. ruber*
0	9.18 ± 1.21	-
0.5	29.84 ± 0.38	-
1	27.82 ± 0.46	-
1.5	127.13 ± 7.32	-
2	-	-
2.5	-	-
3	-	207.21 ± 21.99
3.5	-	126.74 ± 56.82
4	-	136.54 ± 50.15
4.5	-	104.85 ± 10.40
5	-	124.03 ± 6.42
5.5	-	123.52 ± 22.05

The presence of Cl^- + Br^- may be growth-inhibitory

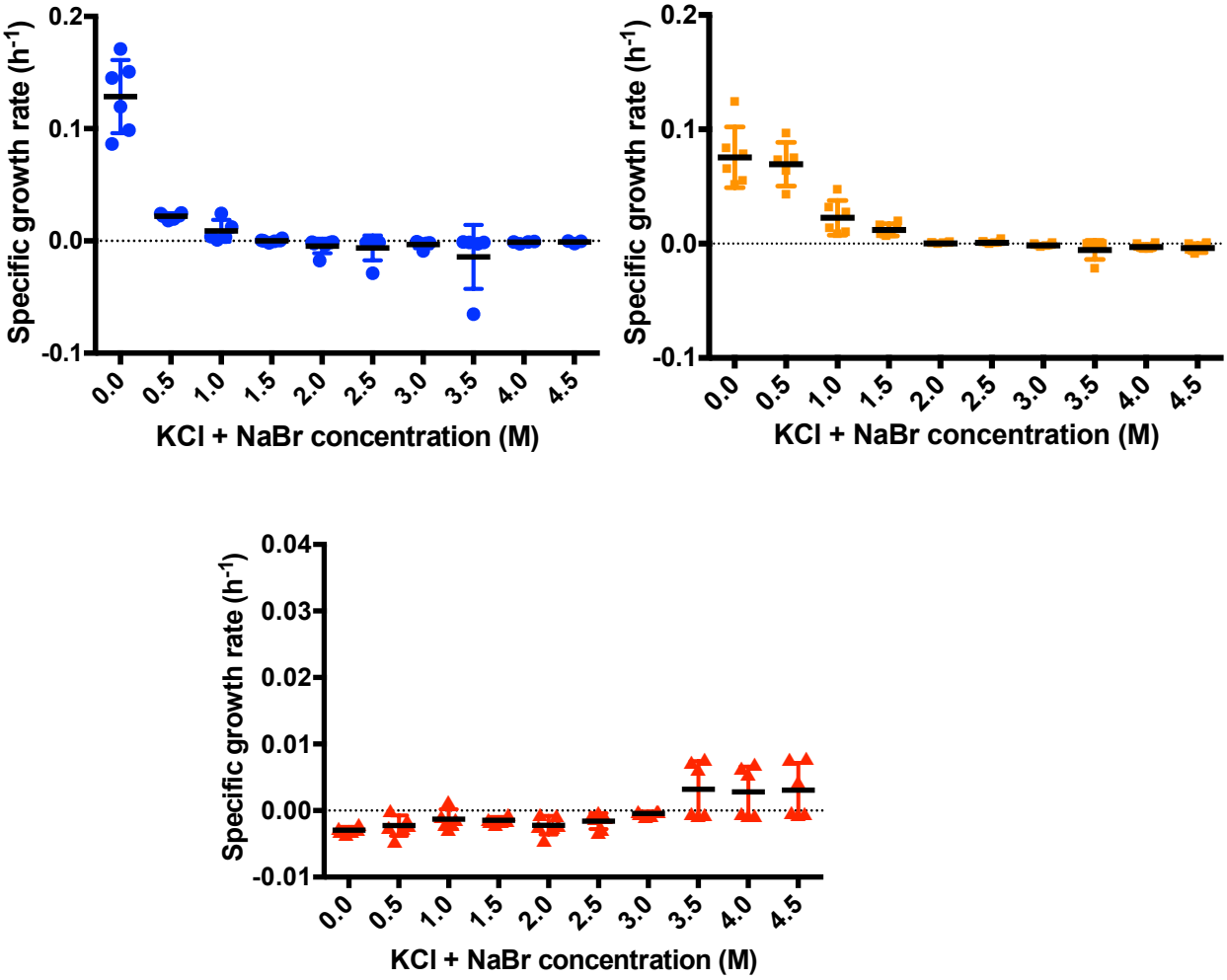

Figure 4.16. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar KCl:NaBr concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (top left – blue circles), *E. vietnamensis* (top right – orange squares) and *S. ruber* (bottom – red triangles) grown in medium containing a range of KCl:NaBr concentrations (x axis). Error bars indicate the standard deviation of individual replicates (6 replicates). Variance between organisms analysed via ANOVA: F (2, 126) = 8.198, p = 0.0004.

Table 4.12. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar KCl:NaBr concentrations. Values refer to the doubling time (hours) of the organism at a given KCl:NaBr concentration, with the SEM of this average value shown (6 replicates per condition). Blank cells indicate that no growth occurred.

KCl:NaBr(M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	31.84 ± 1.66	9.27 ± 0.77	-
1	-	28.69 ± 8.26	-
1.5	-	52.72 ± 10.98	-
2	-	-	-
2.5	-	-	-
3	-	-	-
3.5	-	-	98.97 ± 6.49
4	-	-	111.57 ± 7.49
4.5	-	-	112.28 ± 23.38

S. ruber may require Cl^- for growth when K^+ is present

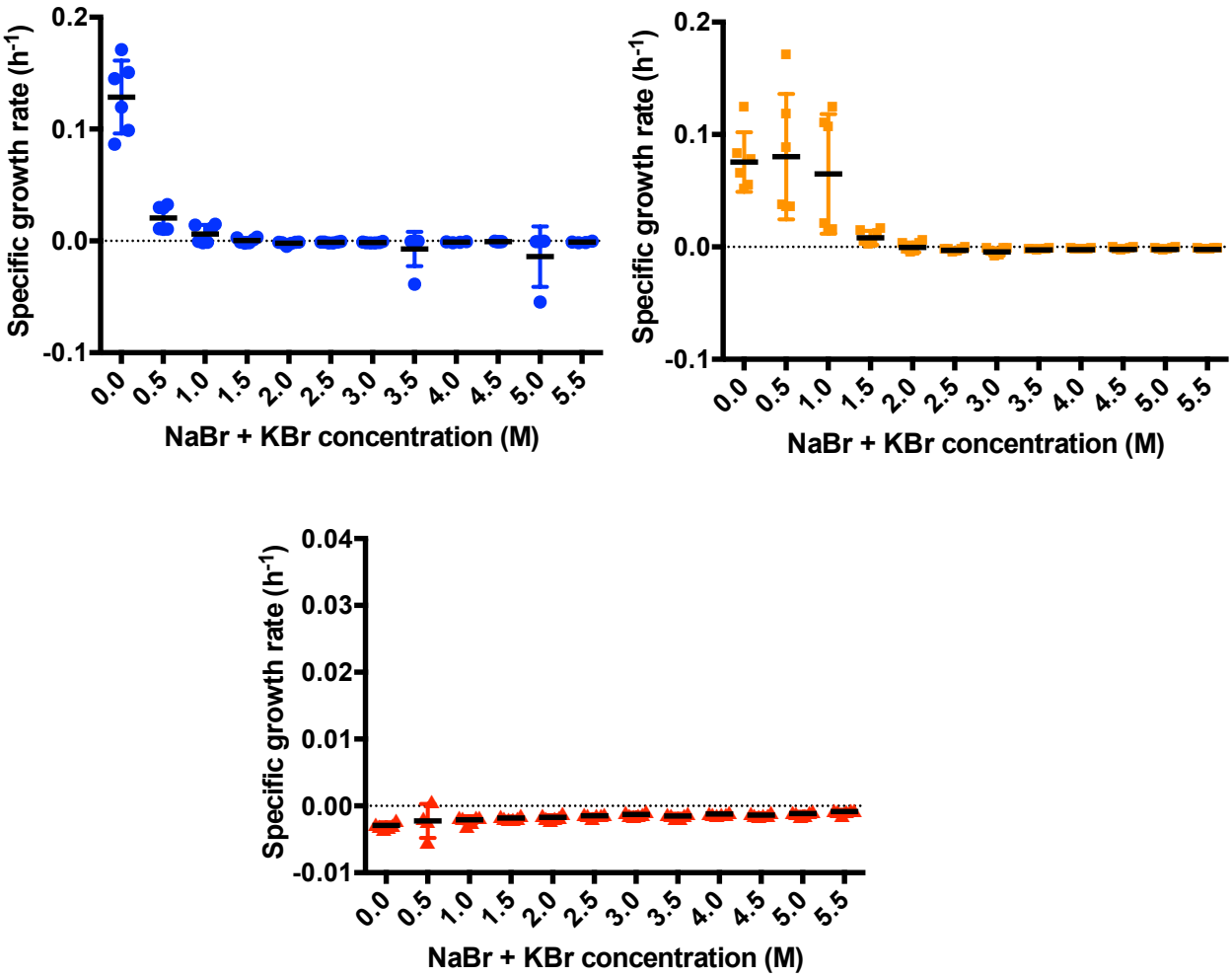

Figure 4.17. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar NaBr:KBr concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in medium containing a range of NaBr:KBr concentrations (x axis). Error bars indicate the standard deviation of individual experiments (6 replicates). Variance between organisms analysed via ANOVA: F (2, 156) = 19.70, $p < 0.0001$.

Table 4.13. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar NaBr:KBr concentrations. Values refer to the doubling time (hours) of the organism at a given NaBr:KBr concentration, with the SEM of this average value shown (6 replicates per condition). Blank cells indicate that no growth occurred.

NaBr:KBr(M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	22.64 ± 0.68	8.63 ± 3.34	-
1	51.51 ± 4.09	10.68 ± 0.284	-
1.5	-	83.76 ± 31.75	-
2	-	-	-
2.5	-	-	-
3	-	-	-
3.5	-	-	-
4	-	-	-
4.5	-	-	-
5	-	-	-
5.5	-	-	-

4.2.5: Growth in presence of the alternative alkali cation lithium

In order to investigate the effects that lithium has on bacterial growth, *E. coli*, *E. vietnamensis* and *S. ruber* were grown in the presence of a range (0M – 4.5M) of LiCl concentrations, as well as equimolar combinations of LiCl + NaCl/KCl/NaBr/KBr. These are shown in Figures 4.18 – 4.22 (specific growth rates) and Tables 4.14 – 4.18 (doubling times).

E. coli was able to tolerate up to 1.5M LiCl (although the doubling time here was 204 hours). This organism is also less tolerant of a KCl:LiCl combination than either of these salts individually. In addition, it is able to tolerate up to 1M equimolar LiCl:NaCl, but struggles to grow at even 0.5M equimolar NaBr:LiCl and does not grow at all in equimolar LiCl:KBr. This is also the case for *E. vietnamensis*, as it cannot grow at all in the presence of equimolar LiCl:KBr and also only grows in up to 1M NaBr:LiCl. *E. vietnamensis* growth in LiCl is better

than in the aforementioned combinations but is still lower than in all previously tested salts, however growth improved in equimolar NaCl:LiCl and KCl:LiCl.

S. ruber cannot grow in the presence of pure LiCl, but can grow in equimolar NaCl:LiCl, NaBr:LiCl as well as LiCl:KCl.

Li^+ is not an effective Na^+ or K^+ replacement

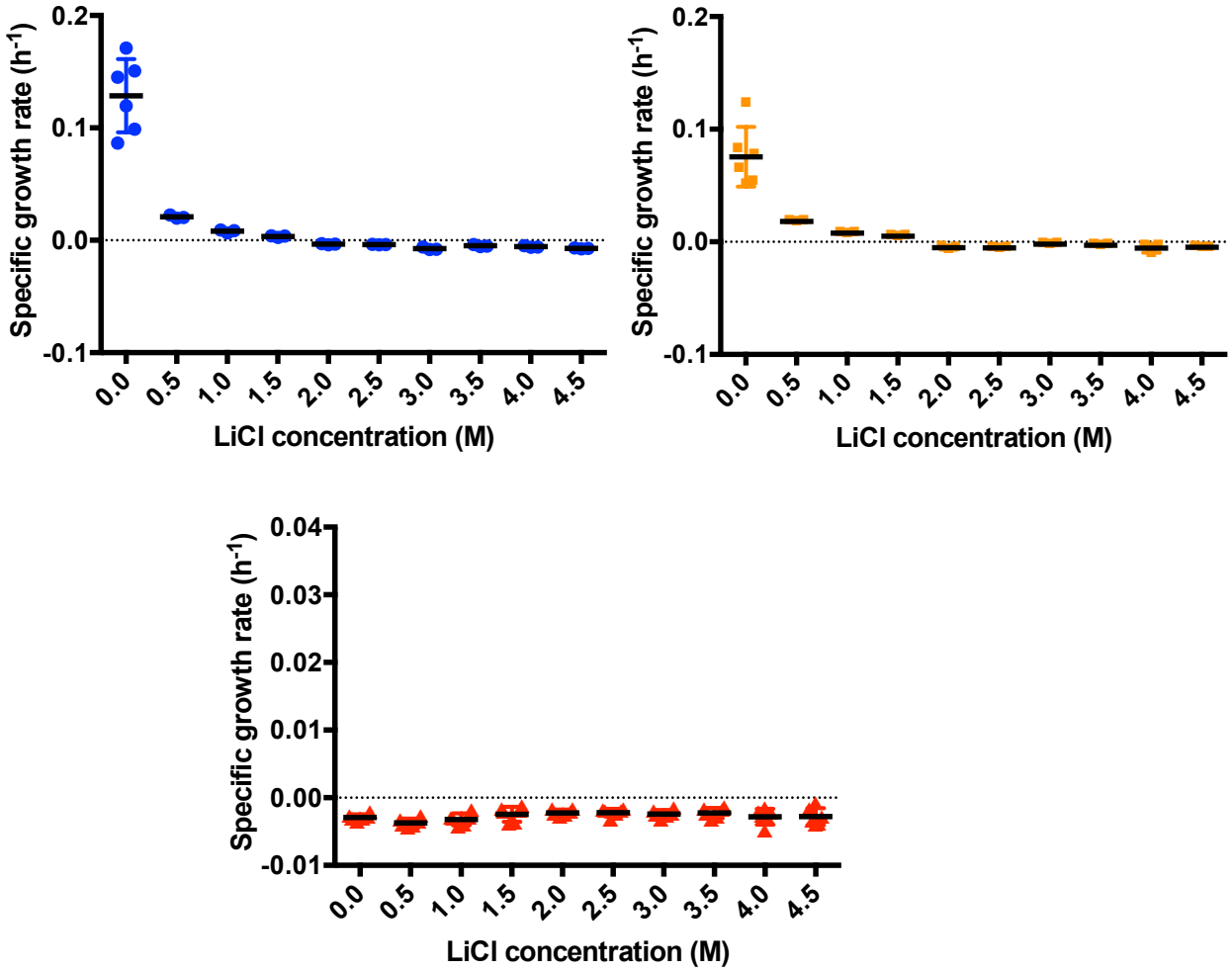

Figure 4.18. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of LiCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in medium containing a range of LiCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3-6 replicates). Variance between organisms analysed via ANOVA: F (2, 54) = 1116, p <0.0001.

Table 4.14. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of LiCl concentrations. Values refer to the doubling time (hours) of the organism at a given LiCl concentration, with the SEM of this average value shown (3 (Ec/Ev) and 6(Sr) replicates per condition). Blank cells indicate that no growth occurred.

LiCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	33.18± 1.36	37.95 ± 0.53	-
1	87.11 ± 9.42	88.82 ± 2.25	-
1.5	210.33 ± 28.35	135.83 ± 3.78	-
2	-	-	-
2.5	-	-	-
3	-	-	-
3.5	-	-	-
4	-	-	-
4.5	-	-	-

Na^+ may reduce growth inhibition caused by Li^+

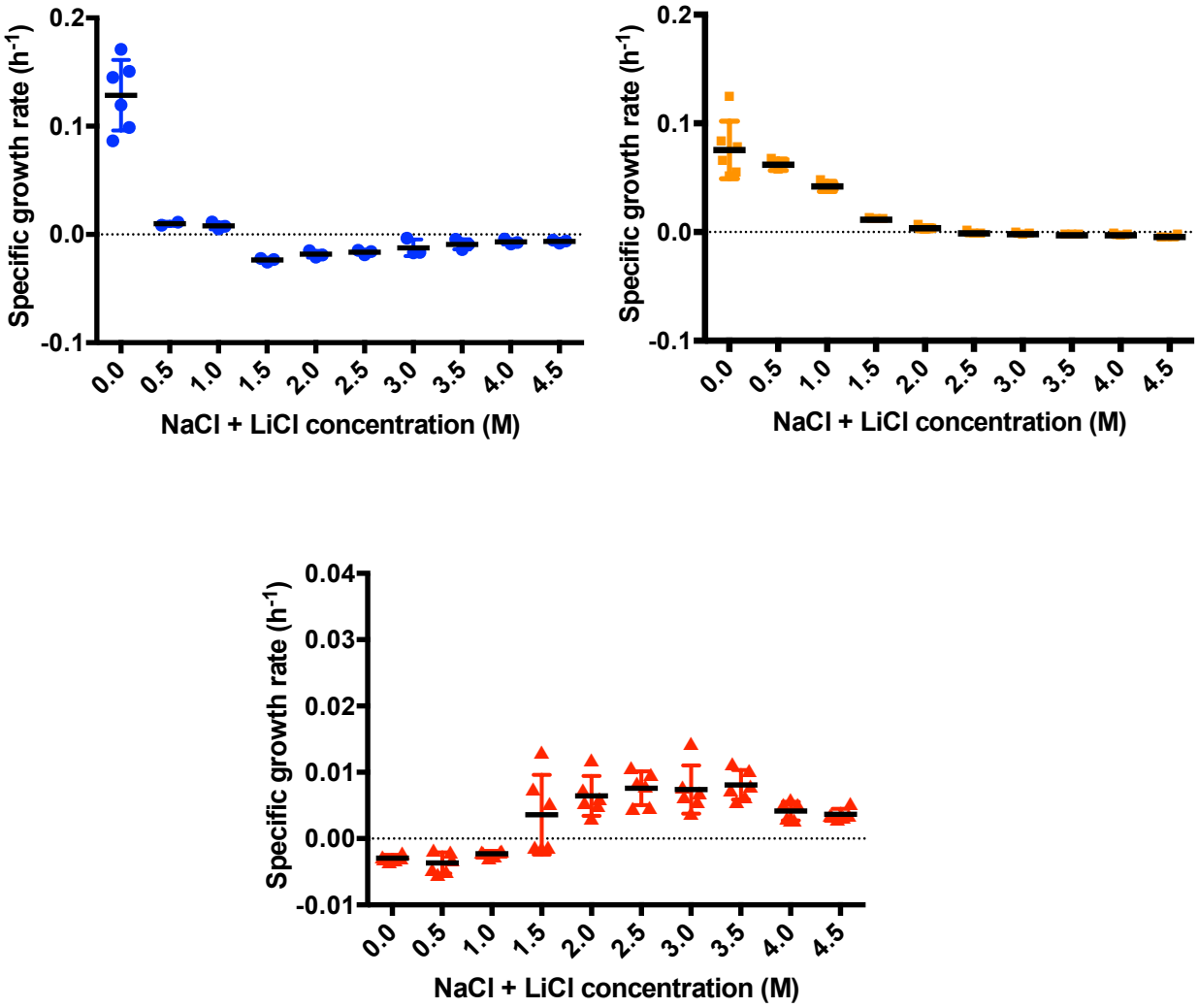

Figure 4.19. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar NaCl:LiCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of NaCl:LiCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3-6 replicates). Variance between organisms analysed via ANOVA: F (2, 79) = 50.15, $p < 0.0001$.

Table 4.15. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar NaCl:LiCl concentrations. Values refer to the doubling time (hours) of the organism at a given NaCl:LiCl concentration, with the SEM of this average value shown (3 (Ec/Ev) and 6(Sr) replicates per condition). Blank cells indicate that no growth occurred.

NaCl:LiCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	71.48 ± 10.09	11.18 ± 0.56	-
1	76.00 ± 15.73	16.58 ± 1.04	-
1.5	-	60.68 ± 2.13	173.40 ± 22.42
2	-	199.38 ± 87.04	106.36 ± 14.11
2.5	-	-	90.99 ± 14.80
3	-	-	94.85 ± 13.02
3.5	-	-	83.93 ± 8.76
4	-	-	170.48 ± 26.12
4.5	-	-	194.81 ± 14.17

Li^+ + Br^+ may be partially toxic

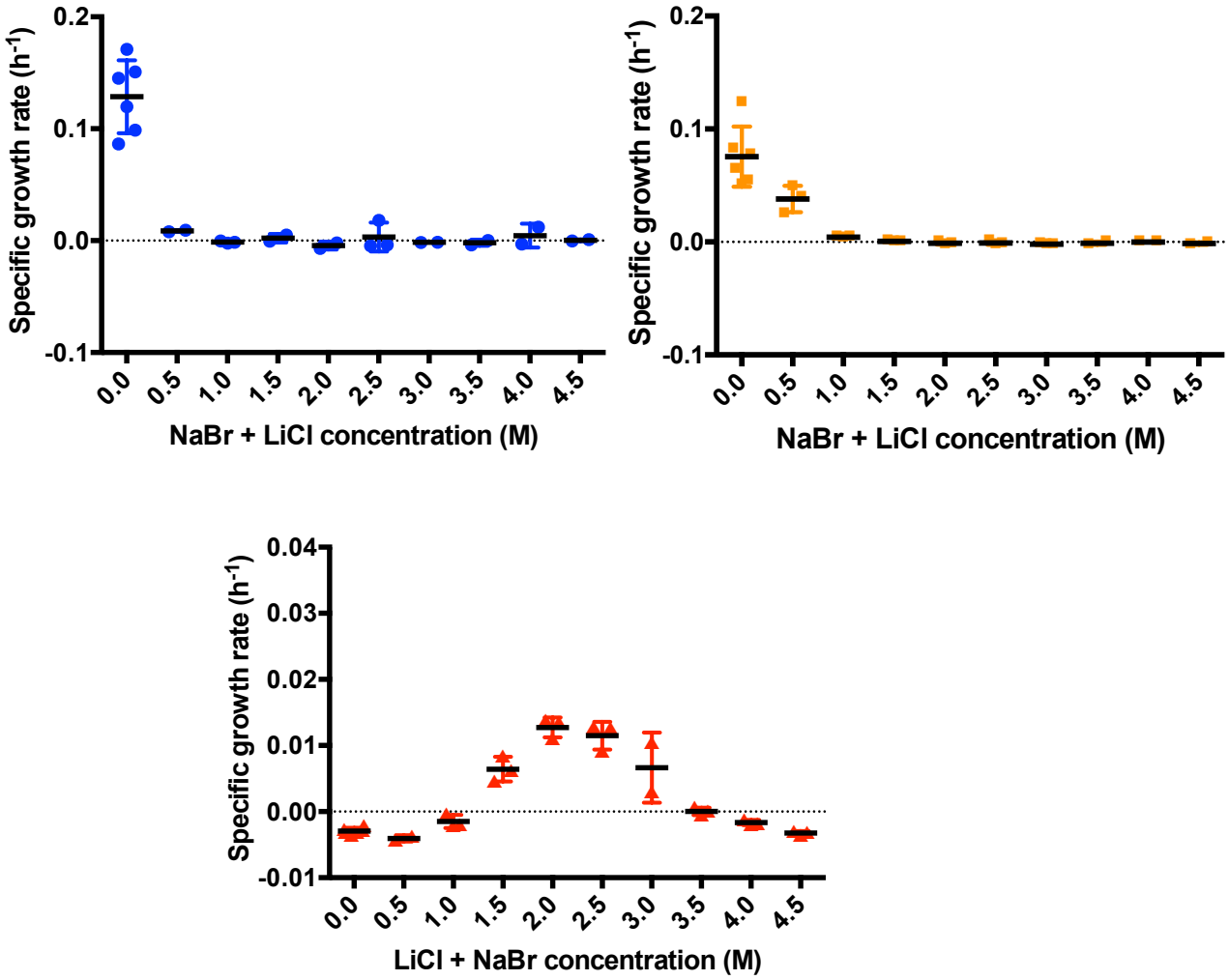

Figure 4.20. ***E. coli*, *E. vietnamensis*** **and** ***S. ruber*** **growth in media containing a range of equimolar NaBr:LiCl concentrations.** Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of NaBr:LiCl concentrations (x axis). Error bars indicate the standard deviation of individual replicates (3 replicates). Variance between organisms analysed via ANOVA: F (2, 42) = 79.27, $p < 0.0001$.

Table 4.16. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar NaBr:LiCl concentrations. Values refer to the doubling time (hours) of the organism at a given NaBr:LiCl concentration, with the SEM of this average value shown (3 replicates per condition). Blank cells indicate that no growth occurred.

NaBr:LiCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	80.52 ± 5.72	19.53 ± 3.85	-
1	-	168.95 ± 11.79	-
1.5	-	-	113.76 ± 19.13
2	-	-	54.72 ± 3.96
2.5	-	-	61.57 ± 7.24
3	-	-	151.69 ± 85.34
3.5	-	-	-
4	-	-	-
4.5	-	-	-

***S. ruber* may not obligately require Na^+ - but growth is hindered significantly in its absence**

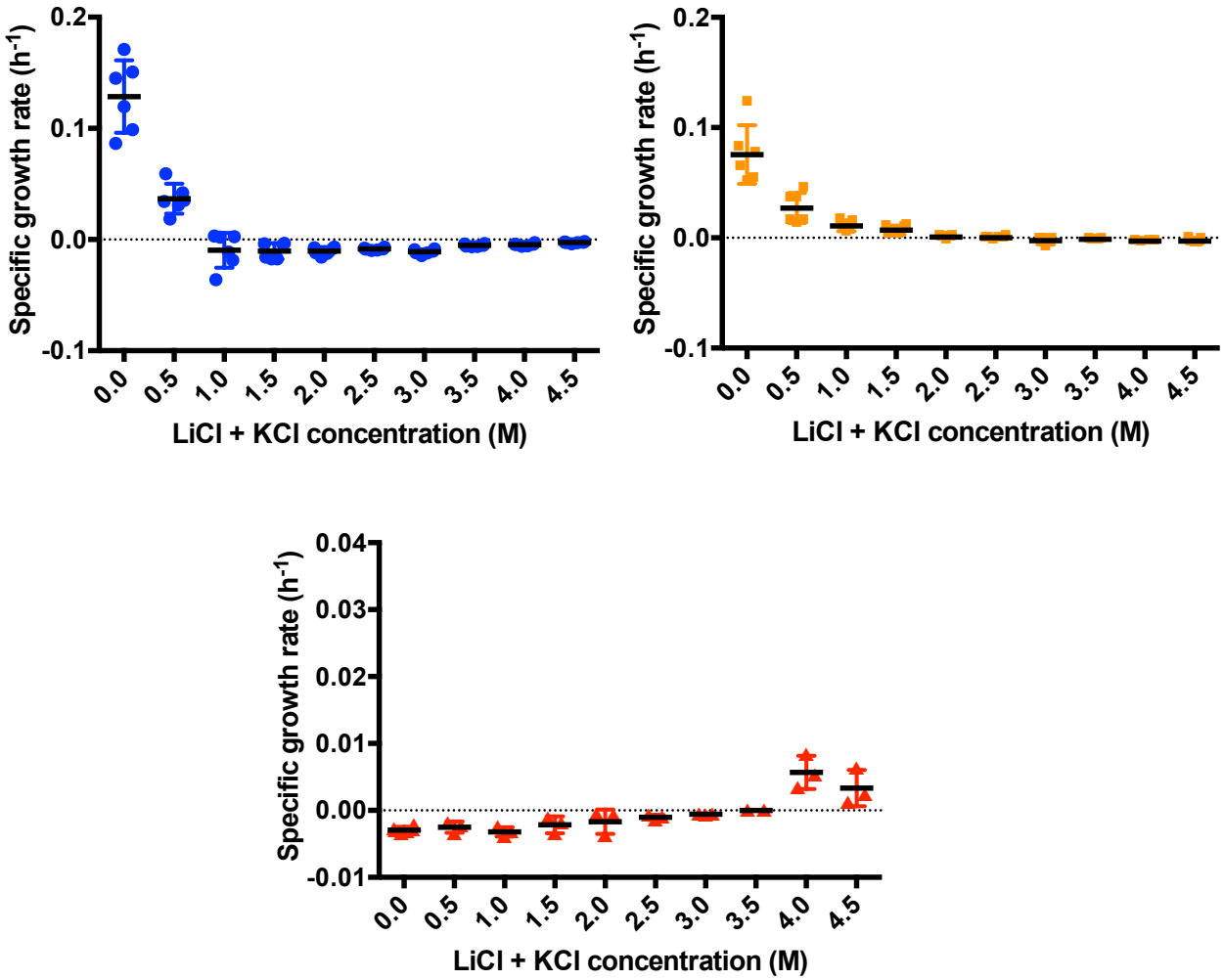

Figure 4.21. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar KCl:LiCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in medium containing a range of KCl:LiCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3-6 replicates). Variance between organisms analysed via ANOVA: F (2, 100) = 15.80, $p < 0.0001$.

Table 4.17. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar KCl:LiCl concentrations. Values refer to the doubling time (hours) of the organism at a given KCl:LiCl concentration, with the SEM of this average value shown (6 (Ec/Ev) and 3 (Sr) replicates per condition). Blank cells indicate that no growth occurred.

KCl:LiCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	18.01 ± 1.84	24.06 ± 6.22	-
1	-	64.31 ± 13.21	-
1.5	-	98.86 ± 33.17	-
2	-	-	-
2.5	-	-	-
3	-	-	-
3.5	-	-	-
4	-	-	106.41 ± 23.71
4.5	-	-	199.20 ± 90.96

LiCl and KBr together may be toxic to bacteria in general

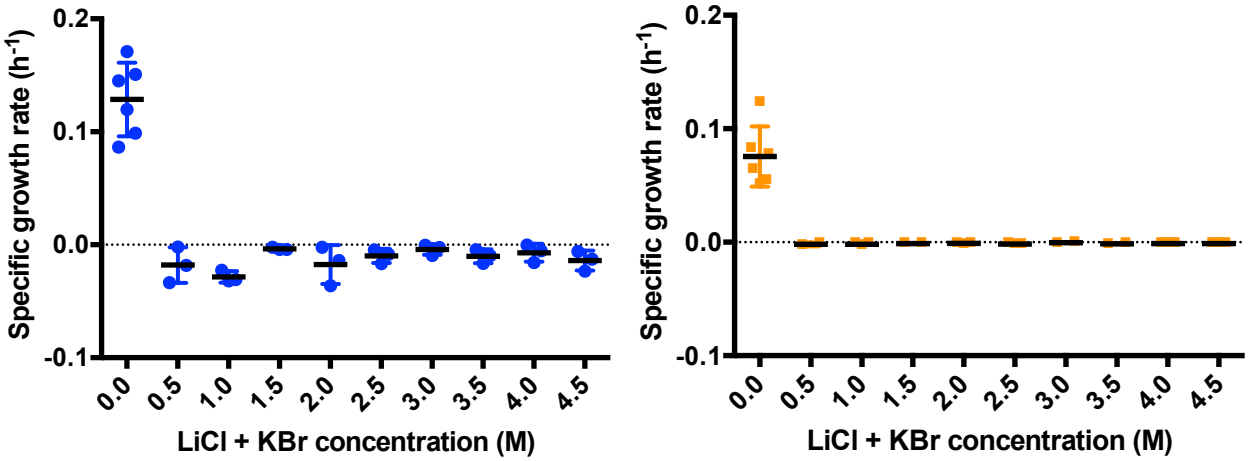

Figure 4.22. *E. coli* and *E. vietnamensis* growth in media containing a range of equimolar KBr:LiCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles) and *E. vietnamensis* (right - orange squares) grown in media containing a range of KBr:LiCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3 replicates). Variance between organisms was analysed via ANOVA, which found that variances were not statistically significant.

Table 4.18. Doubling times (hours) of *E. coli* and *E. vietnamensis* when grown in media containing a range of equimolar KBr:LiCl concentrations. Values refer to the doubling time (hours) of the organism at a given KBr:LiCl concentration, with the SEM of this average value shown (3 replicates per condition). Blank cells indicate that no growth occurred.

LiCl:KBr (M)	*E. coli*	*E. vietnamensis*
0	5.39 ± 0.63	9.19 ± 1.21
0.5	-	-
1	-	-
1.5	-	-
2	-	-
2.5	-	-
3	-	-
3.5	-	-
4	-	-
4.5	-	-

4.2.6: Bacterial tolerance of larger cations

To investigate the effects of Rb^+ and Cs^+ on bacterial growth, *E. coli*, *E. vietnamensis* and *S. ruber* were grown in the presence of a range of media containing RbCl, CsCl and various equimolar combinations of RbCl/CsCl + NaCl/KCl/LiCl. These are shown below in Figures 4.23 – 4.29 (specific growth rates) and Tables 4.19 – 4.25 (doubling times).

E. coli was not able to grow in the presence of either RbCl or CsCl, and growth also did not occur in any of the equimolar combinations of these, with the addition of other salts, except for very slight growth in the presence of equimolar KCl:CsCl. It may also appear as though growth occurred in equimolar NaCl:RbCl but only one *E. coli* replicate out of three showed any growth here, and this was only very slight.

E. vietnamensis was able to tolerate both Rb^+ and Cs^+ - tolerating up to 2.5M RbCl and 3M CsCl. Growth rates improved when NaCl was added to these salts but not when KCl was added.

S. ruber was not able to grow in pure RbCl or CsCl. However, growth could occur from 1.5M equimolar NaCl:RbCl, 1M LiCl:RbCl, and 1M NaCl:CsCl.

RbCl is toxic to *E. coli*, whereas *E. vietnamensis* can tolerate this larger cation

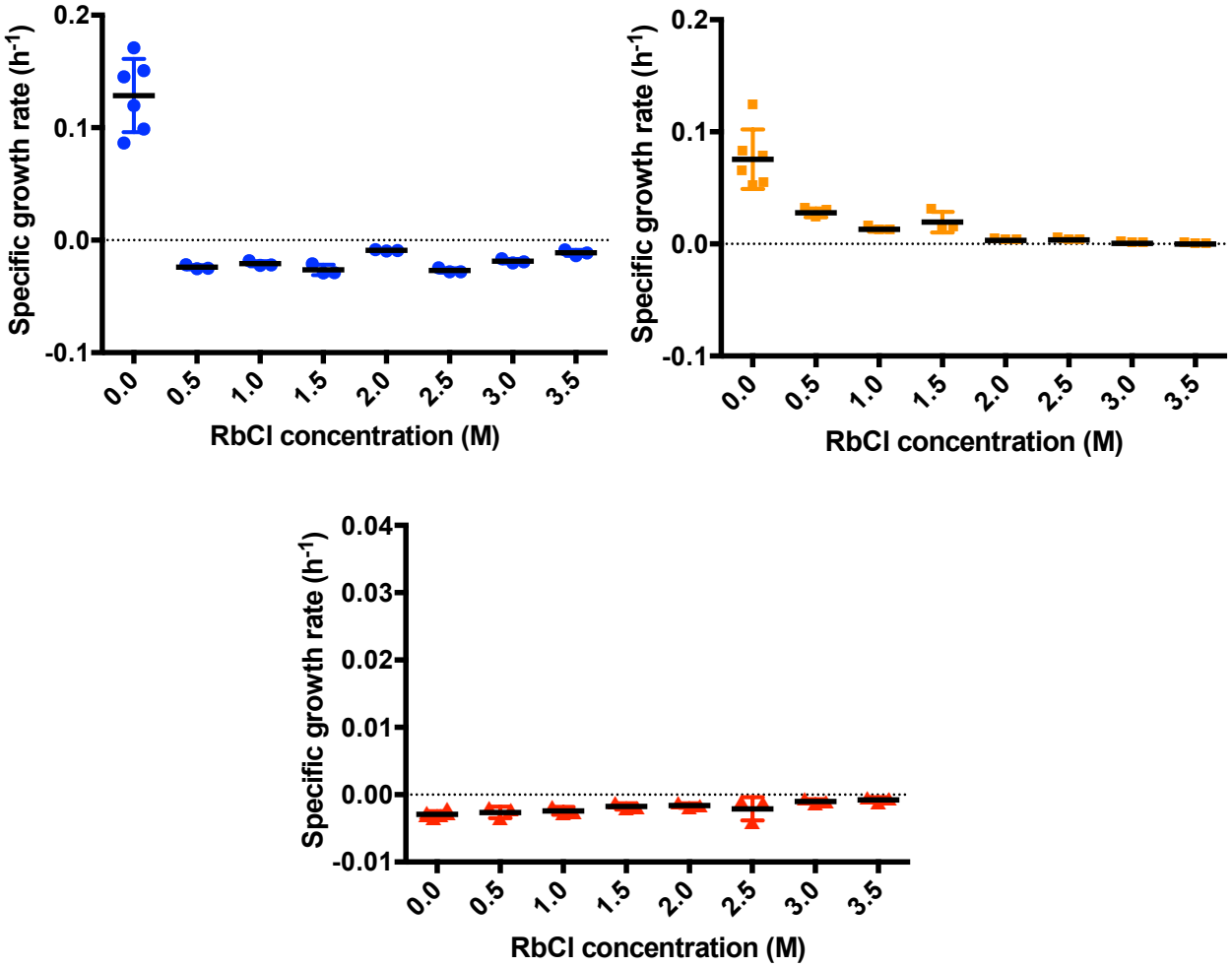

Figure 4.23. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of RbCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and S. *ruber* (bottom – red triangles) grown in medium containing RbCl of different concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3 replicates). Variance between organisms was analysed via ANOVA: F (2, 42) = 129.8, $p < 0.0001$.

Table 4.19. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in media containing a range of RbCl concentrations. Values refer to the doubling time (hours) of the organism at a given RbCl concentration, with the SEM of this average value shown (3 replicates per condition). Blank cells indicate that no growth occurred.

RbCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	-	25.22 ± 2.22	-
1	-	53.94 ± 4.70	-
1.5	-	40.33 ± 8.80	-
2	-	220.92 ± 21.69	-
2.5	-	202.74 ± 30.83	-
3	-	-	-
3.5	-	-	-

Rb^+ is an inhibitor of *E. coli* growth – whereas *E. vietnamensis* and *S. ruber* are not inhibited by its presence

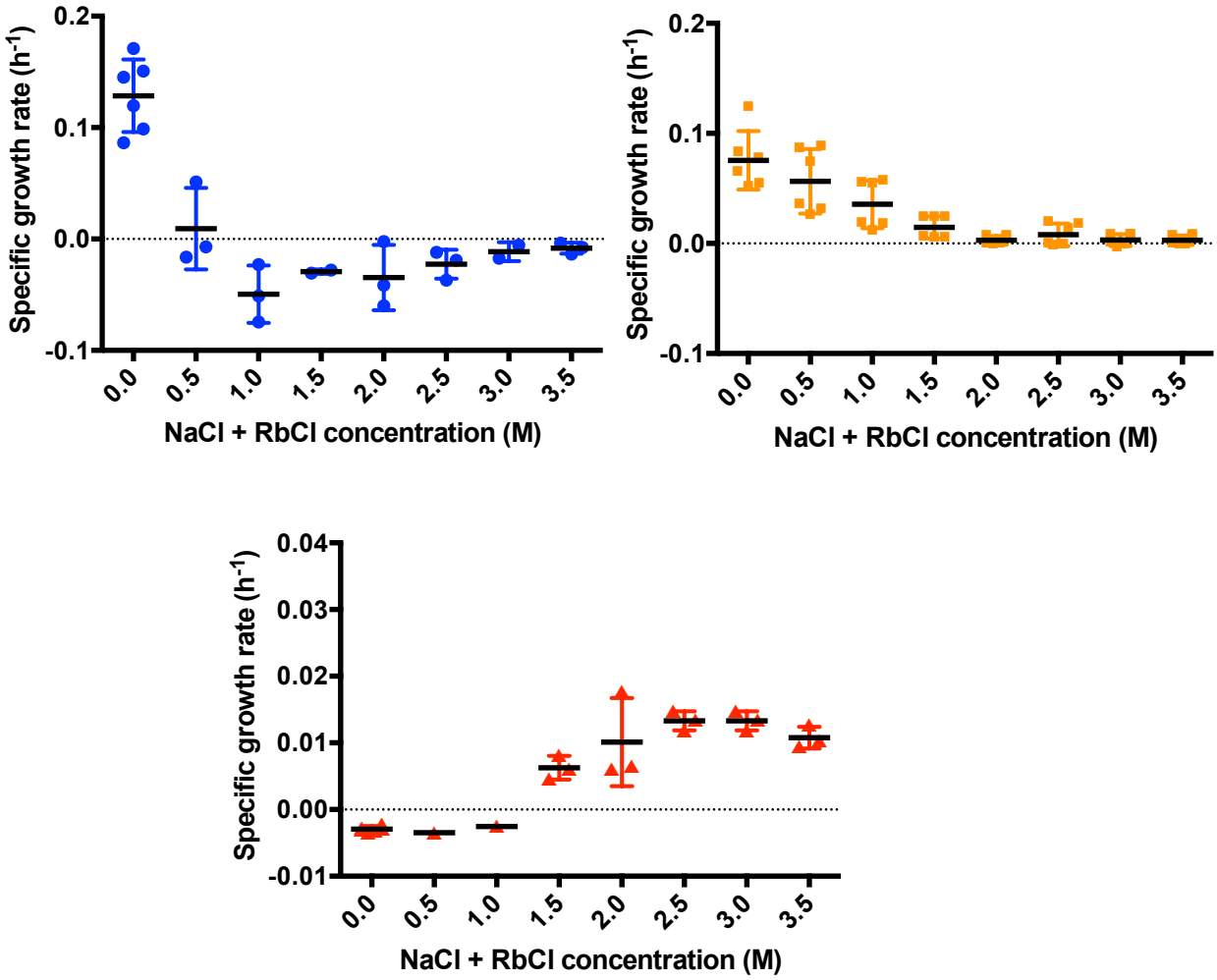

Figure 4.24. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar NaCl:RbCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of NaCl:RbCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3-6 replicates). Variance between the organisms was analysed via ANOVA: F (2, 57) = 33.96, p < 0.0001.

Table 4.20. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in media containing a range of equimolar NaCl:RbCl concentrations. Values refer to the doubling time (hours) of the organism at a given NaCl:RbCl concentration, with the SEM of this average value shown (3 (Ec/Sr) and 6 (Ev) replicates per condition). Blank cells indicate that no growth occurred.

NaCl:RbCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	74.81 ± N/A	13.47 ± 3.15	-
1	-	18.63 ± 6.15	-
1.5	-	50.08 ± 21.00	116.19 ± 18.79
2	-	106.81 ± 2.14	86.18 ± 23.76
2.5	-	41.74 ± 5.08	52.27 ± 3.34
3	-	89.70 ± 0.59	52.27 ± 3.34
3.5	-	103.46 ± 5.50	64.89 ± 5.36

***S. ruber* can grow in equimolar LiCl:RbCl, but not in either salt alone**

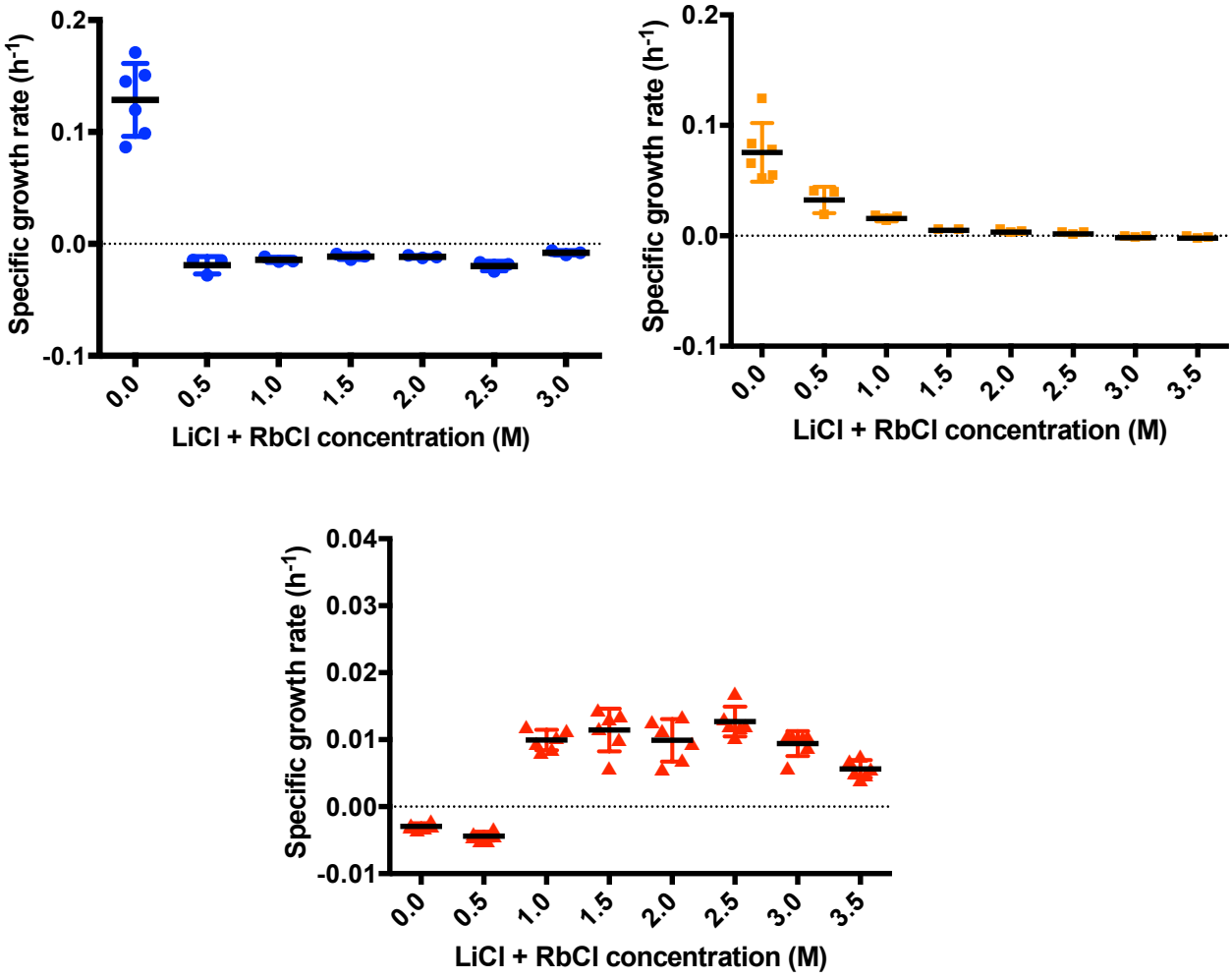

Figure 4.25. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar LiCl:RbCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of LiCl:RbCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3-6 replicates). Variance between organisms was analysed via ANOVA: $F(2, 68) = 60.36$, $p < 0.0001$.

Table 4.21. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in media containing a range of equimolar LiCl:RbCl concentrations. Values refer to the doubling time (hours) of the organism at a given LiCl:RbCl concentration, with the SEM of this average value shown (3 (Ec/Ev) and 6(Sr) replicates per condition). Blank cells indicate that no growth occurred.

LiCl:RbCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	-	24.02 ± 6.47	-
1	-	44.90 ± 4.60	70.70 ± 4.43
1.5	-	142.46 ± 10.23	66.50 ± 11.18
2	-	221.62 ± 42.04	77.22 ± 11.81
2.5	-	310.08 ± 39.81	55.53 ± 3.47
3	-	-	76.74 ± 8.53
3.5	-	-	127.90 ± 12.01

CsCl is toxic to *E. coli*, whereas *E. vietnamensis* can grow in the presence of the E. *coli* growth inhibitor Cs^+

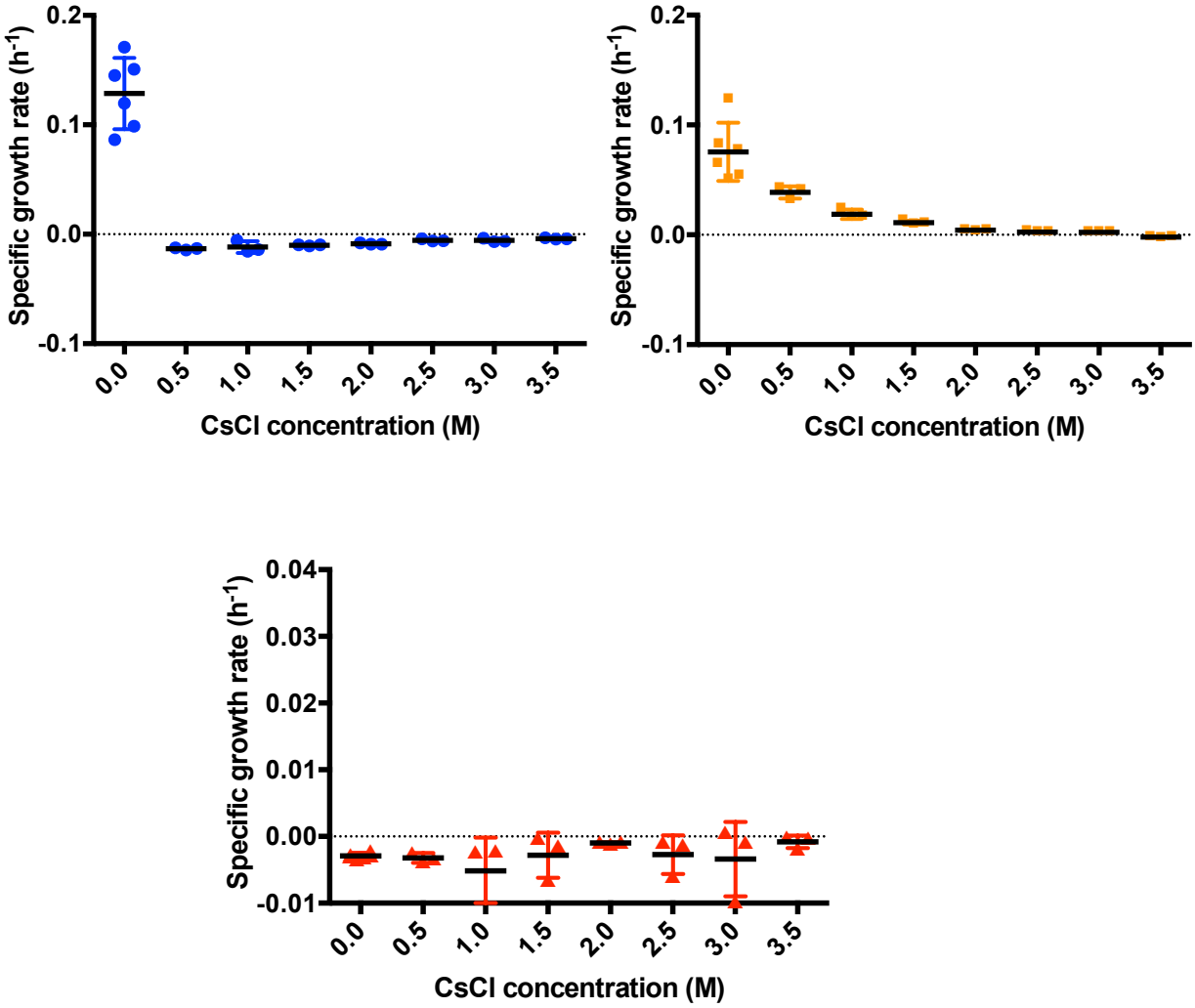

Figure 4.26. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of CsCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of CsCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3 replicates). Variance between organisms was analysed via ANOVA: $F(2, 42) = 339.5$, $p < 0.0001$.

Table 4.22. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in media containing a range of CsCl concentrations. Values refer to the doubling time (hours) of the organism at a given CsCl concentration, with the SEM of this average value shown (3 replicates per condition). Blank cells indicate that no growth occurred.

CsCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	-	18.16 ± 1.66	-
1	-	38.06 ± 4.46	-
1.5	-	63.24 ± 5.53	-
2	-	165.23 ± 10.56	-
2.5	-	277.42 ± 26.73	-
3	-	310.95 ± 8.00	-
3.5	-	-	-

Cs^+ is not toxic towards *E. vietnamensis* or *S. ruber*

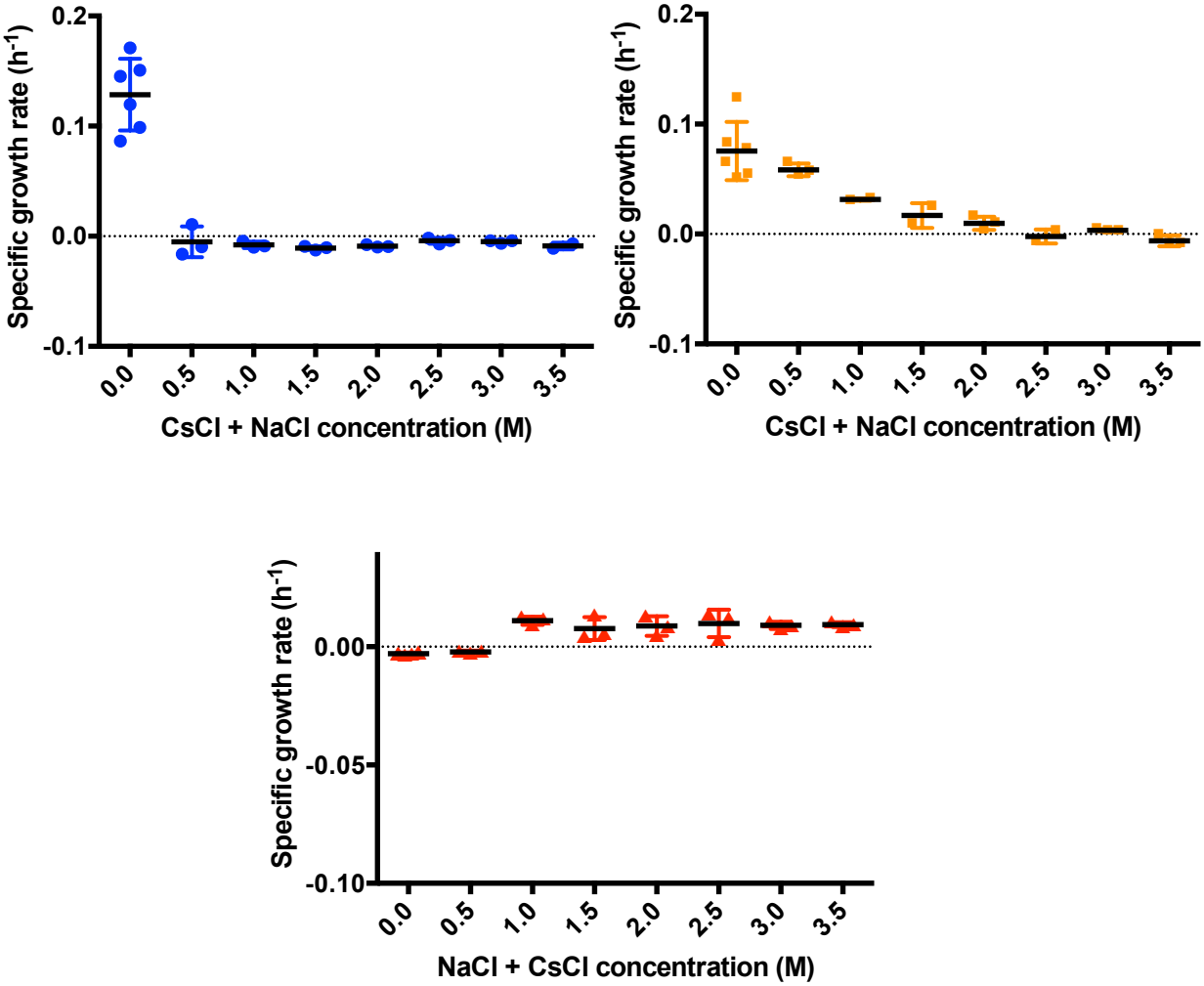

Figure 4.27. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar NaCl:CsCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of NaCl:CsCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3 replicates). Variance between organisms was analysed via ANOVA: F (2, 38) = 74.80, $p < 0.0001$.

Table 4.23. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in media containing a range of equimolar NaCl:CsCl concentrations. Values refer to the doubling time (hours) of the organism at a given NaCl:CsCl concentration, with the SEM of this average value shown (3 replicates per condition). Blank cells indicate that no growth occurred.

NaCl:CsCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	-	11.90 ± 0.66	-
1	-	22.00 ± 0.56	63.85 ± 6.25
1.5	-	52.84 ± 24.98	90.66 ± 38.63
2	-	57.58 ± 13.77	93.26 ± 27.49
2.5	-	-	52.56 ± 3.27
3	-	-	77.82 ± 7.36
3.5	-	-	74.49 ± 4.56

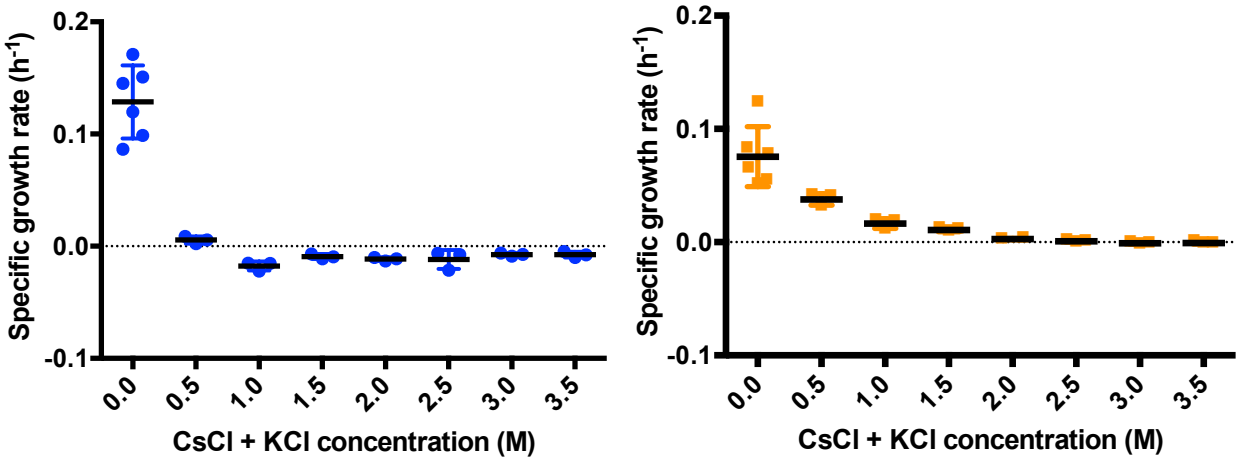

Figure 4.28. *E. coli* and *E. vietnamensis* growth in media containing a range of equimolar KCl:CsCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles) and *E. vietnamensis* (right - orange squares) grown in media containing a range of KCl:CsCl concentrations (x axis). Error bars indicate the standard deviation of individual experiments (3 replicates). Variance between organisms was analysed via ANOVA: $F(1, 27) = 231.6$, $p < 0.0001$.

Table 4.24. Doubling times (hours) of *E. coli* and *E. vietnamensis* when grown in media containing a range of equimolar KCl:CsCl concentrations. Values refer to the doubling time (hours) of the organism at a given KCl:CsCl concentration, with the SEM of this average value shown (3 replicates per condition). Blank cells indicate that no growth occurred.

KCl:CsCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	100.82 ± 21.50	18.55 ± 1.56	-
1	-	44.64 ± 7.98	-
1.5	-	65.12 ± 5.80	-
2	-	254.86 ± 35.67	-
2.5	-	-	-
3	-	-	-
3.5	-	-	-

***S. ruber* can grow in LiCl + CsCl but not in either alone – a potential additive cation effect**

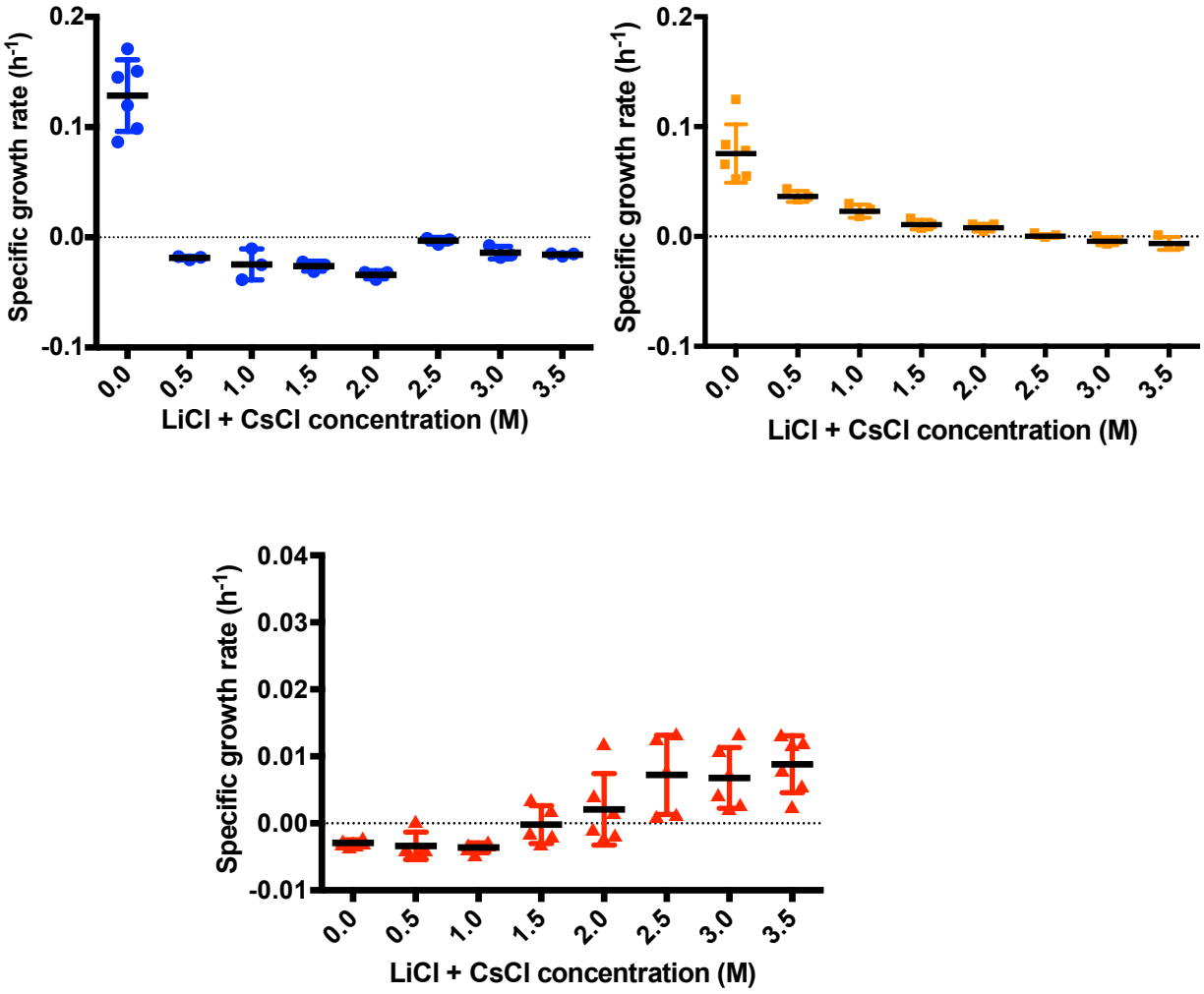

Figure 4.29. *E. coli*, *E. vietnamensis* and *S. ruber* growth in media containing a range of equimolar LiCl:CsCl concentrations. Figures show a scatter distribution about the mean of the specific growth rates (y axis) of *E. coli* (left – blue circles), *E. vietnamensis* (right - orange squares) and *S. ruber* (bottom – red triangles) grown in media containing a range of LiCl:CsCl concentrations (x axis). Error bars indicate the standard deviation (3-6 replicates). Variance between organisms analysed via ANOVA: F (2, 60) = 14.51, $p < 0.0001$.

Table 4.25. Doubling times (hours) of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in media containing a range of equimolar LiCl:CsCl concentrations. Values refer to the doubling time (hours) of the organism at a given LiCl:CsCl concentration, with the SEM of this average value shown (3 (Ec/Ev) and 6 (Sr) replicates per condition). Blank cells indicate that no growth occurred.

LiCl:CsCl (M)	*E. coli*	*E. vietnamensis*	*S. ruber*
0	5.39 ± 0.63	9.18 ± 1.21	-
0.5	-	19.17 ± 1.44	-
1	-	31.46 ± 4.91	-
1.5	-	56.87 ± 12.58	-
2	-	68.66 ± 0.82	215.51 ± 106.35
2.5	-	-	64.83 ± 11.63
3	-	-	69.88 ± 12.60
3.5	-	-	56.02 ± 1.93

4.2.7: Summary

Tables 4.26 – 4.32 show a summary of the results presented in this chapter, displayed as indications of the occurrence or absence of growth in each of the conditions.

Table 4.26. Occurrence of growth of the organisms in the presence of sodium-containing media. Growth is indicated by '✔' and lack of growth is indicated by '✖'.

Salt concentration (M)	NaCl			NaBr			NaCl:NaBr		
	Ec	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*
0	✔	✔	✖	✔	✔	✖	✔	✔	✖
0.5	✔	✔	✖	✔	✔	✖	✔	✔	✖
1	✖	✔	✔	✖	✔	✖	✔	✔	✖
1.5	✖	✔	✔	✖	✔	✔	✖	✔	✖
2	✖	✔	✔	✖	✖	✔	✖	✔	✔
2.5	✖	✔	✔	✖	✖	✔	✖	✖	✔
3	✖	✖	✔	✖	✖	✔	✖	✖	✔
3.5	✖	✖	✔	✖	✖	✔	✖	✖	✔
4	✖	✖	✔	✖	✖	✔	✖	✖	✔
4.5	✖	✖	✔	✖	✖	✔	✖	✖	✔
5	✖	✖	✔	✖	✖	✔	✖	✖	✔
5.5	✖	✖	✔	✖	✖	✖	✖	✖	✔

Table 4.27. Occurrence of growth of the organisms in the presence of potassium-containing media. Growth is indicated by '✔' and lack of growth is indicated by '✖'.

Salt concentration (M)	KCl			KBr			KCl:KBr		
	Ec	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*
0	✔	✔	✖	✔	✔	✖	✔	✔	-
0.5	✔	✔	✖	✔	✔	✖	✔	✔	-
1	✔	✔	✖	✖	✔	✖	✖	✔	-
1.5	✔	✔	✖	✖	✔	✖	✖	✔	-
2	✖	✔	✖	✖	✔	✖	✖	✔	-
2.5	✖	✔	✖	✖	✖	✖	✖	✖	-
3	✖	✔	✖	✖	✖	✖	✖	✖	-
3.5	✖	✖	✖	✖	✖	✖	✖	✖	-
4	✖	✖	✖	✖	✖	✖	✖	✖	-
4.5	✖	✖	✖	✖	✖	✖	✖	✖	-
5	-	-	-	✖	✖	✖	-	-	-
5.5	-	-	-	✖	✖	✖	-	-	-

Table 4.28. Occurrence of growth of the organisms in the presence of sodium + potassium-containing media. Growth is indicated by '✔' and lack of growth is indicated by '✖'.

Salt concentration (M)	NaCl:KCl			NaCl:KBr			KCl:NaBr			NaBr:KBr		
	Ec	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*
0	✔	✔	✖	-	✔	✖	✔	✔	✖	✔	✔	✖
0.5	✔	✔	✖	-	✔	✖	✔	✔	✖	✔	✔	✖
1	✔	✔	✖	-	✔	✖	✔	✔	✖	✔	✔	✖
1.5	✖	✔	✖	-	✔	✖	✖	✔	✖	✖	✔	✖
2	✖	✔	✔	-	✖	✖	✖	✖	✖	✖	✖	✖
2.5	✖	✖	✔	-	✖	✖	✖	✖	✖	✖	✖	✖
3	✖	✖	✔	-	✖	✔	✖	✖	✖	✖	✖	✖
3.5	✖	✖	✔	-	✖	✔	✖	✖	✔	✖	✖	✖
4	✖	✖	✔	-	✖	✔	✖	✖	✔	✖	✖	✖
4.5	✖	✖	✖	-	✖	✔	✖	✖	✔	✖	✖	✖
5	-	-	-	-	✖	✔	-	-	-	✖	✖	✖
5.5	-	-	-	-	✖	✔	-	-	-	✖	✖	✖

Table 4.29. Occurrence of growth of the organisms in the presence of LiCl, NaCl:LiCl and KCl:LiCl media. Growth is indicated by '✔' and lack of growth is indicated by '✖'.

Salt concentration (M)	LiCl			NaCl:LiCl			LiCl:KCl		
	Ec	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*
0	✔	✔	✖	✔	✔	✖	✔	✔	✖
0.5	✔	✔	✖	✔	✔	✖	✔	✔	✖
1	✔	✔	✖	✔	✔	✖	✖	✔	✖
1.5	✔	✔	✖	✖	✔	✔	✖	✔	✖
2	✖	✖	✖	✖	✔	✔	✖	✔	✖
2.5	✖	✖	✖	✖	✖	✔	✖	✖	✖
3	✖	✖	✖	✖	✖	✔	✖	✖	✖
3.5	✖	✖	✖	✖	✖	✔	✖	✖	✖
4	✖	✖	✖	✖	✖	✔	✖	✖	✔
4.5	✖	✖	✖	✖	✖	✔	✖	✖	✔

Table 4.30. Occurrence of growth of the organisms in the presence of LiCl:NaBr and LiCl:KBr media. Growth is indicated by '✔' and lack of growth is indicated by '✖'.

Salt concentration (M)	LiCl:NaBr			LiCl:KBr		
	Ec	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*
0	✔	✔	✖	✔	✔	-
0.5	✔	✔	✖	✖	✖	-
1	✖	✔	✖	✖	✖	-
1.5	✖	✖	✔	✖	✖	-
2	✖	✖	✔	✖	✖	-
2.5	✖	✖	✔	✖	✖	-
3	✖	✖	✔	✖	✖	-
3.5	✖	✖	✖	✖	✖	-
4	✖	✖	✖	✖	✖	-
4.5	✖	✖	✖	✖	✖	-

Table 4.31. Occurrence of growth of the organisms in the presence of RbCl-containing media. Growth is indicated by '✔' and lack of growth is indicated by '✖'.

Salt concentration (M)	RbCl			RbCl:NaCl			RbCl:LiCl		
	Ec	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*
0	✔	✔	✖	✔	✔	✖	✔	✔	✖
0.5	✖	✔	✖	✔✖	✔	✖	✖	✔	✖
1	✖	✔	✖	✖	✔	✖	✖	✔	✔
1.5	✖	✔	✖	✖	✔	✔	✖	✔	✔
2	✖	✔	✖	✖	✔	✔	✖	✔	✔
2.5	✖	✔	✖	✖	✔	✔	✖	✔	✔
3	✖	✔	✖	✖	✔	✔	✖	✔	✔
3.5	✖	✖	✖	✖	✔	✔	✖	✖	✔

Table 4.32. Occurrence of growth of the organisms in the presence of CsCl-containing media. Growth is indicated by '✔' and lack of growth is indicated by '✖'.

Salt concentration (M)	CsCl			CsCl:NaCl			CsCl:KCl			CsCl:LiCl		
	Ec	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*	*Ec*	*Ev*	*Sr*
0	✔	✔	✖	✔	✔	✖	✔	✔	-	✔	✔	✖
0.5	✖	✔	✖	✖	✔	✖	✔	✔	-	✖	✔	✖
1	✖	✔	✖	✖	✔	✔	✖	✔	-	✖	✔	✖
1.5	✖	✔	✖	✖	✔	✔	✖	✔	-	✖	✔	✖
2	✖	✔	✖	✖	✔	✔	✖	✔	-	✖	✔	✔
2.5	✖	✔	✖	✖	✔	✔	✖	✔	-	✖	✔	✔
3	✖	✔	✖	✖	✔	✔	✖	✔	-	✖	✔	✔
3.5	✖	✖	✖	✖	✔	✔	✖	✔	-	✖	✔	✔

4.3: Discussion

Non-halophilic organisms prefer K^+ but halophiles may prefer Na^+ for growth

E. coli, as is typical for non-halophiles, was inhibited above 1M Na^+, which is consistent with previous reports that enteric bacteria such as *E. coli* struggle to grow at above 0.8M NaCl [420]. At this concentration cells have been reported to plasmolyse, hence will show decreased growth at and above 1M salt [104]. Additionally, *E. coli* appeared to grow better in the presence of KCl, as compared to NaCl ($F_{1,80}$ = 4.481, p = 0.0374), which is contrary to a previous study, where it was found that growth in KCl was lower than in NaCl [431]. It should also be noted that the specific growth rate values of individual replicates of *E. coli* grown in the presence of KCl are more dispersed at higher concentrations (unable to grow at equal NaCl concentrations), and this could reflect the 'struggle' of *E. coli* to grow at these concentrations, with some growing faster than others. In addition, the large dispersions seen above 1.5M may show the inaccuracy of negative growth rate values as the cells may be dying/clumping together, as only

the positive growth rate values can be taken with full certainty – values under zero merely indicate a zero growth rate.

The fact that *E. vietnamensis* growth was improved in the presence of 0.5M, 1M and 1.5M KCl, as opposed to 0M, highlights one of the differences between non-halophilic and halotolerant organisms: namely that salt (KCl in this case) can actually prove stimulatory. *E. vietnamensis* was originally isolated from seawater, which has an ionic composition comprising a wide array of elements, but generally has higher Na^+ than K^+ concentrations [298,390]. However, *E. vietnamensis* showed greater growth in the presence of KCl than NaCl (could tolerate up to 3M KCl: $F_{1,233} = 8.737$, $p = 0.0034$), emphasising the potential more 'neutral' effects of K^+ over Na^+ on bacterial growth.

No growth of *S. ruber* occurred in purely potassium containing media, regardless of the potassium concentration. This was unexpected as KCl is one of the most abundant salts in the environment, although in saltern crystalliser ponds (the environment *S. ruber* was isolated from), the sodium concentration is much greater than that of potassium [432]. The lack of *S. ruber* growth when potassium is the main salt appears to be specific to this organism (or may even be halophile-specific in general), as both *E. coli* and *E. vietnamensis* were able to grow in the presence of both NaCl and KCl. This suggests that *S. ruber* may have a requirement for the higher charge density Na^+ over the lower charge density K^+ for growth, perhaps due to its stronger halophilic protein stabilising powers (as has been found for halophilic enzymes previously) [189]. It has been reported that some Gram-negative (*S. ruber* is Gram-negative) halophilic bacteria require Na^+ and cannot utilise K^+ for optimal growth, but this is thought to be due to requiring Na^+ for amino acid uptake [433], which *S. ruber* does not require as it is a salt-in organism. The halophilic archaeon *H. salinarum* has also been found to require Na^+ for growth [434], but the literature on the requirement of halophiles for Na^+ is limited, therefore this requires further investigation.

However, unlike in pure KCl, *S. ruber* was able to grow when NaCl was added, although growth was not as efficient and could not occur at as low

concentrations as in pure NaCl ($F_{1,151}$ = 13.18, $_p$ = 0.0004) - suggesting that Na^+ stimulates growth and K^+ does not have an effect on growth. The fact that *S. ruber* was found to have a clear preference for Na^+ over K^+ may dispute a previous finding that halophilic membranes are no more permeable to Na^+ ions than non-halophilic membranes [165], as there is a clear difference in the ion metabolism between *S. ruber* and that of the other two organisms. Therefore, it is important to gain an insight into the intracellular accumulation preferences of this organism (refer to Chapter 5).

In terms of cation preferences, since NaCl had a more adverse effect on *E. coli* and *E. vietnamensis* than KCl, but *S. ruber* showed a wide range of growth in the presence of NaCl and could not grow at all in KCl, this may suggest that S*. ruber* has a preference for Na^+. This, as previously stated, could be due to the higher charge density of this ion over K^+, which may provide more efficient stabilisation of its acidic proteome [191]. In contrast, the non-halophiles may prefer the lower charge density K^+ over Na^+, in terms of minimising the effects on their internal proteins, as it has been previously found that K^+ is required (in low concentrations) in cells for enzyme stabilisation [435]. In addition, it has been reported that Na^+ is a trigger for glutamate uptake in *E. coli* [436], therefore K^+ may provide a more stable environment and better initial osmotic balance. This may help to explain why the two non-halophiles have a preference for K^+ over Na^+.

The Hofmeister effect may explain differences between halophile and non-halophile growth in specific ions

The fact that *S. ruber* had superior growth in the presence of Na^+ and yet *E. coli* and *E. vietnamensis* had greater growth in the presence of K^+ may imply a difference in the physiology and ion metabolism between these organisms. This difference could be rationalised based on the Hofmeister effect [233]. This may be due to the different water affinities and hence stabilising/destabilising powers of the cations in relation to intracellular proteins [437]. *S. ruber* proteins

may function better in the presence of the higher charge density Na^+ since it will more efficiently stabilise the acidic proteome of this organism, due to its higher affinity for the increased level of carboxylic acid groups on the surface of the proteins in *S. ruber* (section 3.2.2), whereas the non-halophiles will prefer K^+ due to its weaker interactions with their more neutral proteomes, a consequence of its more chaotropic nature [237].

To supplement the general finding of Hofmeister-type differences between the organisms, *S. ruber* was not able to grow in any salt combination that did not contain Na^+ until equimolar KCl:LiCl was tested (variance between NaCl and KCl:LiCl of $F_{1,126} = 27.98$, $p < 0.0001$): suggesting that Li^+ can at least partially replace the effects of Na^+, due to its smaller size than K^+, and hence may provide more effective proteome stabilisation. In addition, *S. ruber* also showed growth in equimolar LiCl + CsCl/RbCl. This was most likely due to a collective Li^+ + $Rb^+/Cs^+/K^+$ effect. It has previously been found, for certain fungi, that growth inhibition by the presence of LiCl can be reversed by the addition of other cations, including both Cs^+ and Rb^+ – so-called 'salt antagonism', where Rb^+ 'antagonises' the inhibitory effects of Li^+ [415]. This may be due to the kosmotropic nature of Li^+ and in lower concentrations (i.e. when used in equimolar proportions with larger cations) it may provide optimal proteome stabilisation but at higher concentrations (pure LiCl) its stronger interaction with cellular proteins results in destabilisation and hence leads to growth deficiency. In comparison to growth in LiCl:RbCl, *S. ruber* growth in LiCl:CsCl is significantly lower, in terms of concentration range as well as growth rates ($F_{1,67} = 66.56$, $p < 0.0001$). Perhaps Rb^+ is more favourable for growth than Cs^+ due to its smaller size [109], in comparison to Cs^+, further supporting the theory of *S. ruber* having a preference for smaller over larger cations. Moreover, *S. ruber* showed no growth in the presence of pure RbCl or in CsCl, as well as KCl. Since both Rb^+ and Cs^+ are below K^+ in the periodic table [438] these cations may not provide the appropriate degree of proteome stabilisation required by the acidic proteome of *S. ruber*, and may hence result in a lack of growth due to enzyme inactivation. It could therefore be hypothesised that *S. ruber* prefer smaller cations (Na^+ and Li^+) over larger cations (K^+, Rb^+ and Cs^+), although it may have a

partial requirement for Na^+ as the only pure salts *S. ruber* could grow in were NaCl and NaBr.

E. coli growth in pure KCl was better than in equimolar NaCl:KCl – which emphasises the general finding that Na^+ has stronger growth inhibitory effects on *E. coli* growth than K^+ does. As an alternative explanation, the fact that *E. coli* had a particularly low growth rate in equimolar KCl:NaBr implies that perhaps there are combined ion effects, or that *E. coli* has a low tolerance for multiple ions in the growth medium. Since Na^+ as well as both Cl^- and Br^- are protein-destabilising ions [439], according to the Hofmeister effect, perhaps these ions lead to an interference of cellular function, which may result in lower growth. This is further supported by the fact that *E. vietnamensis* growth in the presence of both sodium and potassium is lower than in the presence of either alone – specific ion effects have been found to be additive previously, which may decrease the rate of growth [440].

In addition to specific cation effects (the main aim of the current study) there also appeared to be varying effects on growth as a result of the presence of different anions. Br^- generally appeared to result in a greater level of growth inhibition (or a lower level of growth stimulation, in the case of *S. ruber*) than Cl^- (for example, variance for *S. ruber* NaCl vs NaBr: $F_{1,186} = 7.671$, $p = 0.0062$; *E. vietnamensis* NaCl vs NaBr: $F_{1,141} = 7.181$, $p = 0.0082$). The various effects of different anions on the growth of *S. aureus* and *Pseudomonas aeruginosa* in terms of Hofmeister effects on the whole organism has been reported previously [9], where it was found that Br^- resulted in a greater level of growth inhibition than Cl^-, which may be explained by the lower charge density and hence lower water structure making activities of Br^-, hence meaning it would be more likely to interact directly with intracellular proteins (i.e. binding to the protein surface), which is according to the Hofmeister effect [226,235]. Additionally, it has been shown that Cl^- is more beneficial for bacterial growth than Na^+ paired with alternative anions, and it has also previously been found that Br^- concentrations of above 1.5M can inhibit halophile growth, thus suggesting that it is equally as growth inhibitory towards non-halophiles [441,442]. The fact that Br^- is

inhibitory (although perhaps not to the same extent) towards both halophiles and non-halophiles to the same extent could be rationalised based on the theory of LMWA (section 1.5), since Br^- will interact with chaotropic NH_4^+ groups on protein surfaces, hence affecting their stability.

Therefore, specific ion effects on whole bacteria could be related to the order presented in the Hofmeister series, which varies between different organisms and may vary between halophiles and non-halophiles, in terms of the cation, but in terms of the anion these effects may be more universal. It is suggested that halophiles (or at least *S. ruber*) have a general preference for higher charge density (and more highly hydrated) cations, whereas non-halophiles show a preference for lower charge density (more weakly hydrated) cations.

From the data presented in this chapter, the effects on growth in relation to the Hofmeister series are as follows (from least to most inhibitory):

- *E. coli*: $K^+ > Na^+ > Li^+ > Rb^+ > Cs^+$
- *E. vietnamensis*: $K^+ > Na^+ > Rb^+ \sim Cs^+ \sim Li^+$
- *S. ruber*: $Na^+ > Rb^+ \sim Cs^+ > Li^+ > K^+$

Cs^+ has different levels of toxicity in different organisms and its effects may be due to channel block, cellular accumulation or K^+ depletion (which may also apply to Rb^+)

The *E. coli* growth inhibition by Rb^+/Cs^+ suggests cation specific effects, since growth could occur in Na^+, K^+ and Li^+. Rb^+ and Cs^+ are not physiological salts, and are not generally found inside the *E. coli* normal environment [423], so it is not unexpected that *E. coli* cannot tolerate the presence of Rb^+ and Cs^+. However, *E. coli* was able to tolerate Li^+, despite the fact that it too is not a physiologically relevant cation [406]. In addition, various studies have found that Cs^+ is more toxic than Rb^+ and certainly than K^+, with Rb^+ even being reported to sometimes have the ability to act as an effective K^+ replacement [443]. The similarity between K^+ and Rb^+ is highlighted by the difficulty in analysing spectral lines

(from flame spectrometry) of mixtures of K^+ and Rb^+, whereas K^+ and Cs^+ spectral lines can be more easily separated, thus highlighting the more similar chemistry and potentially superior 'K^+ replacement' powers of Rb^+ [405]. Furthermore, the fact that *E. coli* growth is so low in equimolar CsCl:KCl, but the organism does manage to grow very slightly implies that K^+ may interact with the Cs^+ or with the cell to 'block' these toxic effects, at least partially. This could be due to the similarity of K^+ with Cs^+ [444], and hence involve transport into the cell. Bossemeyer *et al* found that the *E. coli* K^+ transporter kup (TrkD) can also transport Cs^+ [445], so it could be the case that Cs^+ and K^+ compete for the same entry site (i.e. kup), which could result in less Cs^+ entering the cell/interacting with kup when K^+ is also present, which may decrease its toxic effects. Furthermore, the fact that only *E. coli* was found to contain this kup transporter in the analysis of cation transporters carried out in section 3.2.4 perhaps explains why this toxicity is specific towards *E. coli*.

The inhibition of the growth of microbes by Cs^+ has been reported previously, and this toxicity seems to vary for different organisms [446]. It has been found by several different studies that generally Rb^+ is able to act as a partial K^+ replacement, but Cs^+ is not as effective [447]. As mentioned previously, this is thought to be due to Rb^+ having a more similar ionic radius to K^+ and both ions are hydrated to more similar degrees. However, this does not explain the fact that Rb^+ was also detrimental to *E. coli* growth. Regarding the specific nature of Cs^+ toxicity (towards *E. coli*), it has previously been found that Cs^+ may accumulate to different extents in different cells [405]. This ability to accumulate Cs^+ has been found to be dependent on the presence of specific ion transport systems within the cell membrane, in organisms other than *E. coli* [126], with the extent of growth inhibition dependent on the levels of cellular accumulation [109]. Cs^+ is not toxic to all organisms, and it is likely that its toxicity is primarily determined by its passage into and out of the cells, i.e. cells that lack specific membrane proteins will not be inhibited by this ion, as is illustrated by the data for *E. vietnamensis* and *S. ruber* presented in the current study. The cellular analysis in Chapter 5 should clarify the link between the cellular accumulation of these cations and toxicity.

In addition to its accumulation potentially causing toxicity, Cs^+ has previously been reported as being a potassium channel blocker, and due to this it has even been used in order to reduce the growth rate of cancerous glioma cells, where growth rates were reduced by Cs^+ induced K^+ channel block [448]. K^+ channel block by Cs^+ has also been reported for outward K^+ channels in squid axonal cells (applying to Rb^+ as well as Cs^+), resulting in these cations (and K^+) not being able to efflux from the cell as effectively [449]. In an earlier study it was found that (in the squid giant axon) around 50% of the K^+ channels were blocked by Cs^+ concentrations of 1M – comparable to the current study in terms of concentrations used [450]. Therefore, the detrimental effects observed on *E. coli* growth could be due to a large proportion of the K^+ channels in this organism becoming blocked.

It was found that even with increased K^+ concentration, if the Cs^+ concentration is equally as high, then *E. coli* growth still barely occurs (variance between CsCl:KCl and CsCl did not show significant differences in growth rates: $F_{1,\ 14}$ = 0.04006, p = 0.8442). It has previously been reported that if the K^+ concentration is increased, then this Cs^+ channel block can be overcome [451], but clearly this is not the case from the current study. This suggests that the detrimental effects may be due to either an irreversible channel block or specific intracellular effects induced by the presence of high Cs^+ concentrations – effects that may not be able to be reversed by the addition of K^+. It has been found that Cs^+ can cause certain cellular components to become unstable, such proteins and the ribosomes [126]. This potentially disruptive effect caused by Cs^+ on cellular proteins may be rationalised based on its stronger adsorption to protein surfaces (dispersion forces) due to its larger polarisibility [213]. Conversely, it has been reported that even if the Cs^+ accumulation inside cells alone is not toxic, the fact that this often results in K^+ depletion ultimately results in growth inhibition – i.e. it may be the Cs^+:K^+ ratio that determines cellular toxicity [126]. A 50:50 ratio of Cs^+/Rb^+ with another cation was not enough to restore cell growth in *E. coli*. However, the fact that very slight growth occurred in equimolar CsCl:KCl and RbCl:NaCl (although these data did not significantly differ from the pure salts, for example variance between RbCl and RbCl:NaCl was $F_{1,14}$ = 0.3289, p = 0.5754) suggests that the

addition of these other cations may reduce the amount of Cs^+ cellular accumulation, as was found in the cyanobacterium *Synechocystis*, which showed increased rate of growth as well as decreased Cs^+ accumulation when NaCl or KCl was also added [109]. Similar effects have been observed for *B. subtilis* [128].

As an alternative potential explanation to compliment the above Cs^+ effects observed for *E. coli* in the current study, Cs^+/Rb^+ have been proposed for cancer therapy, on the basis of these elements 'raising the pH' of cells, which will decrease the membrane potential across the membrane of the cell [452]. In relation to *E. coli*, an altered membrane potential could result in a decreased rate of growth due to a decreased level of energy generation. The mechanism of this altered membrane potential was thought to be due channel block and the resultant K^+-depletion – with the pH inside the cell being the cause of the growth inhibition [453]. Therefore, the adverse effects on *E. coli* could be due directly to channel block, K^+ depletion (with Cs^+/Rb^+ unable to act as effective replacements) or perhaps due to the pH inside the cell becoming sub-optimal, due to a change in membrane-potential.

To summarise, the main theories behind the specific Cs^+ (and Rb^+) toxicity in *E. coli* are: K^+ replacement by Cs^+ and the subsequent interference of enzymatic activities; K^+ depletion; K^+-channel block; and alterations in membrane potential – all caused by entry via the kup membrane transporter.

Rb^+ can be transported into the cells by K^+ transporters and may result in cell toxicity

A previous study found that Rb^+ can replace K^+ and assist in the growth of K^+-depleted *E. coli* cells [454]. However, these findings are severely disputed in the current study as Rb^+ was found to not be an effective K^+ replacement. As mentioned above, Rb^+ (as well as Cs^+) proved to be specifically toxic towards *E. coli*. The fact that the range of growth of *E. vietnamensis* in the presence of NaCl:RbCl is greater than for any other salt/salt combination tested suggests that

RbCl is not toxic towards *E. vietnamensis*, and that this toxicity (with regards to the current study) is specific for *E. coli*.

Therefore, the mechanisms of Rb^+ toxicity may be similar to that of Cs^+: destabilisation of internal proteins and/or K^+ channel block. It has been found that Rb^+ being present in the environment can cause K^+ channels to slow down their rate of closing, caused by Rb^+ binding to the K^+ channel from the cytoplasmic side (i.e. when inside the cell) [455]. Furthermore, it has previously been found using skeletal muscle from a frog, that Rb^+ can both stimulate K^+ exit as well as inhibit K^+ exit from the cell, and this may in part be due to Rb^+ 'replacing' or 'acting like' a K^+ ion [456]. Therefore, adverse affects on *E. coli* of Rb^+ may be a consequence of its similarity with K^+ and may be due to channel block or K^+ replacement with Rb^+, similar to the theories behind the observed Cs^+ toxicity.

In addition to the above explanations, RbCl can be used in order to prepare competent bacterial cells for artificial transformations, which means that the cells are made to be able to take up DNA through their membranes [457]. This suggests that perhaps RbCl could have an effect on the membrane structure, potentially causing the lack of growth seen in *E. coli*, either directly or by introducing other unwanted solutes into the cell. The RbCl transformation method used by Ren *et al* suggests that this protocol can be used for all bacterial species [457], however, adverse affects of RbCl presence were only observed for *E. coli* in the current study. Moreover, the concentrations of RbCl reported to have been used in these transformations are 10mM - 100mM [458], were much lower than was used in the current study. Since all three organisms used in this study are Gram-negative, one would expect that both *E. vietnamensis* and *S.ruber* would be equally as affected by Rb^+, if membrane structural alterations were the case. Therefore, this may be an unlikely cause of the growth inhibition, and instead it is more likely due to a similar mechanism as for Cs^+.

Effects of lithium may vary for different organisms

E. coli was able to grow in up to 1.5M LiCl, the same range as for KCl (although growth was significantly lower in LiCl). However, the fact that *E. coli* does grow considerably better in KCl ($F_{1,37}$ = 5.105, p = 0.0298) could be due to the effects of Li^+ being more disruptive intracellularly than K^+. Cebrian *et al* found that the effects of LiCl on a range of non-halophilic bacteria were more growth inhibitory than the other solutes tested, which included NaCl, glycerol and sucrose [404]. This further suggests LiCl can have some degree of toxicity and its effects may be quite different to the more 'neutral' NaCl and KCl.

Both *E. coli* and *E. vietnamensis* appeared to grow better in the presence of equimolar NaCl:LiCl than in pure LiCl, especially for *E. vietnamensis* (*E. vietnamensis*: $F_{1,45}$ = 7.467, p = 0.0089; *E. coli*: $F_{1,29}$ = 1.756, p = 0.1955) – therefore suggesting that Na^+ is less inhibitory towards growth than Li^+, i.e. Li^+ is not an effective Na^+ replacement. This could be due to the lower concentration of Li^+ (in the equimolar combination), due to an effect referred to in a historic study as 'ion antagonism', whereby the addition of one 'harmless' salt to a toxic salt results in a lower level of toxicity [391]. The greater level of inhibition (or lower level of stimulation, in the case of *S. ruber*) caused by Li^+ (as compared to Na^+ or K^+) could be due to the higher charge density of this ion, meaning its chemistry is similar to that of magnesium - it has been reported that lithium may compete with magnesium for binding sites on proteins and may provoke its biological effects in this way [411].

The fact that Li^+ has very different biological effects to that of Na^+ is further emphasised by the fact that *S. ruber* is unable to grow at all in the presence of pure LiCl. This could either point to an obligate requirement for Na^+ by *S. ruber*, as has been reported for the halophile *H. salinarum*, or perhaps growth inhibition caused by lithium, as has been reported for many bacterial species [424,425,434]. However, the fact that *S. ruber* cannot grow in the presence of pure LiCl but can grow when Na^+ is added may imply a collaborative effect of Li^+ + Na^+, since growth in equimolar NaCl:LiCl is able to occur at a Na^+ concentration

as low as 0.75M NaCl, whereas in all other experiments the NaCl concentration was higher than this for growth to commence. Moreover, the fact that *S. ruber* was able to grow in equimolar LiCl:KCl but not in either salt alone may suggest that Li^+ is perhaps able to partially (but not fully) replace the effects of Na^+ ($F_{1,126}$ = 27.98, $p < 0.0001$), possibly due to the lower concentrations of Li^+ present (as compared to in pure LiCl) being more stabilising towards intracellular proteins (i.e. it is stabilising when used at lower concentrations). Additionally, growth in this salt combination may be due Na^+/K^+ antiport (where $Na^+(Li^+)$ is extruded in exchange for K^+). This ion combination was found to be partially inhibitory towards both *E. coli* and *E. vietnamensis*, so this tolerance may show some aspect specific to halophilism, which allows for growth in this combination. Growth can only occur when each ion is at a minimum of 2M, suggesting that Na^+ may be required for growth at lower concentrations.

The fact that *S. ruber* cannot grow in pure LiCl but can when other salts are added suggests that Li^+ may affect *E. coli* and *E. vietnamensis* differently from *S. ruber*. The cation transport analysis carried out in section 3.2.4 found that both *E. coli* and *E. vietnamensis* contain Nha-transporters, which are able to transport both Na^+ and Li^+, in exchange for H^+ [143], but this was found, from the current study, to be lacking in *S. ruber*. Therefore, this cation transport system may be crucial as to why Li^+ affects these organisms differently.

Cl^- + Br^- together may be partially inhibitory towards bacterial growth

Since *E. coli* slightly showed better growth in KBr than in NaCl (although this was a small difference: variance analysis was insignificant), this highlights that the cation may have a more significant effect on growth than the anion. However, the anion effect is still significant as growth in KBr was slightly decreased in comparison to KCl ($F_{1,31}$ = 3.014, p = 0.0925). This was also the case for *E. vietnamensis* as it cannot tolerate above 2M KBr, and at this concentration growth was so low it is almost negligible (for example, variance between *E.*

vietnamensis KCl and KBr was $F_{1,153} = 9.935$, $p = 0.0020$). Non-halophilic organisms have previously been demonstrated to require Cl^- when faced with high sodium concentrations, and growth has been reported to be adversely affected by the replacement of Cl^- with other anions [8]. Therefore this chloride requirement of bacteria may also be the case for growth at high potassium concentrations.

Moreover, more extreme than the increased level of growth inhibition observed in the presence of Br^- as compared with Cl^-, was the fact that growth in the presence of both of these anions was significantly lower than growth when only Br^- was present. *E. vietnamensis* cannot grow up to an as high concentration of NaCl:NaBr (and KCl:KBr) as these salts individually, thus implying that this is most likely due to the presence of both of these anions resulting in greater growth inhibition. This inhibitory multiple anion effect is supported by the *S. ruber* data - *S. ruber* growth in equimolar NaCl:NaBr is also lower than when only Br^- is present as the anion (pure NaBr) ($F_{1,117} = 13.26$, $p = 0.0004$). This was also shown for *E. coli*, as its growth was worse in equimolar KCl:KBr than the salts individually. Clearly, both Cl^- and Br^- being present within the culture medium has some sort of adverse affect. This could potentially be due to Hofmeister-type effects, involving the chaotropic $Cl^- + Br^-$ ions, perhaps having some sort of additive effect. Two weakly hydrated anions [439] causing destabilisation of intracellular proteins or perhaps even the membrane proteins - leading to an additive effect on cell growth.

LiCl + KBr bacterial toxicity – a potential antiseptic tool

The adverse effects on growth of $Cl^- + Br^-$ is further exemplified by the fact that no growth whatsoever, for both *E. coli* and *E. vietnamensis*, occurred when KBr was used in combination with LiCl. Since growth in NaBr:LiCl was extremely low, it seems reasonable to assume that there may be some sort of adverse interactions between $Li^+ + Br^- + Cl^-$ and these interactions may be made more severe when K^+ (instead of Na^+) is also added to the medium. It remains to be

determined whether growth is merely inhibited or the cells are actually dead [104]. Regardless, it is clear that the LiCl + KBr combination is strongly unfavourable towards bacterial growth. The cells (*E. coli* and *E. vietnamensis*) are clearly adversely affected by these ions in combination with one another. This could be a case of 'ion antagonism', where the Li^+ ion interferes with the function of potassium and perhaps also the Br^- ion interferes with the function of Cl^- [459]. This is based on the fact that Cl^- has been found to be essential for non-halophilic bacteria growing at high salt concentrations (as was mentioned previously), and K^+ is required by non-halophilic organisms for the maintenance of cell turgor as well as various cellular processes, so interference of these interactions may lead to growth inhibition and (potentially) cell death [342,442]. This interference may be due to Cl^- channel block by Br^- (hence lowered Cl^- concentrations in the cell) or altered membrane potential caused by the lower ability of Li^+ removal from the cell, resulting in decreased K^+ uptake [147].

It has previously been reported that using both Cl^- and Br^- together was more effective at killing bacteria (even chlorine resistant bacteria) than using either of the two alone [460]. Of course, the organisms were able to grow (albeit at a low level) in LiCl + NaBr (*E. coli* variance between these salts was $F_{1,29} = 14.49$, $p = 0.0007$, *E. vietnamensis* variance was $F_{1,18} = 32.74$, $p < 0.0001$), so clearly the K^+ also has an effect, as well as the Li^+. In addition, since the Hofmeister effect states that anions tend to be more destabilizing than cations, then perhaps this could provide an additional theory to at least partially explain the LiCl + KBr effects - two low charge density anions (chaotropes) + Li^+ (high charge density - kosmotrope) [213,221] - together they may result in higher levels of protein destabilization than either alone, hence leading to a lack of growth.

Due to the level of effectiveness of this particular salt combination at inhibiting bacterial growth, a patent application for the use of this salt combination as an antimicrobial agent has been filed.

The halotolerant classification of *E. vietnamensis* should be re-considered

E. vietnamensis has a very large range of growth, from 0M up to as great as 3.5M, which is dependent on the specific salt(s) present. It is however unusual for halotolerant organisms to grow in such a large range of salt concentrations [400]. *E. vietnamensis* does, however, grow better in the absence of salts, as compared to when salt was present, but is able to grow efficiently at 0.5M and 1M in many salts. Even though *E. vietnamensis* grows optimally at lower salt concentrations, it could be considered to be an 'extremely halotolerant' organism, given its large range of growth within multiple salts. Oren (2008) described an organism to be extremely halotolerant if it is able to grow in salt concentrations above 2.5M [84]. Using this criterion, and since *E. vietnamensis* is able to grow (dependent on the specific salt) at or above this 2.5M concentration, it is suggested that this organism be re-classified as extremely halotolerant. Note that there is no doubt that *E. vietnamensis* is halotolerant as opposed to halophilic, as it clearly grows more efficiently (mostly) at the lowest salt concentrations, and so its classification as a halotolerant organism is not disputed.

In addition, the fact that *E. vietnamensis* growth could occur in the presence of RbCl and CsCl emphasises the ability of this organism to tolerate a wide range of ions, even those that are only found as minor trace elements in its natural environment, that of seawater [390]. It would therefore appear that the large cations are not toxic to all bacteria, and that the differences between *E. coli* and *E. vietnamensis* may be one of the pivotal factors to this observed toxicity in *E. coli*. The fact that *E. vietnamensis* appears to be tolerant to many different cations may be a consequence of its environment (seawater)[298]. It is more tolerant towards a range of ions (as well as having the ability to grow at a wide range of concentrations) and does not appear to be as specific in terms of cation preferences as both *E. coli* and *S. ruber*.

S. ruber 'extreme halophile' classification should be re-considered

S. ruber was found from the current study to have a large range of growth, larger than is typical for any single category of halophile [86], i.e. in NaCl and NaBr it was able to commence growth at as low as 1M, which is in contrast to previous work, which has stated it cannot grow below 2.6M NaCl [172]. This is lower than most salt-in halophiles (generally require at least 1.7M for optimal growth), similar to the lower concentration boundary of optimal growth for moderate halophiles, but also similar to the higher concentration end for extreme halophiles [84,94]. The fact that the growth of *S. ruber* at 5.5M is particularly low indicates that even halophilic organisms have limits in terms of salt tolerance (NaCl concentrations reaching saturation). In addition, the optimal NaCl concentration for *S. ruber* growth has previously been reported as 3.4M – 5.1M [202]. However, the fact that in the currently study *S. ruber* was able able to grow at 1M strongly disputes this, and is also not characteristic of extreme halophiles.

According to the general scale of halophile classification, organisms that have optimal growth from 0.5-2.5M are moderate halophiles, those that grow best between 1.5-4M are borderline extreme halophiles and those that grow best in 2.5-5.2M are extreme halophiles [84,266]. Since *S. ruber* was shown to have optimal growth at 3M (NaCl) but growth could occur at as low as 1M NaCl, it is suggested that *S. ruber* should be re-classified as a borderline extreme halophile.

S. ruber does not appear to require Cl^- for growth but its presence may be stimulatory

Given that *S. ruber* has previously been described as requiring Cl^- [181], the result of the experiments in NaBr disputes this as growth here was similar to that in NaCl, therefore suggesting that *S. ruber* does not in fact require Cl^-, as has been previously suggested. However, Br^- is in fact present in many halophilic environments, albeit at much lower concentrations than Cl^-, so *S. ruber* may be 'optimised' to be able to function in the presence of this alternative anion [441].

Conversely, the fact that *S. ruber* was not able to grow in the presence of K^+ when only Br^- was present as the anion (NaBr:KBr) clearly implies that the anion does have a significant effect on the growth of *S. ruber*, even if it is not obligately required. *S. ruber* appears to require Cl^- when K^+ is also present, but does not require it when K^+ is not present. A membrane transporter that transports Cl^- coupled to Na^+ has been found in the *Halobacteriaceae* [461]. Since it has already been found that *S. ruber* accumulates Cl^-, then perhaps its requirement for Na^+ is at least partially dependent on Cl^-, hence its larger requirement for Cl^- when K^+ is present, i.e. it requires Cl^- when Na^+ is less readily available, in order to import Na^+ into the cell [181].

Specifically, in terms of anion effects on *S. ruber*, considering the growth of *S. ruber* in the presence of equimolar NaCl:KBr, growth cannot occur until the Na^+ concentration reaches 1.5M, which is also the case for NaBr. This may imply an effect of Cl^- on Na^+ transport into the cell – which may mean that at lower Cl^- concentrations Na^+ is less readily transported into the cell, hence the concentration in the environment has to be greater for growth to commence, when Cl^- is at a lower concentration in the environment. Additionally, it has previously been found that if NaCl is replaced by Na-gluconate, *S. ruber* cannot grow, but since the current study has found that it can grow in the presence of NaBr, this could have merely been due to a toxic effect of gluconate on the bacteria [461]. Therefore, it is likely that Cl^- can stimulate *S. ruber* growth, but is not strictly required for growth to occur.

Clearly, the effect of the cation is much more significant for *S. ruber* growth than the effect of the anion, as no growth occurred at all in the presence of K^+ instead of Na^+, whereas growth could occur in NaBr. However, the previous work that suggests *S. ruber* requires Cl^- for growth is disputed in the current study, so this strict requirement of Cl^- should be re-evaluated [461].

General comments on the growth rates

As was expected for *E. coli*, it was found to have a relatively fast doubling time within the current study, although doubling times as rapid as the widely quoted 20 minutes for *E. coli* grown in LB medium [462] were not found here (just over 3 hours was the doubling time for *E. coli* grown in LB medium), although this 20 minute doubling time has not always been quoted in the literature, depending on the *E. coli* strain used [462,463]. The slower growth seen in the current study could have been due to the continuous sub-culturing of the organisms or sub-optimal pH/lack of nutrient availability [464,465]. In addition, the strain of *E. coli k12* used here, *DH5α*, is known to have a slower rate of growth than is typical for other *E. coli* strains, which may be exaggerated in the general medium (sub-optimal media composition) [466]. Moreover, the fact that *E. coli* growth is lower in the General Medium than LB medium implies that the salt composition of LB medium may be more favourable to *E. coli* than that of the General Medium (refer to section 2.2 for details). The main differences between these two media are that LB medium does not contain any magnesium salts, and since magnesium has shown antibacterial activity against *E. coli* in concentrations as low as around 6mM [467], this may be the reason for the decreased growth rate of *E. coli* in the General Medium.

With a doubling time of just over 8 hours, the growth of *E. vietnamensis* in MB medium is slower than that of *E. coli* in LB medium. No existing growth rate data exists for *E. vietnamensis,* or indeed other *Echinicola* species [298,468]. Further characterisation of the growth rates of this organism should be performed, however, the results from the current study suggest that *E. vietnamensis* growth may generally be slower than that of *E. coli*.

Also to note is the lower scatter of individual replicates of *E. vietnamensis* grown in MB medium, in comparison to *E. coli* grown in LB medium. This suggests that *E. coli* growth in LB medium is more variable than *E. vietnamensis* growth in MB medium. It has been shown that the growth rate of *E. coli* can fluctuate and this fluctuation may be related to the cell density and the maintenance of a constant

culture which sub-cultures are taken from [469], which was the case in the current study. Tan *et al.* discussed 'unbalanced growth' in which the growth rate of a certain bacterial species fluctuates throughout an experiment, but that this is not necessarily a problem and may merely reflect the unique physiology of that bacterium [470], particularly considering that this particular stock of *E. coli* has been sub-cultured countless times and stored within the laboratory at -80^{o}C for potentially many years. This may additionally explain some of the larger scatters shown in the data for *E. vietnamensis*, as compared to *E. coli*, as this may just be a unique feature of the growth characteristics of this organism, which may be dependent upon the media composition.

The doubling time of *S. ruber* in Salinibacter medium indicates that it has a dramatically lower growth rate in comparison to both *E. coli* and *E. vietnamensis*. The doubling time of just under 108 hours found in the present study is significantly greater than that quoted in the literature [199,471]. The fact that *S. ruber* was grown at sub-optimal conditions in the current study (lower temperature) should be taken into account, as its optimal growth temperature has been reported to be 40^{o}C [199], which could potentially explain its slower rate of growth in the present study as compared with previous studies.

Even though the growth of the organisms was not as rapid in the General Medium as compared to their optimal media, it was of vital importance to grow the three organisms in the same medium as even the same strain of bacteria can grow at different rates in slightly different media [472]. It was not surprising that *S. ruber* did not grow in this medium with no added salts. However, since the General Medium composition was very similar to the Salinibacter medium (see section 2.2), it was known that it could grow in this medium, most likely at higher salt concentrations.

Next steps

The data from the current study suggests that specific ion effects are crucial for the response of bacteria towards hypersaline conditions – i.e. the specific ion

present has a major impact on the survival of the organism. The effects on growth of the organisms are not only due to molecular level effects (due to interactions of ions with cellular proteins) but may be determined based on the presence of specific ion transporters. Therefore, in order to understand the effects that each cation has on the bacterial cells, in order to investigate the mechanisms of halo-adaptation, bacterial salt tolerance, and cation effects on whole organisms, it is important to compare the data presented in this chapter to that presented in Chapter 5 - where the cell contents were analysed in order to determine the levels of ion accumulation, and to relate this to the specific growth effects observed in the present chapter.

Chapter 5: Elemental Analysis of Accumulated Cations within Bacterial Cells

5.1: Introduction

5.1.1: Bacterial ion accumulation

As mentioned in section 1.2, all bacterial cells, at least initially, will accumulate ions for osmotic balance with the external media. However, growth of a bacterium within a salt does not necessarily mean that the salt ions will enter the cell, due to the presence of selective transporters and channels in the cell membrane [110]. Hyperosmotic conditions result in rapid loss of water from the bacterial cell (see Figure 4.1), which needs to be balanced with the accumulation of inorganic ions and the subsequent uptake of compatible solutes [395,473]. Regarding the general cation balance found within the cytoplasm of most bacteria, K^+ is in fact the most abundant ion within bacterial cells, as well as the majority of living cells on the planet, due to its essential biological functions [342]. Predominantly, for bacterial cells, Na^+ is excluded from the cytoplasm [423]. Due to this, biological membranes have to be selective in the passage of ions into and out of the cell. For a description of bacterial ion transport, refer to sections 1.2 and 3.2.4.

It has been found that bacteria will generally accumulate K^+ in proportion to the external salinity, which may be a factor in determining the maximim salinity that an organism is able to grow within [163]. The initial response of a non-halophilic bacterium when grown in the presence of a high salt concentration is to accumulate K^+, which then triggers the synthesis of compatible solutes [106]. Once the external environment osmolarity is balanced by the accumulation of compatible solutes, K^+ will be released from the cytoplasm [398]. Glutamate is one of the main osmotic solutes accumulated in non-halophilic bacteria after the

initial accumulation of K^+ [474]. It has also been found that the uptake of several amino acids and sugars by *E. coli*, such as proline, glutamate and melibiose, is dependent on Na^+, and the formation of a Na^+ gradient [414]. In addition, sodium-substrate co-transport is a common method of accumulating compatible solutes, via the transport of Na^+ into the cell at the same time as the solute, such as glutamate or melibiose - the energy generated from Na^+ entry into the cell (down its concentration gradient) is used to drive the entry of the substrate into the cell [475]. The establishment of electrochemical gradients via Na^+/H^+ antiporters is also essential for bacteria [151]. This removal of Na^+ from the cytoplasm indicates that a bacterium will generally contain a Na^+ concentration lower than that of its environment [162]. However, Shabala *et al.* found that as the external NaCl concentration was increased, *E. coli* cells would gradually increase their concentrations of Na^+ as compared to K^+ (i.e. the ratio of Na^+:K^+ would increase – although K^+ concentrations still exceeded that of Na^+), which suggests that the nature of the salt in the environment can have an effect with regards to the intracellular levels of specific ions [476].

The accumulation of cations within a bacterium may vary depending on its level of salt tolerance. The responses of halotolerant organisms to specific salts have not been studied in as much detail as for non-halophiles and halophiles, however they have generally also been reported to maintain lower levels of Na^+ than K^+ [477]. *E. vietnamensis* is of particular interest since it can grow in a very wide range of salts (refer to Chapter 4) and can also tolerate a wide range of salt concentrations, sometimes as high as would be considered moderately halophilic, even though it is classified as halotolerant. Nagata *et al.* investigated the effects of a sudden osmotic upshock on the ion accumulation inside the halotolerant bacterium *Brevibacterium sp*. They found that the intracellular Na^+ concentrations were consistently low, whereas the K^+ concentration increased in response to an increase in external salt concentration [478]. In addition, other halotolerant organisms have also been found to keep their internal Na^+ concentrations lower than their K^+ concentrations [479]. Therefore, an analysis of the cytoplasmic composition of this organism may give insight into its survival strategies, as this has so far not been characterised [298]. Since *E. vietnamensis*

was found to tolerate a large range of cations, its accumulation of specific cations may reflect this.

Since *S. ruber* is an extreme halophile that uses the salt-in strategy of adaptation, it would be expected that it would contain very high ion concentrations, higher than for the other two organisms (*E. coli* and *E. vietnamensis*). As far back as 1970, it was reported that the extreme archaeal halophile *H. marismortui* accumulated higher levels of K^+ within its cytoplasm than Na^+, although the Na^+ concentrations were still found to be in the molar range [480]. Therefore, this suggests that the general K^+ preference seen for non-halophiles may also be the case for many halophilic organisms. Moreover, a previous analysis of the ionic composition of *S. ruber* (after the cells had been grown in the presence of medium containing predominately NaCl – at 3.3M) found that the cells contained more K^+ than Na^+ [181]. Additionally, Anton *et al*. analysed the concentrations of K^+ within *S. ruber* and found that they contained very high levels of K^+, but in this particular study they did not measure Na^+ concentrations, so whether or not it accumulated more or less Na^+ than K^+ is unknown from their study [199]. Therefore, the cation concentrations within this organism have not been well studied or assessed in response to changing salt concentrations, or within the presence of alternative cations.

Due to the extremely high ionic concentrations in their environment, it is of increased importance for halophiles to maintain an adequate proton electrochemical gradient across their cell membranes, via the removal of sodium (via Na^+/H^+ antiporters) and the accumulation of potassium, due to the higher Na^+ concentrations within the environment [10]. Regarding the high internal K^+ concentration that has been found in some halophiles, it is thought this is due partly to passive transport as well as the utilization of ATP in order to actively transport K^+ into the cell (via the Trk system), against a concentration gradient, as has been found for *H. volcanii* [481]. However, since *S. ruber* has been found to require Na^+ for growth (in the present study – refer to Chapter 4) and to not be able to grow (mostly) when Na^+ is replaced by K^+, perhaps its intracellular ion preferences may reflect this.

5.1.2: Principles of Elemental Analysis of Bacterial Cells

5.1.2.1: Ionic Strength

Ionic strength is of extreme importance towards biological systems, where enzymatic processes and osmotic responses will be determined based on the ionic composition of the surroundings [482]. The ionic strength is a means of expressing the total ionic effects of a solution, taking into account the fact that some ions will make larger contributions than others, due to their more significant interactions with other ions. For example, divalent ions will make a larger contribution towards the total ionic strength than monovalent ions [483]. Therefore, ionic strength is a measure of the contributions of each ion in a solution as a function of both its concentration and charge. Ionic strength can be calculated by the formula in Equation 5.1 [484].

$$I = \frac{1}{2}\sum_{i} m_i z_i^2$$

Equation 5.1. Where I is the ionic strength, m_i is the concentration of the ion (in M) and z_i is the charge number of that ion. The sum is carried out for all ions within the solution. Taken from de Vicente, 2004.

However, since only the cation concentrations are of interest in the current study, and since compatible solutes will also make contributions towards the ionic strength [485,486], the ionic strength was not used for the current study, and instead ion ratios were used. In addition, since all of the salts used in the current study consist of monovalent cations, which hence have a charge number of +1 and monovalent anions, which hence have a charge number of -1, the ionic strength is not expected to vary between solutions (of equal molarity), according to the above equation, making it effectively redundant for the current purposes.

5.1.2.2: Inversely Coupled Plasma Mass Spectroscopy

Inversely Coupled Plasma Mass Spectrometry (ICP-MS) has the ability to analyse the concentration of almost all known elements, making it a particularly powerful form of MS [487]. Currently, ICP-MS is particularly utilized within the analysis of trace elements, due to its ability to detect very low concentrations: having the capability to detect elements in the parts per trillion range, which other commonly used elemental analysis techniques, such as x-ray microanalysis, are not able to do [488]. Additionally, ICP-MS is able to analyse the concentrations of multiple ions simultaneously, meaning that the concentrations of multiple elements can be measured within the same sample [489]. Most of the ions that ICP-MS is able to detect are positively charged, although it has been reported that it can also detect some negatively charged ions [318].

A schematic is shown in Figure 5.1, giving an overview of the ICP-MS instrument and how it works [489]. The sample is introduced into the instrument as an aerosol (via the nebulizer) and is then ionized by argon plasma, resulting in all components within the sample, including proteins, being broken down into their constituent ions [490]. The ions are then removed from the argon plasma and are focused into the mass spectrometer, where the ions are separated based on their mass to charge ratio (m/z). The ions are counted and this is loaded into the data analysis software to obtain concentrations of each of the ions of interest in the sample, by the comparison with a reference sample.

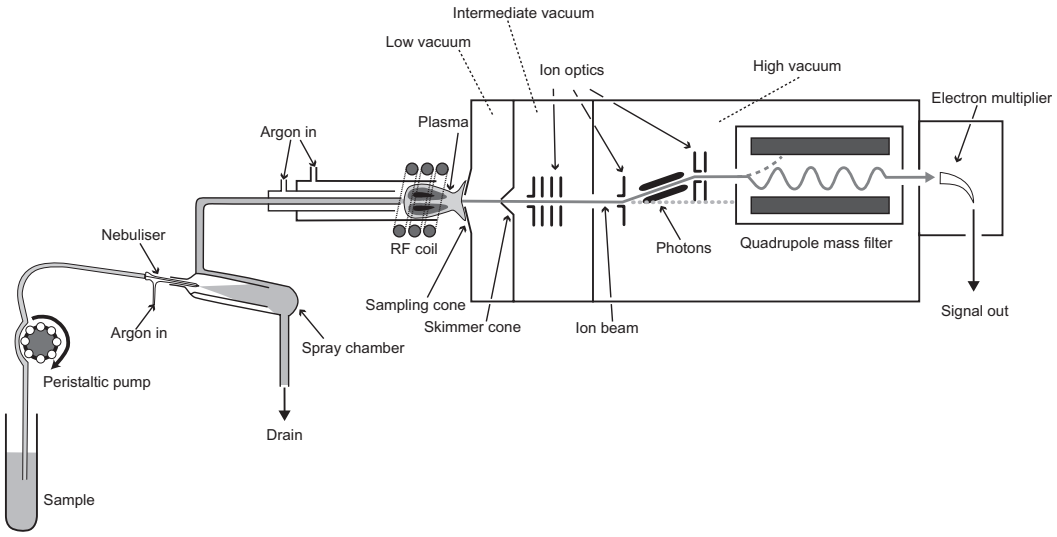

Figure 5.1. Schematic of an ICP-MS instrument. The sample is introduced into the instrument via the nebuliser (as an aerosol), where it is desolvated and converted to a gas, before it is ionised by the plasma (argon in this case). The ions are then passed onto the mass spectrometer, where they are separated by their mass to charge ratio (m/z), and the concentration of each ion calculated. Adapted from Linge, 2009.

5.1.2.3: Specific ion accumulation

Since the effects of non-essential cations on bacterial growth were analysed in Chapter 4, the effects of these intracellularly need to be analysed, in order to understand variations in their metabolism. Lithium (Li^+) is the smallest of the alkali metal ions and has been reported to have reactivity similar to that of magnesium, due to its small ionic radius and large charge density [411]. Lithium is a non-essential element, with an average abundance in humans of around 7mg (for a 70kg human) [407]. It is a logical 'Na^+-replacement', due to its position above sodium in the periodic table. It has been found previously that growth in the presence of lithium is largely dependent upon the presence of NhaA and NhaB genes: lack of these will lead to a lack of Li^+ expulsion from the cell and will be toxic [491]. Regarding the current study, it was found that growth in the presence of lithium was variable between the three organisms, but was generally able to occur (refer to Chapter 4). However, *S. ruber* could not grow in pure LiCl, suggested to be a consequence of its lack of the Nha $Na^+(Li^+)/H^+$ antiporters (refer to section 3.2.4) which remove $Na^+(Li^+)$ from the cell, and have been

reported to be responsible for tolerance to Li^+ [419], whereas *E. coli* and *E. vietnamensis* do contain this transport system, and were also able to grow in the presence of pure LiCl. Therefore, it needs to be determined if the concentrations of Li^+ within each of the cells will be directly related to both its ability to grow in Li^+ as well as the presence or absence of these Nha transporters.

Rubidium (Rb^+) and cesium (Cs^+) are two of the largest of the alkali metal cations (excluding francium) [406]. Cesium is a non-essential element for most cells and has a mass of around 6mg in a 70kg human [407]. Rubidium is also non-essential for most cells and its abundance in humans is much greater than that of cesium, with a mass of around 680mg per 70kg human. This difference in abundance suggests that these elements may enter cells in separate ways, resulting in different intracellular levels. When the growth effects of Cs^+ and Rb^+ were analysed within the current study (Chapter 4), their effects were found to vary somewhat between the three organisms. It has been found that organisms unaffected by Cs^+ toxicity generally maintain low intracellular concentrations of this ion, whereas the accumulation of this cation has been linked to toxicity [128,429]. Therefore, it needs to be determined whether the observed toxic effects of both rubidium and cesium have occurred due to an increased accumulation in *E. coli*, in comparison to the other organisms, which do not show this growth inhibition in the presence of these cations.

5.1.3: Rationale for current study

In order to obtain a better understanding on how the ions analysed in Chapter 4 affect the organisms in terms of growth, it is essential to have a look inside the cells to understand how each specific ion is accumulated and how this varies in relation to the other ions, as well as to relate this to the results presented in section 3.2.4 regarding the presence or absence of specific ion transport systems. This should help to elucidate the physical/chemical and biological mechanisms behind the observed growth effects. Due to its sensitivity and ability to detect all

of the cations of interest, ICP-MS was used for the elemental analysis of the ionic compositions of *E. coli*, *E. vietnamensis* and *S. ruber*.

For *E. coli* and *E. vietnamensis* in their optimal media (LB and MB), just like in their natural environments, they are not experiencing significant osmotic stress (although *E. vietnamensis* is naturally exposed to much higher osmotic pressure than *E. coli*), and so it is important to compare these conditons with that where these organisms are experiencing osmotic stress in order to determine how cation accumulation changes during growth in hypersaline conditions [298,492]. Therefore, organisms were grown in their optimal media as well as a multitude of media containing NaCl, KCl, LiCl, RbCl and CsCl, as well as equimolar combinations of these. An important factor to note when interpreting the levels of cation accumulation inside the cells is that the cation ratios of these media are reflected by the composition of the salt – i.e. in pure salts the external medium will be predominately composed of one of Na^+, K^+, Li^+, Rb^+ or Cs^+, and in salt combination media the external medium will be a 50:50 ratio of the cations used. Therefore, by comparing the internal ion concentrations to the concentration within the medium, one can gain insight into how the cellular ionic composition varies from the ionic composition of the medium.

Organisms were grown within media containing the salt concentrations that they were determined to grow within from the data presented in Chapter 4. Exponentially growing cells were harvested (media removed and cells dried) and the cellular lysates of these were analysed by ICP-MS (refer to section 2.3). The ions of interest were Na^+, K^+, Li^+, Rb^+ and Cs^+.

It should be noted that *S. ruber* is fastidious in the laboratory and consequently there was little biomass present in cultures to extract and analyse the cellular compositions. For this reason, and because of temporal constraints, only 7 conditions are shown in this chapter for *S. ruber*. In addition, because of the low cell densities of *S. ruber*, as well as the low cell densities of both *E. coli* and *E. vietnamensis* in several salts, protein concentrations were not measured and data

are given as ratios, with samples normalized based on OD_{600} and estimated cell number (refer to section 2.3.4).

5.2: Results

5.2.1: Baseline media ion accumulation

Intracellular analysis (via ICP-MS) was carried out on the cellular extracts of *E. coli*, *E. vietnamensis* and *S. ruber* after they were grown in the presence of LB, Marine Broth and Salinibacter medium, respectively. Additionally, *E. coli* and *E. vietnamensis* were grown in the General Medium (refer to section 2.2) with no salts added (excluding magnesium salts). *S. ruber* was not grown for this experiment as it cannot grow in the absence of salts. The data for this are presented in Figures 5.2 and 5.3 in terms of the ratios of Na^+:K^+, where values greater than 1 indicate that the Na^+ concentration is greater than the K^+ concentration, and values lower than 1 mean the K^+ concentration is greater than the Na^+ concentration. Tables 5.1 and 5.2 show the estimated ion concentrations per cell (refer to section 2.3 for details).

The composition of LB medium contains more NaCl than KCl (see section 2.2.1), and the data indicate that *E. coli* accumulated more K^+ than Na^+. *E. vietnamensis* contained more Na^+ than K^+ and *S. ruber* accumulated slightly more K^+ than Na^+. Even though the ratio of Na^+:K^+ in *E. vietnamensis* is higher than that of *S. ruber*, S*. ruber* accumulates much larger concentrations of both Na^+ and K^+ than *E. vietnamensis* - 479 times more Na^+ and 990 times more K^+ was present in *S. ruber* than in *E. vietnamensis*.

Overall, when no salts are added to the medium the level of ion accumulation for *E. coli* and *E. vietnamensis* is very similar. Both organisms accumulate more K^+ and significantly less Na^+, with *E. vietnamensis* containing slightly more K^+ and *E. coli* containing slightly more Na^+.

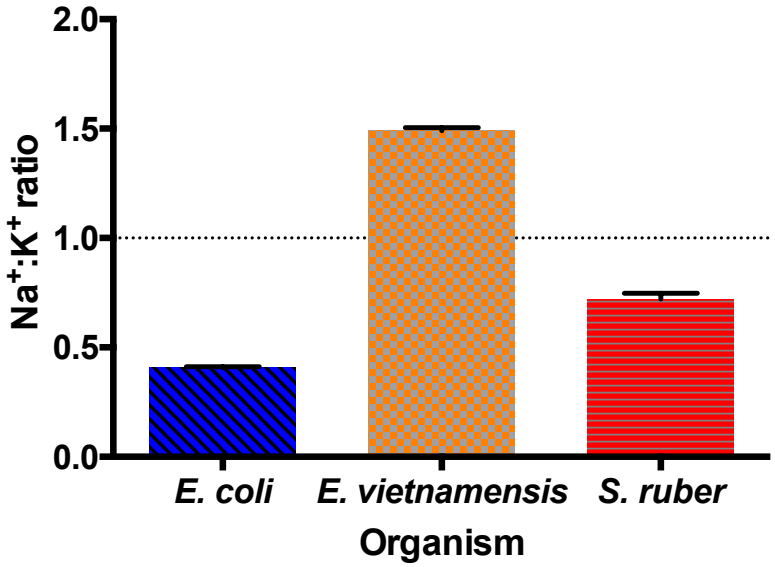

Figure 5.2. Intracellular ion ratios of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in their optimal media. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in LB, MB and Salinibacter medium, respectively. Shown is the ratio of Na^+:K^+ ions within the cells (Y axis) against the organism (X axis), where ratios lower than 1 equate to a higher K^+ concentration and those greater than 1 equate to a higher Na^+ concentration. Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.1. Ion concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in their optimal media. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in LB, MB and Salinibacter medium, and the estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

Organism	Na^+ (g/L)	K^+ (g/L)
E. coli	0.017 ± 5E-5	0.041 ± 5E-4
E. vietnamensis	0.094 ± 9E-4	0.063 ± 6E-4
S. ruber	44.971 ± 1.030	62.325 ± 2.990

Cation preferences in non-halophiles may be similar

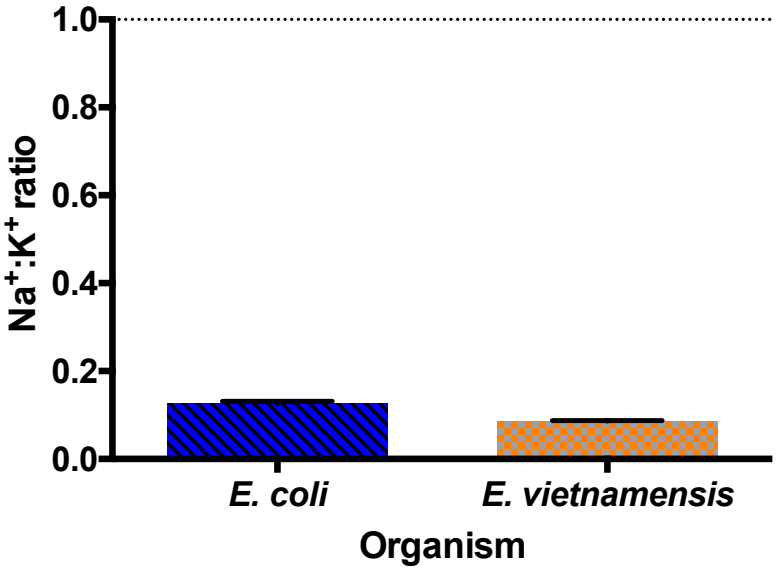

Figure 5.3. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the General Medium with no added salts. *E. coli* and *E. vietnamensis* were grown in the General Medium containing no added salts (0M). Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the organism (X axis). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.2. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in 0M salts. *E. coli* and *E. vietnamensis* were grown in the General Medium with no added salts and the estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

Organism	Na^+ (g/L)	K^+ (g/L)
E. coli	0.002 ± 1E-4	0.017 ± 2E-4
E. vietnamensis	0.002 ± 5E-5	0.020 ± 2E-4

5.2.2: Effects of sodium on ion accumulation

E. coli, *E. vietnamensis* and *S. ruber* were grown in media containing a range of NaCl, NaBr and equimolar NaCl:NaBr concentrations and ICP-MS analysis was carried out on the cellular lysates from cells harvested after growth in these media. The data for this, expressed as the ratio of Na^+:K^+, are shown in Figures

5.4 - 5.6 and estimated intracellular concentrations are shown in Tables 5.3 - 5.5. Additionally, a summary of these results is shown in Table 5.6.

E. coli accumulated higher levels of K^+ as compared with Na^+. *E. vietnamensis* intracellular ion concentration increases linearly with increasing medium salt concentration. *S. ruber* mostly accumulates Na^+ in excess of K^+ (except for 3 – 4M NaCl). All three organisms accumulate K^+, even though it was not added to the medium.

S. ruber may prefer Na^+ over K^+ for osmotic balance

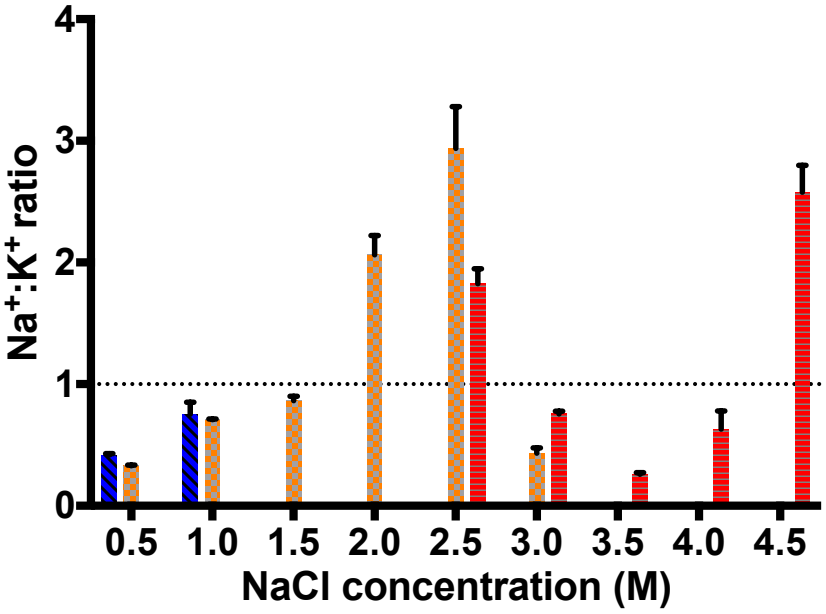

Figure 5.4. Intracellular ion ratios of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in the presence of a range of NaCl concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 4.5M NaCl. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the NaCl concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*; orange bars (checkered); *S. ruber*: red bars (horizontal stripes). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.3. Ion concentrations (g/L) o f *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of NaCl concentrations. *E. coli* (EC), *E. vietnamensis* (EV), and *S. ruber* (SR) were grown in media containing 0.5 – 4.5M NaCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl (M)	Na^+ (g/L)			K^+ (g/L)		
	EC	***EV***	***SR***	***EC***	***EV***	***SR***
0.5	0.010 ± 3E-4	0.010 ± 1E-4	-	0.024 ± 5E-4	0.031 ± 5E-4	-
1	0.140 ± 0.017	0.032 ± 4E-4	-	0.186 ± 0.025	0.046 ± 2E-4	-
1.5	-	0.018 ± 0.001	-	-	0.021 ± 2E-4	-
2	-	0.275 ± 0.003	-	-	0.133 ± 0.019	-
2.5	-	0.225 ± 0.008	1.552 ± 0.054	-	0.076 ± 0.015	0.850 ± 0.085
3	-	17.634 ± 1.305	33.424 ± 0.555	-	40.737 ± 5.129	44.308 ± 2.003
3.5	-	-	4.420 ± 0.047	-	-	17.108 ± 1.600
4	-	-	36.476 ± 6.295	-	-	57.832 ± 17.275
4.5	-	-	27.321 ± 0.798	-	-	10.597 ± 1.511

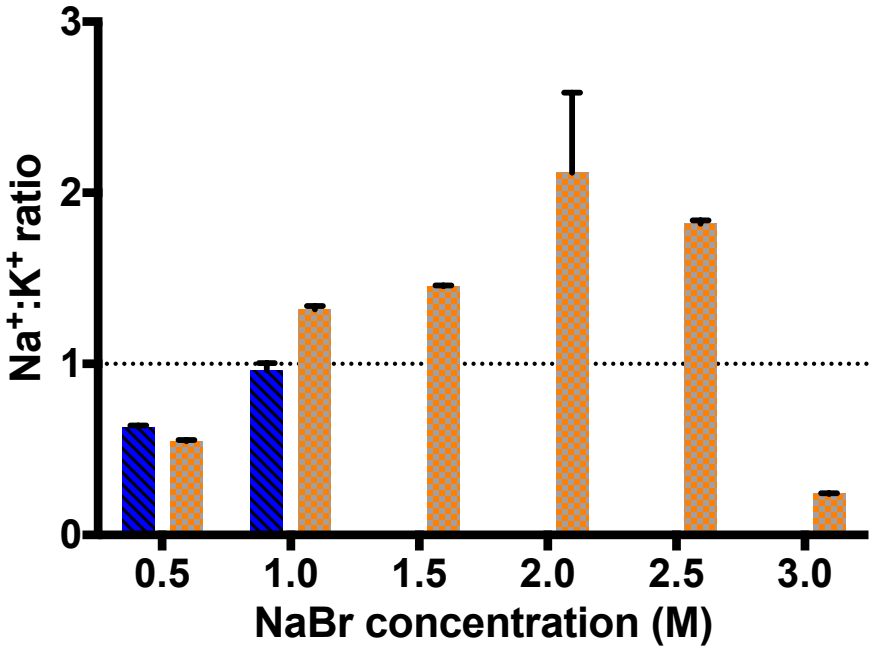

Figure 5.5. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of NaBr concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 3M NaBr. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the NaBr concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*; orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.4. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of NaBr concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 3M NaBr and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaBr (M)	Na^+(g/L)		K^+(g/L)	
	E. coli	*E. vietnamensis*	*E. coli*	*E.vietnamensis*
0.5	0.010 ± 1E-4	0.011 ± 5E-5	0.024 ± 4E-4	0.020 ± 4E-4
1	0.140 ± 0.006	0.024 ± 7E-5	0.186 ± 0.007	0.018 ± 5E-4
1.5	-	0.066 ± 2E-4	-	0.045 ± 9E-5
2	-	7.072 ± 1.080	-	3.340 ± 0.966
2.5	-	7.039 ± 0.083	-	3.869 ± 0.038
3	-	183.797 ± 5.606	-	769.046 ± 7.498

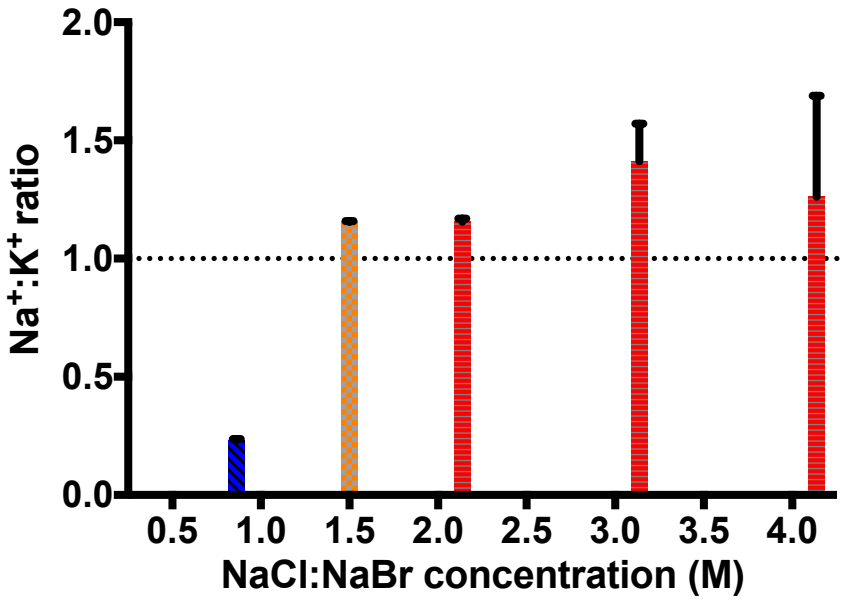

Figure 5.6. Intracellular ion ratios of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in the presence of a range of equimolar NaCl:NaBr concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 4M NaCl:NaBr. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the NaCl:NaBr concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered); *S. ruber*: red bars (horizontal stripes). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.5. Ion concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of equimolar NaCl:NaBr concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 4M NaCl:NaBr and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:NaBr (M)	Na^+ (g/L)			K^+ (g/L)		
	EC	***EV***	***SR***	***EC***	***EV***	***SR***
0.5	0.055 ± 3E-4	-	-	0.239 ± 0.002	-	-
1	-	-	-	-	-	-
1.5	-	0.057 ± 3E-4	-	-	0.050 ± 1E-4	-
2	-	-	3.755 ± 0.019	-	-	3.249 ± 0.058
2.5	-	-	-	-	-	-
3	-	-	20.332 ± 0.894	-	-	14.400 ± 2.607
3.5	-	-	-	-	-	-
4	-	-	57.290 ± 31.509	-	-	45.410 ± 5.767

Summary

Table 5.6. Cation accumulated to the highest levels in the cells. The table shows the cation which was accumulated to the highest levels for the samples analysed in Figures 5.4 – 5.6.

Salt concentration (M)	NaCl			NaBr		NaCl:NaBr		
	Ec	***Ev***	***Sr***	***Ec***	***Ev***	***Ec***	***Ev***	***Sr***
0	K^+	K^+	-	K^+	K^+	K^+	K^+	-
0.5	K^+	K^+	-	K^+	K^+	-	-	-
1	K^+	K^+	-	K^+	Na^+	K^+	-	-
1.5	-	K^+	-	-	Na^+	-	Na^+	-
2	-	Na^+	-	-	Na^+	-	-	Na^+
2.5	-	Na^+	Na^+	-	Na^+	-	-	-
3	-	K^+	K^+	-	K^+	-	-	Na^+
3.5	-	-	K^+	-	-	-	-	-
4	-	-	K^+	-	-	-	-	Na^+
4.5	-	-	Na^+	-	-	-	-	-

5.2.3: Ion accumulation in the presence of potassium

E. coli and *E. vietnamensis* were grown in media containing a range of concentrations of KCl, KBr and equimolar KCl:KBr, and the cellular lysates of these were analysed via ICP-MS. The ratios of Na^+:K^+ present in the cells are shown in Figures 5.7 - 5.9, and the estimated intracellular concentrations are shown in in Tables 5.7 - 5.9, with a summary of these results shown in Table 5.10. Since *S. ruber* could not grow in the presence of KCl or KBr, experiments analysing the replacement of Na^+ with K^+ on intracellular accumulation within *S. ruber* could not be performed on this organism. In addition, cell densities of *E. coli* grown in KBr were low and so cells could not be harvested in sufficient levels for analysis.

It is clear from the data presented that *E. coli* accumulates significantly more K^+ than Na^+; this becomes particularly evident as the KCl concentration increases. This is also the case for *E. vietnamensis*, as it generally accumulates more K^+ than

Na^+. However, at the highest concentration of KCl (3M) the Na^+ concentration in *E. vietnamensis* is higher than the K^+ concentration.

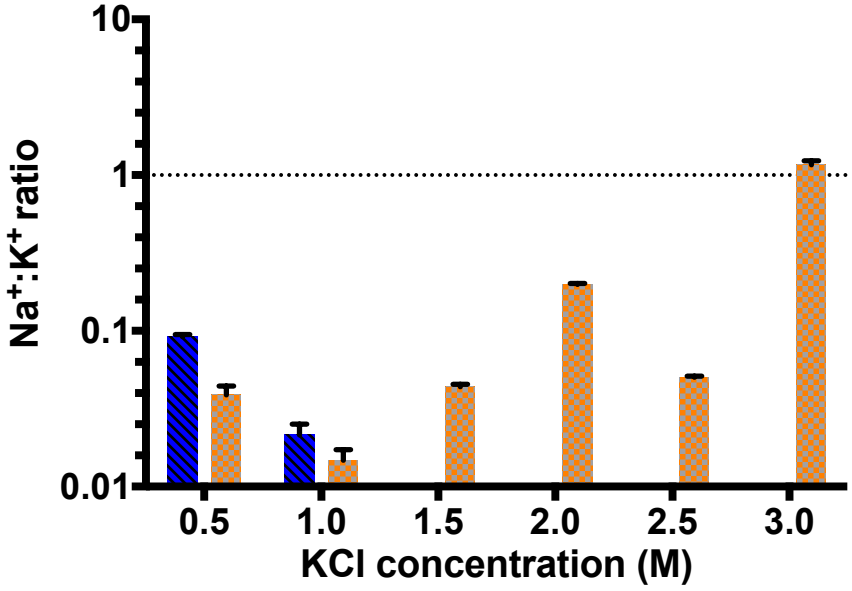

Figure 5.7. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of KCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 3M KCl. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the KCl concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.7. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of KCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 3M KCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl (M)	Na^+(g/L)		K^+(g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.015 ± 6E-4	0.001 ± 1E-4	0.159 ± 0.006	0.038 ± 0.006
1	0.003 ± 6E-4	0.001 ± 2E-4	0.124 ± 0.019	0.057 ± 0.008
1.5	-	0.032 ± 2E-4	-	0.735 ± 0.003
2	-	1.835 ± 0.028	-	9.242 ± 0.160
2.5	-	0.398 ± 0.008	-	7.914 ± 0.091
3	-	27.975 ± 1.457	-	23.995 ± 1.554

***E. vietnamensis* may be able to accumulate molar levels of K^+**

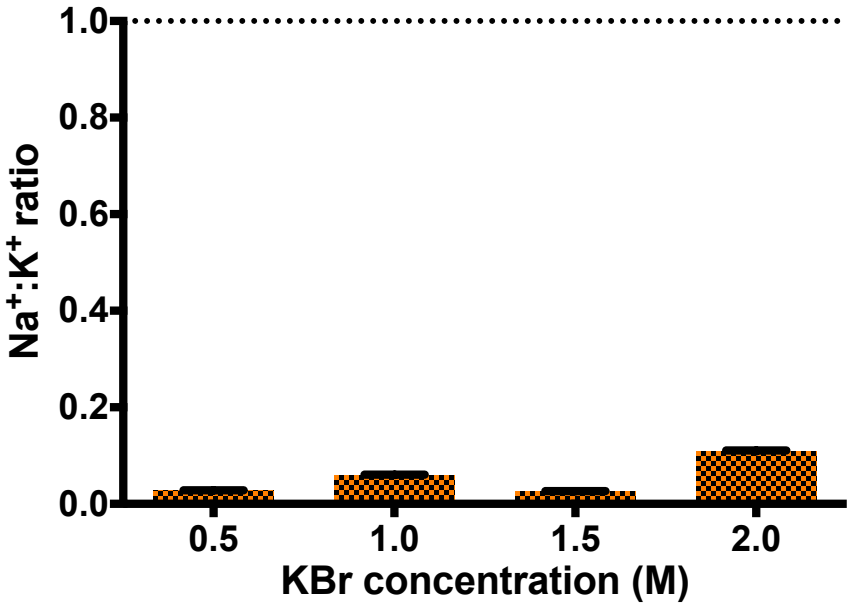

Figure 5.8. Intracellular ion ratios of *E. vietnamensis* when grown in the presence of a range of KBr concentrations. *E. vietnamensis* was grown in media containing 0.5 – 2M KBr. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the KBr concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.8. Ion concentrations (g/L) of *E. vietnamensis* when grown in the presence of a range of KBr concentrations. Cells were grown in media containing 0.5 – 2M KBr and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KBr (M)	Na^+ (g/L)	K^+ (g/L)
0.5	0.001 ± 3E-5	0.028 ± 3E-4
1	0.004 ± 5E-5	0.060 ± 3E-4
1.5	0.043 ± 4E-4	1.648 ± 0.017
2	4.645 ± 0.142	42.853 ± 0.436

E. vietnamensis has a greater cation accumulation capacity than *E. coli*

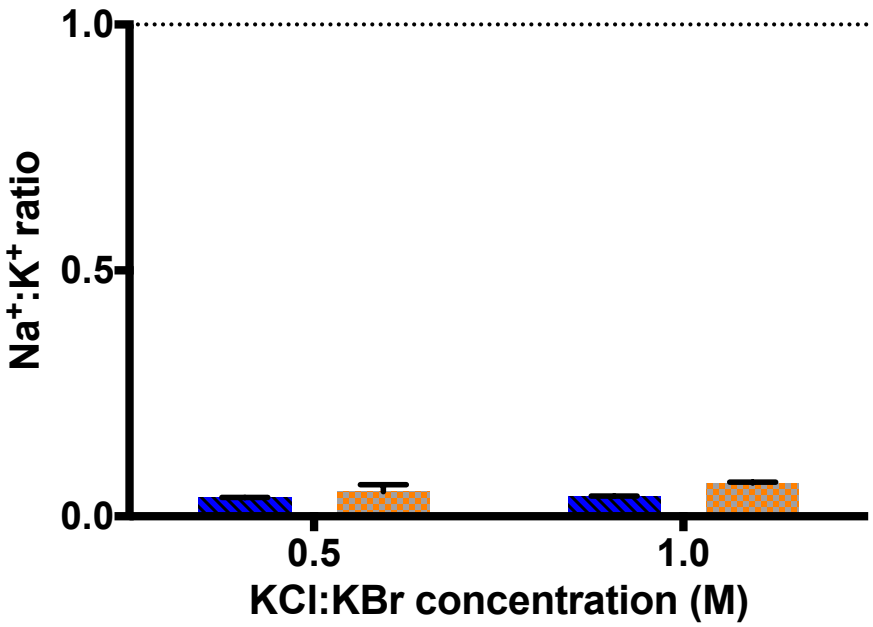

Figure 5.9. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar KCl:KBr concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 1M KCl:KBr. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the KCl:KBr concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.9. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar KCl:KBr concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 1M KCl:KBr and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl:KBr (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.002 ± 6E-5	0.004 ± 0.001	0.044 ± 2E-4	0.084 ± 0.023
1	0.004 ± 4E-5	0.011 ± 3E-4	0.090 ± 0.001	0.167 ± 0.004

Summary

Table 5.10. Cation accumulated in the highest levels in the cells. The table shows the cation which was accumulated to the highest levels for the samples analysed in Figures 5.7 – 5.9.

Salt concentration (M)	KCl		KBr	KCl:KBr	
	Ec	***Ev***	***Ev***	***Ec***	***Ev***
0	K^+	K^+	K^+	K^+	K^+
0.5	K^+	K^+	K^+	K^+	K^+
1	K^+	K^+	K^+	K^+	K^+
1.5	-	K^+	K^+	-	-
2	-	K^+	K^+	-	-
2.5	-	K^+	-	-	-
3	-	Na^+	-	-	-

5.2.4: Ion accumulation in the presence of sodium and potassium

E. coli and *E. vietnamensis* were grown in media containing various equimolar salt combinations of Na^+ and K^+. ICP-MS analysis was carried out on the cellular lysates from these and the ratios of Na^+:K^+ and the estimated intracellular ionic concentrations are shown in Figures 5.10 – 5.13 and Tables 5.11 – 5.14, respectively. Additionally, Table 5.15 shows a summary of these data. *S. ruber* cellular analysis was not performed insufficient biomass being present when grown in these salt combinations (i.e. cell pellets were close to negligible after harvesting).

At lower concentrations (0.5M/1M), cation accumulation within both *E. coli* and *E. vietnamensis* appears similar. In addition, both organisms accumulate higher levels of K^+ than Na^+. However, *E. vietnamensis* appears to have a greater ability for ion accumulation as this increases linearly with increasing media salt concentration. However, in 0.5M equimolar NaBr:KBr, *E. coli* contains much

higher Na^+ concentrations than K^+ (and also higher than *E. vietnamensis*), whereas at 1M it contains a very low Na^+ concentration (below the axis limits).

Non-halophiles may preferentially accumulate K^+ over Na^+ for osmotic balance

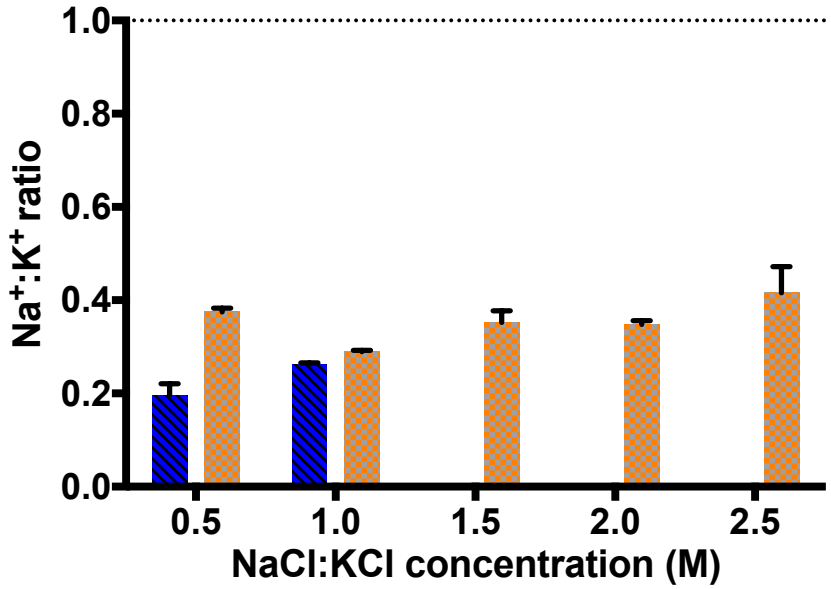

Figure 5.10. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar NaCl:KCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 2.5M NaCl:KCl. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the NaCl:KCl concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.11. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar NaCl:KCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 2.5M NaCl:KCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:KCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.009 ± 0.001	0.018 ± 5E-4	0.043 ± 0.006	0.048 ± 7E-4
1	0.017 ± 6E-5	0.019 ± 3E-4	0.067 ± 0.001	0.067 ± 1E-4
1.5	-	0.273 ± 0.018	-	0.776 ± 0.102
2	-	1.230 ± 0.013	-	3.533 ± 0.128
2.5	-	0.257 ± 0.034	-	0.617 ± 0.085

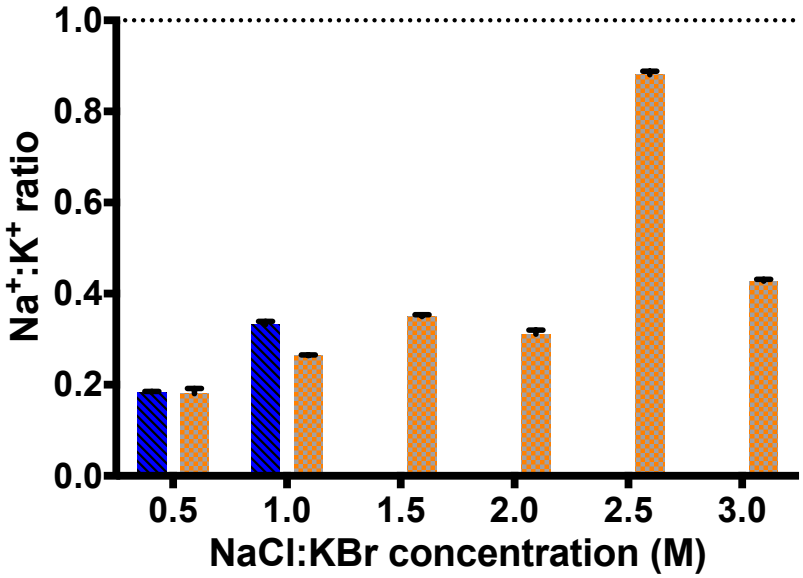

Figure 5.11. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar NaCl:KBr concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 3M NaCl:KBr. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the NaCl:KBr concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.12. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar NaCl:KBr concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 3M NaCl:KBr and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:KBr (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.008 ± 5E-5	0.009 ± 5E-4	0.042 ± 5E-4	0.048 ± 0.003
1	0.034 ± 0.007	0.152 ± 0.001	0.101 ± 0.020	0.576 ± 0.003
1.5	-	0.023 ± 3E-4	-	0.066 ± 8E-4
2	-	0.497 ± 0.005	-	1.598 ± 0.076
2.5	-	10.450 ± 0.136	-	11.866 ± 0.047
3	-	167.478 ± 0.787	-	391.796 ± 5.172

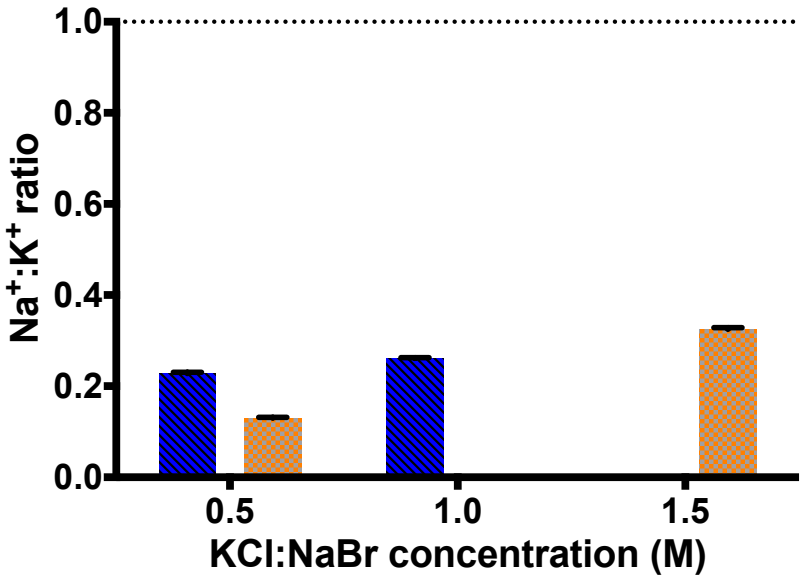

Figure 5.12. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar KCl:NaBr concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 1.5M KCl:NaBr. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the KCl:NaBr concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.13. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar NaBr:KCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 1.5M NaBr:KCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaBr:KCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.009 ± 1E-4	0.008 ± 5E-5	0.040 ± 5E-4	0.062 ± 0.001
1	0.031 ± 4E-4	-	0.117 ± 9E-4	-
1.5	-	0.138 ± 0.002	-	0.425 ± 0.003

Chloride may be required to control intracellular sodium levels

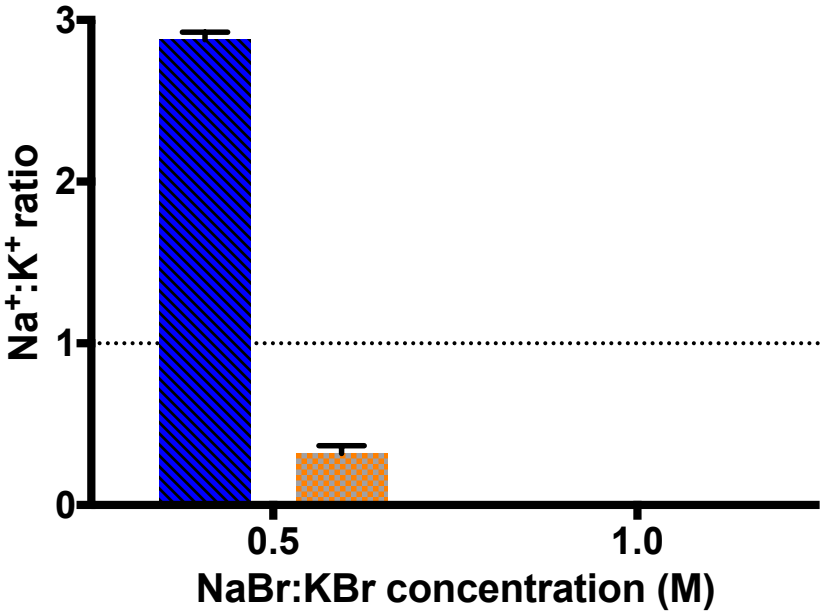

Figure 5.13. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar NaBr:KBr concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 and 1M NaBr:KBr. Data are plotted as the ratio of Na^+:K^+ ions within the cells (Y axis) against the NaBr:KBr concentration (M) (X axis). Values greater than 1 show a greater Na^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.14. Ion concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar NaBr:KBr concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 1M NaBr:KBr and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaBr:KBr (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.123 ± 0.002	0.043 ± 0.007	0.043 ± 8E-4	0.059 ± 0.009
1	0.000	-	0.266 ± 0.002	-

Summary

Table 5.15. Cation accumulated to the highest levels in the cells. The table shows the cation which was accumulated to the highest levels for the samples analysed in Figures 5.10 – 5.13.

Salt concentration (M)	NaCl:KCl		NaCl:KBr		KCl:NaBr		NaBr:KBr	
	Ec	***Ev***	***Ec***	***Ev***	***Ec***	***Ev***	***Ec***	***Ev***
0	K^+	K^+	K^+	K^+	K^+	K^+	K^+	K^+
0.5	K^+	K^+	K^+	K^+	K^+	K^+	Na^+	K^+
1	K^+	K^+	K^+	K^+	K^+	-	K^+	-
1.5	-	K^+	-	K^+	-	K^+	-	-
2	-	K^+	-	K^+	-	-	-	-
2.5	-	K^+	-	K^+	-	-	-	-
3	-	-	-	K^+	-	-	-	-

5.2.5: Effects of lithium on ion accumulation

E. coli, *E. vietnamensis* and *S. ruber* were grown in media containing a range of LiCl concentrations, as well as equimolar combinations of LiCl + NaCl, KCl and NaBr. ICP-MS analysis was performed on the cellular lysates from these and the Na^+:K^+ and Li^+:K^+ ratios are shown in Figures 5.14 – 5.17, and estimated intracellular concentrations are shown in Tables 5.16 – 5.22, with a summary of these results shown in Tables 5.23 and 5.24. Since *S. ruber* could not grow in the presence of pure LiCl, it was excluded from the analysis of this salt. However, only *S. ruber* data is shown for equimolar NaBr:LiCl, since insufficient biomnass was obtained for *E. coli* and *E. vietnamensis* grown in these media.

E. coli accumulates K^+ to the greatest extent (as compared with Na^+ and Li^+) in all salts. *E. vietnamensis* accumulates higher levels of K^+ than both Li^+ and Na^+ in pure LiCl, although the Na^+:K^+ ratio increases with increasing medium salinity. In addition to this, *E. vietnamensis* also accumulates increasing levels of Li^+ with increasing medium salinity, as is also the case for *S. ruber*. *S. ruber*, like the other two organisms, accumulates larger K^+ than Na^+ concentrations in all conditions

except for equimolar NaBr:LiCl – where the Na^+:K^+ ratio is significantly greater than 1.

E. vietnamensis may be able to utilize Li^+ for osmotic balance

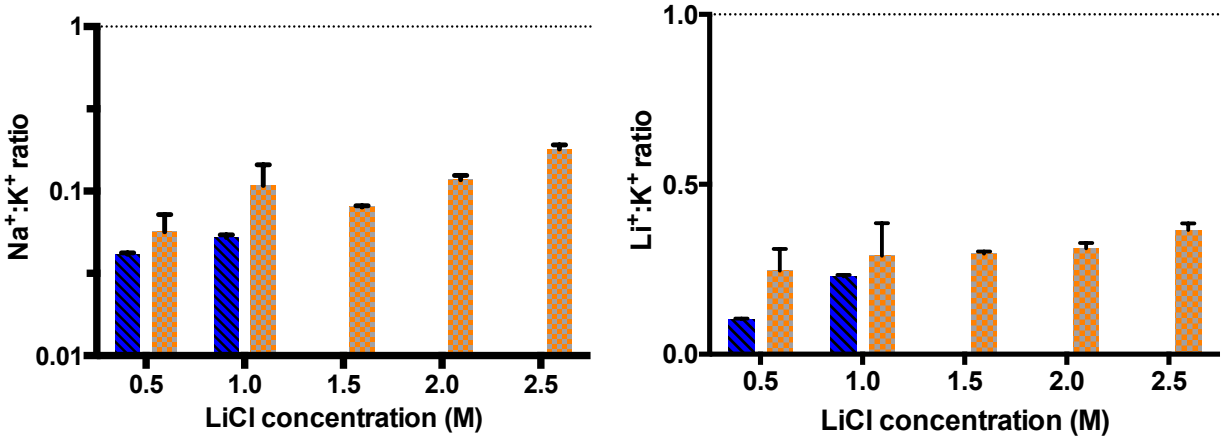

Figure 5.14. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of LiCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 2.5MLiCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Li^+:K^+ ions (right) against the LiCl concentration (M). Values greater than 1 show a greater Na^+/Li^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.16. Na^+ and K^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of LiCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 2.5M LiCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

LiCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	*EV*	*EC*	*EV*
0.5	0.013 ± 4E-4	0.002 ± 6E-4	0.316 ± 0.004	0.027 ± 0.007
1	0.008 ± 5E-4	0.005 ± 0.002	0.146 ± 8E-4	0.044 ± 0.015
1.5	-	0.014 ± 2E-4	-	0.171 ± 0.004
2	-	0.443 ± 0.035	-	3.780 ± 0.177
2.5	-	0.698 ± 0.041	-	3.874 ± 0.220

Table 5.17. Li^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of LiCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 2.5M LiCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

LiCl (M)	Li^+ (g/L)	
	EC	***EV***
0.5	0.033 ± 6E-4	0.007 ± 0.002
1	0.033 ± 0.001	0.013 ± 0.004
1.5	-	0.051 ± 9E-4
2	-	1.179 ± 0.064
2.5	-	1.416 ± 0.070

S. ruber specific ion preference is, from highest to lowest: $Na^+ > K^+ > Li^+$

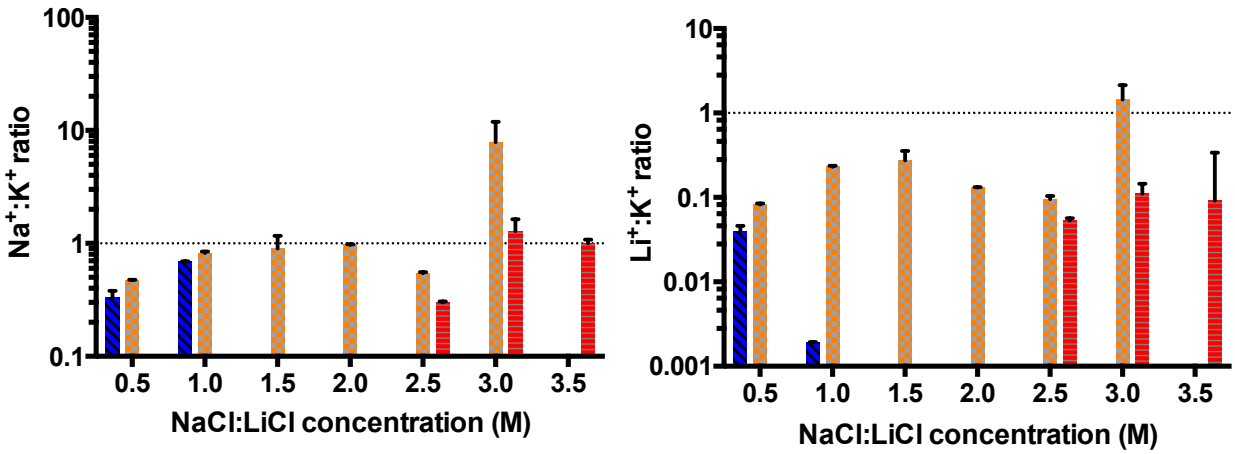

Figure 5.15. Intracellular ion ratios of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in the presence of a range of equimolar NaCl:LiCl concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 3.5M NaCl:LiCl.. Data are plotted as the ratio of Na^+:K^+ ions (left) and Li^+:K^+ ions (right) against the NaCl:LiCl concentration (M). Values greater than 1 show a greater Na^+/Li^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered); *S. ruber*: red bars (horizontal stripes). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.18. Na^+ and K^+ concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of equimolar NaCl:LiCl concentrations. *E. coli* (EC), *E. vietnamensis* (EV) and *S. ruber* (SR) were grown in media containing 0.5 – 3.5M LiCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:LiCl (M)	Na^+ (g/L)			K^+ (g/L)		
	EC	EV	SR	EC	EV	SR
0.5	0.037 ± 0.005	0.009 ± 3E-5	-	0.113 ± 0.016	0.020 ± 3E-4	-
1	0.034 ± 3E-4	0.018 ± 5E-4	-	0.050 ± 1E-4	0.022 ± 8E-4	-
1.5	-	0.045 ± 0.013	-	-	0.050 ± 0.015	-
2	-	0.610 ± 0.005	-	-	0.629 ± 0.008	-
2.5	-	1.469 ± 0.432	4.242 ± 0.054	-	2.739 ± 0.028	14.188 ± 0.312
3	-	21.283 ± 9.152	91.597 ± 6.412	-	2.701 ± 1.580	71.302 ±14.439
3.5	-	-	185.910 ± 3.346	-	-	185.140 ± 24.624

Table 5.19. Li^+ concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of equimolar NaCl:LiCl concentrations. *E. coli* (EC), *E. vietnamensis* (EV) and *S. ruber* (SR) were grown in media containing 0.5 – 3.5M LiCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:LiCl(M)	Li^+ (g/L)		
	EC	EV	SR
0.5	0.004 ± 7E-4	0.002 ± 8E-5	-
1	0.000	0.005 ± 3E-4	-
1.5	-	0.014 ± 0.004	-
2	-	0.082 ± 0.001	-
2.5	-	0.261 ± 0.049	0.752 ± 0.085
3	-	3.895 ± 1.515	7.876 ± 3.382
3.5	-	-	16.882 ± 6.100

S. ruber* intracellular concentrations of Li^+ are higher than for *E. coli* and *E. vietnamensis

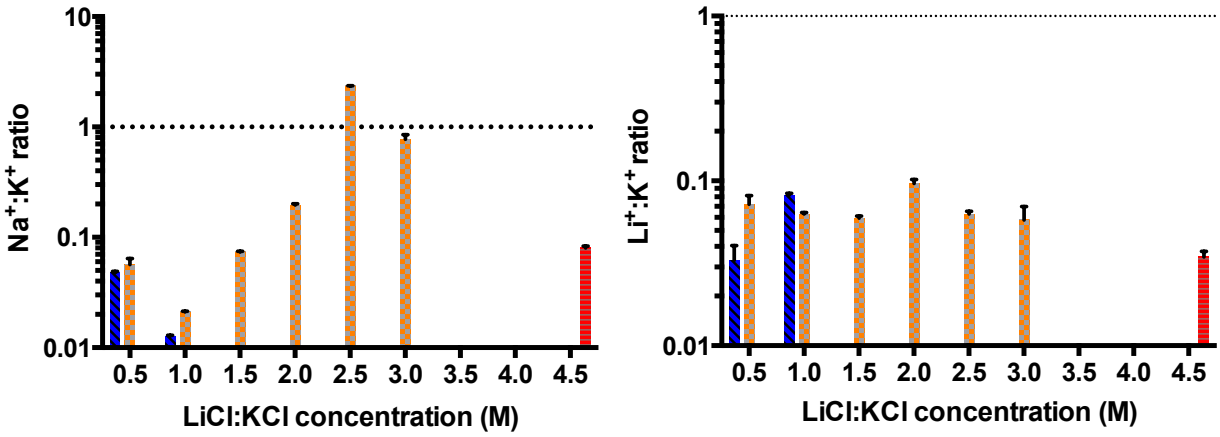

Figure 5.16. Intracellular ion ratios of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in the presence of a range of equimolar KCl:LiCl concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 4.5M NaCl:LiCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Li^+:K^+ ions (right) against the KCl:LiCl concentration (M). Values greater than 1 show a greater Na^+/Li^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered); *S. ruber*: red bars (horizontal stripes). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.20. Na^+ and K^+ concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of equimolar KCl:LiCl concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 4.5M KCl:LiCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl:LiCl (M)	Na^+(g/L)			K^+ (g/L)		
	EC	EV	SR	EC	EV	SR
0.5	0.004 ± 0.001	0.003 ± 4E-4	-	0.094 ± 0.022	0.046 ± 0.006	-
1	0.001 ± 2E-5	0.001 ± 2E-5	-	0.070 ± 9E-4	0.068 ± 4E-4	-
1.5	-	0.008 ± 1E-4	-	-	0.109 ± 0.001	-
2	-	1.150 ± 0.049	-	-	5.881 ± 0.083	-
2.5	-	12.284 ± 0.119	-	-	5.215 ± 0.152	-
3	-	25.864 ± 1.629	-	-	33.448 ± 4.482	-
3.5	-	-	-	-	-	-
4	-	-	-	-	-	-
4.5	-	-	18.541 ±0.927	-	-	231.389 ± 6.942

Table 5.21. Li^+ concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of equimolar KCl:LiCl concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 4.5M KCl:LiCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl:LiCl(M)	Li^+ (g/L)		
	EC	EV	SR
0.5	0.003 ± 7E-4	0.003 ± 4E-4	-
1	0.006 ± 2E-4	0.004 ± 1E-4	-
1.5	-	0.006 ± 3E-4	-
2	-	0.568 ± 0.054	-
2.5	-	0.329 ± 0.015	-
3	-	1.937 ± 0.545	-
3.5	-	-	-
4	-	-	-
4.5	-	-	7.989 ± 1.070

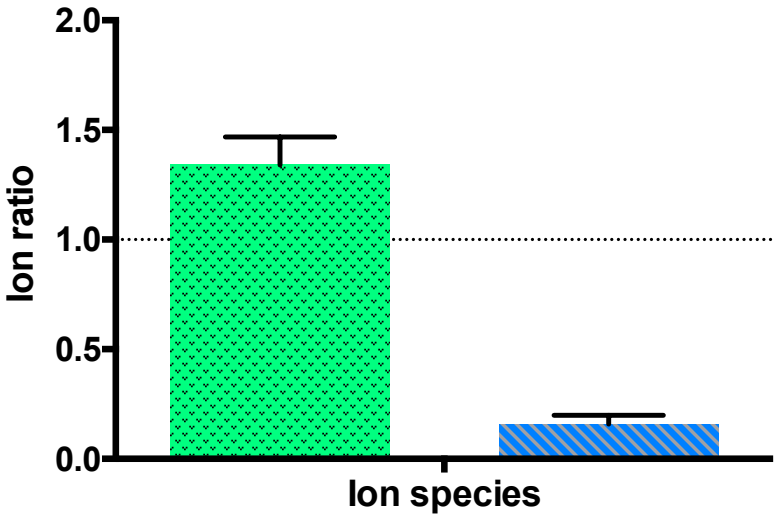

Figure 5.17. Intracellular on ratios of *S. ruber* cells, when grown in the presence of 4.5M equimolar NaBr:LiCl. *S. ruber* was grown in medium containing 4.5M NaBr:LiCl. Data are plotted as the ratio of Na^+:K^+ ions (left – green/dotted bar) and Li^+:K^+ ions (right – blue/striped bar). Values greater than 1 show a greater Na^+/Li^+ concentration than K^+ concentration within the cells. Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.22. Ion concentrations (g/L) of *S. ruber* when grown in the presence of 4.5M equimolar NaBr:LiCl. Estimated cellular ion concentrations are expressed as g/L per cell, with the standard deviation shown (3 measurements).

NaBr:LiCl (M)	Na^+ (g/L)	K^+ (g/L)	Li^+ (g/L)
4.5	115.367 ± 2.928	86.072 ± 14.202	13.647 ± 4.625

Summary

Table 5.23. Levels of cation accumulation in the cells. The table shows a summary to depict the order (highest to lowest) in which cations were accumulated for each of the results presented in Figures 5.14 and 5.15.

Salt concentration (M)	LiCl		NaCl:LiCl		
	Ec	***Ev***	***Ec***	***Ev***	***Sr***
0	$K^+>Na^+>Li^+$	$K^+>Na^+>Li^+$	$K^+>Na^+>Li^+$	$K^+>Na^+>Li^+$	-
0.5	$K^+>Li^+>Na^+$	$K^+>Li^+>Na^+$	$K^+>Na^+>Li^+$	$K^+>Na^+>Li^+$	-
1	$K^+>Li^+>Na^+$	$K^+>Li^+>Na^+$	$K^+>Na^+>Li^+$	$K^+>Na^+>Li^+$	-
1.5	-	$K^+>Li^+>Na^+$	-	$K^+>Na^+>Li^+$	-
2	-	$K^+>Li^+>Na^+$	-	$K^+>Na^+>Li^+$	-
2.5	-	$K^+>Li^+>Na^+$	-	K+>Na+>Li+	$K^+>Na^+>Li^+$
3	-	-	-	$Na^+>Li^+>K^+$	$Na^+>K^+>Li^+$
3.5	-	-	-	-	$Na^+>K^+>Li^+$

Table 5.24. Levels of cation accumulation in the cells. The table shows a summary to depict the order (highest to lowest) in which cations were accumulated for each of the results presented in Figures 5.16 and 5.17.

Salt concentration (M)	LiCl:KCl		NaBr:LiCl
	Ec	***Ev***	***Sr***
0	$K^+>Na^+>Li^+$	$K^+>Na^+>Li^+$	-
0.5	$K^+>Na^+>Li^+$	$K^+>Na^+\sim Li^+$	-
1	$K^+>Li^+>Na^+$	$K^+>Li^+>Na^+$	-
1.5	-	$K^+>Na^+>Li^+$	-
2	-	$K^+>Na^+>Li^+$	-
2.5	-	$Na^+>K^+>Li^+$	-
3	-	$K^+>Na^+>Li^+$	-
3.5	-	-	-
4	-	-	-
4.5	-	-	$Na^+>K^+>Li^+$

5.2.6: Effects of larger cations on ion accumulation

E. coli and *E. vietnamensis* were grown in media containing RbCl, CsCl and equimolar combinations of RbCl/CsCl + NaCl/KCl. *S. ruber* cells were able to be

harvested after being grown in the presence of equimolar NaCl + RbCl, but no other combination resulted in enough biomass for analysis. It should also be noted that even though *E. coli* did not actively grow within these media cell density was high enough for cells to be harvested and analysed. ICP-MS analysis was performed on the cellular lysates from these, which are shown as Na^+:K^+, Rb^+:K^+ and Cs^+:K^+ ratios, in Figures 5.18 – 5.23 and the estimated intracellular concentrations are shown in Tables 5.25 – 5.36, with summaries of these data shown in Tables 5.37 and 5.38.

Within media containing RbCl, *E. coli* accumulates Rb^+ to levels higher than *E. vietnamensis* (at the same medium concentration). However, *E. vietnamensis* has the ability to accumulate large levels of Rb^+ (almost 6 times the level of K^+ accumulated), especially at higher external salinity and when Rb^+ is the only cation present.

When grown in the presence of CsCl, Cs^+ accumulation in E*. coli* is higher than K^+. *E. vietnamensis* Cs^+:K^+ ratios at 0.5M are lower than for *E. coli*, but it accumulated increasing levels with increasing external salinity. Moreover, *E. coli* accumulated higher levels of all 3 ions in equimolar NaCl:CsCl than *E. vietnamensis* (as well as containing larger Cs^+:K^+ ratios than *E. vietnamensis*) – in contradiction to all of the previous findings. Additionally, *S. ruber* accumulated both Na^+ and K^+ (preference of Na^+) to high levels, and also accumulates significant levels of Rb^+.

Rb^+:K^+ ratio may be a major factor for cellular toxicity – accumulation of Rb^+ may inhibit growth

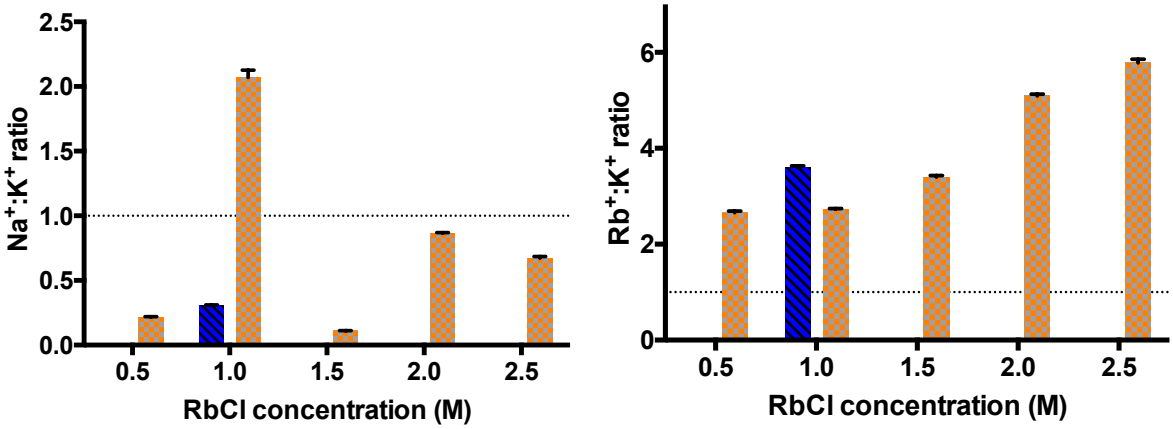

Figure 5.18. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of RbCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 2.5M RbCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Rb^+:K^+ ions (right) against the RbCl concentration (M). Values greater than 1 show a greater Na^+/Rb^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.25. Na^+ and K^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of RbCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 2.5M RbCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

RbCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	-	0.004 ± 5E-5	-	0.020 ± 3E-4
1	0.031 ± 5E-4	0.078 ± 0.004	0.101 ± 0.002	0.037 ± 2E-4
1.5	-	0.013 ± 6E-5	-	0.114 ± 0.002
2	-	2.181 ± 0.007	-	2.516 ± 0.018
2.5	-	3.418 ± 0.068	-	5.081 ± 0.096

Table 5.26. Rb^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of RbCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 2.5M RbCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

RbCl (M)	Rb^+ (g/L)	
	EC	***EV***
0.5	-	0.054 ± 4E-4
1	0.364 ± 0.001	0.102 ± 7E-4
1.5	-	0.389 ± 6E-4
2	-	12.821 ± 0.076
2.5	-	29.387 ± 0.240

Cs^+ toxicity may related to levels of intracellular accumulation – as well as being organism specific

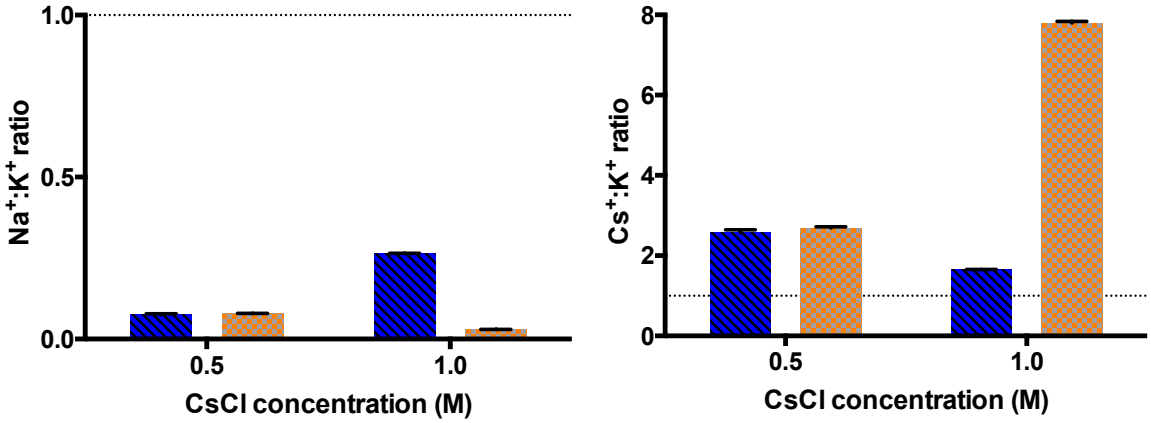

Figure 5.19. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of CsCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 1M CsCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Cs^+:K^+ ions (right) against the CsCl concentration (M). Values greater than 1 show a greater Na^+/Cs^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal strpes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.27. Na^+ and K^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of CsCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 1M CsCl and cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

CsCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.004 ± 3E-5	0.003 ± 7E-6	0.049 ± 0.002	0.036 ± 4E-4
1	0.024 ± 3E-5	0.001 ± 3E-5	0.090 ± 4E-4	0.025 ± 4E-5

Table 5.28. Cs^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of CsCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 1M CsCl and cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

CsCl (M)	Cs^+ (g/L)	
	EC	***EV***
0.5	0.126 ± 9E-4	0.096 ± 0.001
1	0.149 ± 4E-4	0.195 ± 0.002

Adding Na^+ to Rb^+ decreases the level of Rb^+ accumulation

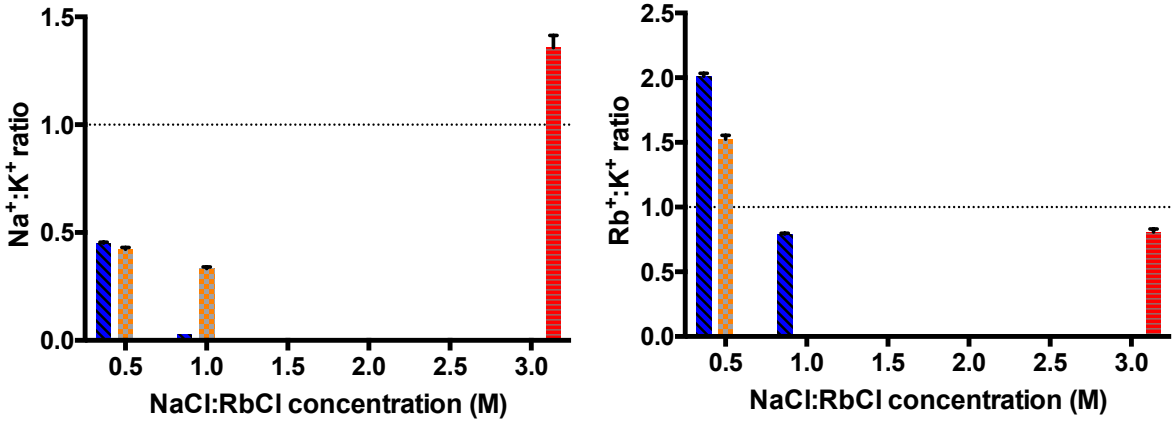

Figure 5.20. Intracellular ion ratios of *E. coli*, *E. vietnamensis* and *S. ruber*, when grown in the presence of a range of equimolar NaCl:RbCl concentrations. *E. coli*, *E. vietnamensis* and *S. ruber* were grown in media containing 0.5 – 3M NaCl:RbCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Rb^+:K^+ ions (right) against the NaCl:RbCl concentration (M). Values greater than 1 show a greater Na^+/Rb^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered); *S. ruber*: red bars (horizontal stripes). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.29. Na^+ and K^+ concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of equimolar NaCl:RbCl concentrations. *E. coli* (EC), *E. vietnamensis* (EV) and *S. ruber* (SR) were grown in media containing 0.5 – 3M NaCl:RbCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:RbCl (M)	Na^+ (g/L)			K^+ (g/L)		
	EC	EV	SR	EC	EV	SR
0.5	0.013 ± 1E-4	0.009 ± 4E-4	-	0.029 ± 3E-4	0.022 ± 6E-4	-
1	0.002 ± 2E-5	0.006 ± 1E-4	-	0.088 ± 0.004	0.019 ± 5E-4	-
1.5	-	-	-	-	-	-
2	-	-	-	-	-	-
2.5	-	-	-	-	-	-
3	-	-	50.377 ± 1.511	-	-	37.096 ± 1.914

Table 5.30. Rb^+ concentrations (g/L) of *E. coli*, *E. vietnamensis* and *S. ruber* when grown in the presence of a range of equimolar NaCl:RbCl concentrations. *E. coli* (EC), *E. vietnamensis* (EV) and *S. ruber* (SR) were grown in media containing 0.5 – 3M NaCl:RbCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:RbCl(M)	Rb^+ (g/L)		
	EC	EV	SR
0.5	0.058 ± 7E-4	0.033 ± 4E-4	-
1	0.064 ± 6E-4	0.000	-
1.5	-	-	-
2	-	-	-
2.5	-	-	29.970 ± 0.168
3			

Cs^+ can accumulate inside *E. coli* cells at higher levels than K^+

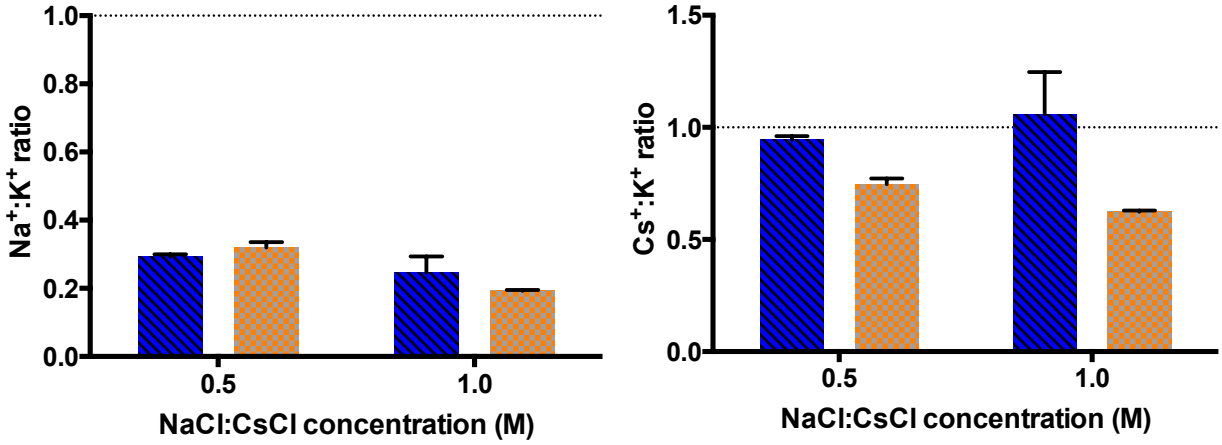

Figure 5.21. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar NaCl:CsCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 1M NaCl:CsCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Cs^+:K^+ ions (right) against the NaCl:CsCl concentration (M). Values greater than 1 show a greater Na^+/Cs^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.31. Na^+ and K^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar NaCl:CsCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 and 1M NaCl:CsCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:CsCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.021 ± 5E-4	0.027 ± 0.001	0.072 ± 0.002	0.086 ± 0.004
1	0.058 ± 0.012	0.010 ± 2E-4	0.230 ± 0.046	0.052 ± 5E-4

Table 5.32. Cs^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar NaCl:CsCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 and 1M NaCl:CsCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

NaCl:CsCl (M)	Cs^+ (g/L)	
	EC	***EV***
0.5	0.068 ± 8E-4	0.064 ± 0.001
1	0.248 ± 0.040	0.032 ± 3E-4

Rb^+ is accumulated in *E. coli* to much higher levels than in *E. vietnamensis*

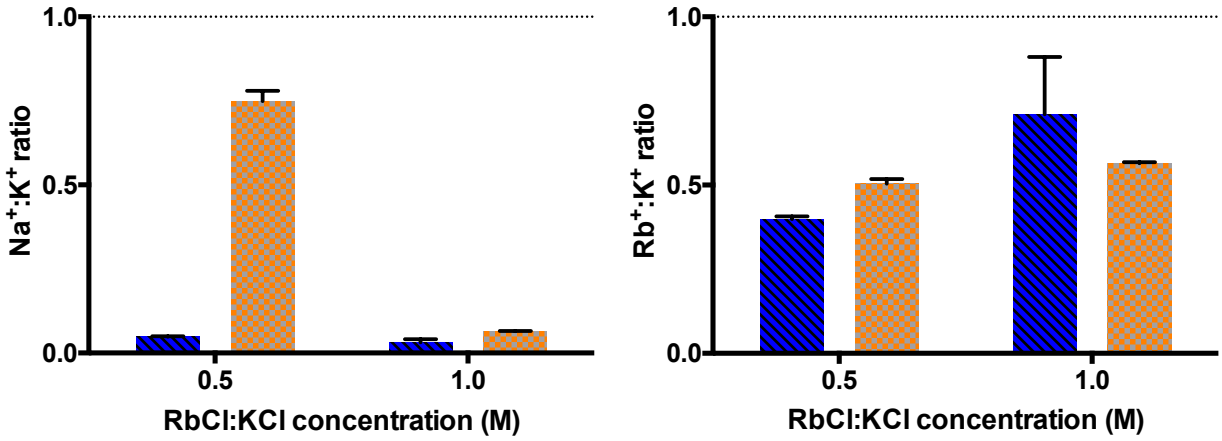

Figure 5.22. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar KCl:RbCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 and 1M KCl:RbCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Rb^+:K^+ ions (right) against the KCl:RbCl concentration (M). Values greater than 1 show a greater Na^+/Rb^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.33. Na^+ and K^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar KCl:RbCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 and 1M KCl:RbCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl:RbCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.005 ± 3E-4	0.022 ± 0.001	0.104 ± 0.002	0.030 ± 0.001
1	0.005 ± 0.002	0.002 ± 2E-5	0.153 ± 0.039	0.034 ± 3E-4

Table 5.34. Rb^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar KCl:RbCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 and 1M KCl:RbCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl:RbCl (M)	Rb^+ (g/L)	
	EC	***EV***
0.5	0.042 ± 8E-4	0.015 ± 4E-4
1	0.108 ± 0.025	0.019 ± 4E-5

Cs^+ cellular accumulation may be linked to toxicity – but seems to be organism-specific

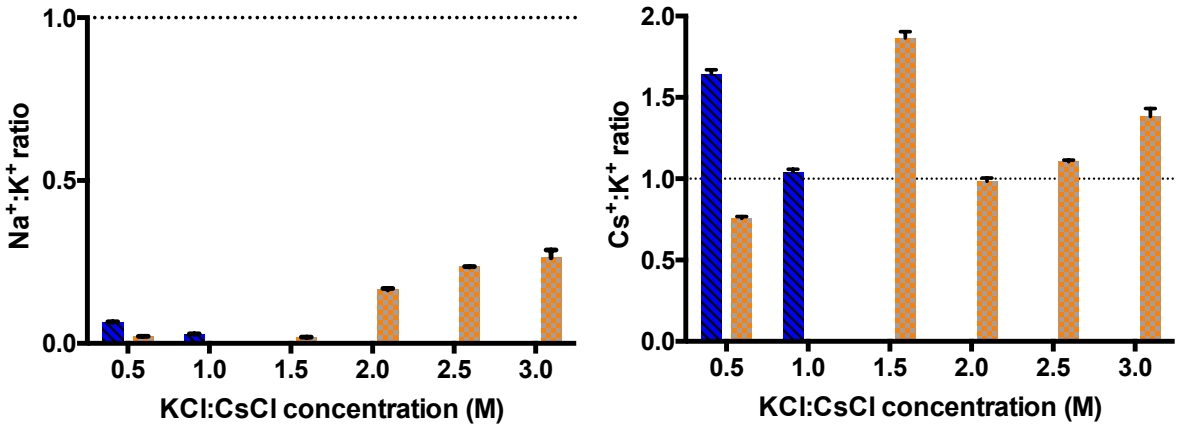

Figure 5.23. Intracellular ion ratios of *E. coli* and *E. vietnamensis*, when grown in the presence of a range of equimolar KCl:CsCl concentrations. *E. coli* and *E. vietnamensis* were grown in media containing 0.5 – 3M KCl:CsCl. Data are plotted as the ratio of Na^+:K^+ ions (left) and Cs^+:K^+ ions (right) against the KCl:CsCl concentration (M). Values greater than 1 show a greater Na^+/Cs^+ concentration than K^+ concentration within the cells. *E. coli*: blue bars (diagonal stripes); *E. vietnamensis*: orange bars (checkered). Error bars refer to the standard deviation of the ion ratios in the samples.

Table 5.35. Na^+ and K^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar KCl:CsCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 3M KCl:CsCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl:CsCl (M)	Na^+ (g/L)		K^+ (g/L)	
	EC	***EV***	***EC***	***EV***
0.5	0.009 ± 3E-4	0.001 ± 1E-5	0.138 ± 0.002	0.050 ± 0.002
1	0.010 ± 3E-4	-	0.341 ± 0.002	-
1.5	-	0.002 ± 7E-5	-	0.080 ± 3E-4
2	-	1.161 ± 0.043	-	0.980 ± 0.013
2.5	-	0.264 ± 0.039	-	1.125 ± 0.017
3	-	3.404 ± 0.234	-	12.952 ± 1.443

Table 5.36. Cs^+ concentrations (g/L) of *E. coli* and *E. vietnamensis* when grown in the presence of a range of equimolar KCl:CsCl concentrations. *E. coli* (EC) and *E. vietnamensis* (EV) were grown in media containing 0.5 – 3M KCl:CsCl and estimated cellular concentrations (g/L) are shown, with the standard deviation shown (3 measurements).

KCl:CsCl (M)	Cs^+ (g/L)	
	EC	***EV***
0.5	0.227 ± 2E-4	0.038 ± 0.001
1	0.355 ± 0.002	-
1.5	-	0.150 ± 0.001
2	-	0.964 ± 0.004
2.5	-	1.244 ± 0.002
3	-	17.911 ± 0.042

Summary

Table 5.37. Levels of cation accumulation in the cells when grown in Rb^+ containing media.
The table shows a summary to depict the order in which cations were accumulated (highest to lowest) for each of the results presented in Figures 5.18, 5.20 and 5.22.

Salt concentration (M)	RbCl		RbCl:NaCl			RbCl:KCl	
	Ec	***Ev***	***Ec***	***Ev***	***Sr***	***Ec***	***Ev***
0	$K^+>Na^+>Rb^+$	$K^+>Na^+>Rb^+$	$K^+>Na^+>Rb^+$	$K^+>Na^+>Rb^+$	-	$K^+>Na^+>Rb^+$	$K^+>Na^+>Rb^+$
0.5	-	$Rb^+>Na^+>K^+$	$Rb^+>K^+>Na^+$	$Rb^+>K^+>Na^+$	-	$K^+>Rb^+>Na^+$	$K^+>Na^+>Rb^+$
1	$Rb^+>K^+>Na^+$	$Rb^+>K^+>Na^+$	$K^+>Rb^+>Na^+$	$K^+>Na^+>Rb^+$	-	$K^+>Rb^+>Na^+$	$K^+>Rb^+>Na^+$
1.5	-	$Rb^+>K^+>Na^+$	-	-	-	-	-
2	-	$Rb^+>K^+>Na^+$	-	-	-	-	-
2.5	-	$Rb^+>K^+>Na^+$	-	-		-	-
3	-	-	-	-	$Na^+>K^+>Rb^+$	-	-

Table 5.38. Levels of cation accumulation in the cells when grown in Cs^+ containing media.
The table shows a summary to depict the order in which cations were accumulated (highest to lowest) for each of the results presented in Figures 5.19, 5.21 and 5.23.

Salt concentration (M)	CsCl		CsCl:NaCl		CsCl:KCl	
	Ec	***Ev***	***Ec***	***Ev***	***Ec***	***Ev***
0	$K^+>Na^+>Cs^+$	$K^+>Na^+>Cs^+$	$K^+>Na^+>Cs^+$	$K^+>Na^+>Cs^+$	$K^+>Na^+>Cs^+$	$K^+>Na^+>Cs^+$
0.5	$Cs^+>K^+>Na^+$	$Cs^+>K^+>Na^+$	$K^+>Cs^+>Na^+$	$K^+>Cs^+>Na^+$	$Cs^+>K^+>Na+$	$K^+>Cs^+>Na^+$
1	$Cs^+>K^+>Na^+$	$Cs^+>K^+>Na^+$	$Cs^+>K^+Na^+$	$K^+>Cs^+>Na^+$	$Cs^+>K^+>Na^+$	-
1.5	-	-	-	-	-	$Cs^+>K^+>Na^+$
2	-	-	-	-	-	$Na^+>K^+>Cs^+$
2.5	-	-	-	-	-	$Cs^+>K^+>Na^+$
3	-	-	-	-	-	$Cs^+>K^+>Na^+$

5.3: Discussion

General comments

Overall, when no salts were added to the medium the level of ion accumulation for *E. coli* and *E. vietnamensis* is very similar, which suggests that both organisms have similar cytoplasmic ion contents when no osmotic stress is present, i.e. similar osmotic responses. The fact that these organisms both occupy very different niches [298,492] is indicative of a general response in common to all non-halophilic organisms, at least regarding lower salinities. It has been reported previously that the K^+ content of *E. coli* will increase linearly with osmotic stress (under lower salt conditions, i.e. below 1M), so when osmotic stress is not present then K^+ concentrations inside the cell will be relatively low [474]. In addition, glutamate synthesis in *E. coli* cells is dependent upon the presence of K^+ in the medium [8], so this could perhaps explain why *E. coli* (and *E. vietnamensis*) growth is better in KCl than in NaCl, as well as the general preference of these organisms to accumulate higher concentrations of K^+ over Na^+ (note that these media contain greater Na^+ than K^+ concentrations). Moreover, from the data presented here the external salinity appears to be the main factor for K^+ accumulation within *E. coli*, even if the salinity is a consequence of a non-potassium containing solute, such as NaCl, which has been reported previously [163].

Halophilic ion accumulation may not be as simple as originally thought

S. ruber accumulates high levels of cations within its cytoplasm when grown at higher salt concentrations. In the 'optimal media', *S. ruber* was found to contain several hundred times the level of Na^+ and K^+ as *E. vietnamensis*. This is consistent with *S. ruber* being a salt-in halophile, described to accumulate inorganic ions in their cytoplasm in excess of the concentration in the

environment, as opposed to using compatible solutes [361]. It has been found previously that *S. ruber* does not use compatible solutes for osmotic balance [493]. Since the ion concentrations inside *S. ruber* cells are generally many times higher than in the non-halophilic cells, this is consistent with it being a salt-in halophile [84]. Important to note however is that *E. vietnamensis* also appears to have the ability to accumulate large concentrations of cations, when the external salt concentration is very high (2.5 – 3M external salinity) – this will be discussed in more detail later on.

It is clear that the ratio between the different ions in *S. ruber* is different from the other two organisms, with *S. ruber* accumulating more Na^+. However, *E. vietnamensis* often also contains larger Na^+:K^+ ratios than *E. coli*, similar to that of *S. ruber*. Moreover, *S. ruber* does not always accumulate Na^+ in excess of K^+ - in fact, in several experiments the K^+ concentration exceeded that of the Na^+ concentration (refer to Figures 5.2, 5.4 and 5.16). However, the ion ratios within *S. ruber* are different from *E. coli* and it would appear that S*. ruber* uses Na^+ for osmotic balance preferentially over K^+, but K^+ may still have a (perhaps less significant) role in the osmotic balance of this organism, but cannot replace Na^+ in terms of growth effects. This is in contrast to what has previously been found for the salt-in halophiles, and for this organism [181,494]. From a molecular perspective, it is intriguing that halophilic organisms have been reported to exclude Na^+ and accumulate K^+, since the malate dehydrogenase from the halophile *H. marismortui* has been found to be more stable in the presence of NaCl than in KCl [208]. This follows from the fact that the LMWA predicts that Na^+ will interact more strongly with the increased level of COO^- groups present on the surfaces of halophilic proteins, whereas K^+ would not be able to provide as efficient stabilisation due to its different water affinity to that of the COO^- groups [226,240]. However, at higher cytoplasmic concentrations, Na^+ could result in greater protein destabilisation than K^+. It is therefore suggested that the preference of *S. ruber* for Na^+ over K^+ may due to the lower optimal salinity required for the growth of *S. ruber*, as compared to other salt-in halophiles (refer to Chapter 4). This may hence affect its specific ion metabolism – i.e. lower levels of Na^+ accumulation are needed for osmotic balance at lower external salt

concentrations, therefore avoiding potentially destabilizing effects of increased concentrations of this cation within its cytoplasm. This may be why other salt-in halophiles have been reported to mostly accumulate K^+, as they require to accumulate an increased level of this cation due to their higher optimal salinity for growth. On the other hand, K^+ concentrations within *S. ruber* are often higher than the external medium, suggesting that *S. ruber* may also actively sequester this cation. Therefore, this study suggests that Na^+ may be more essential to the halophilic lifestyle than previously thought, and the salinity growth range of an organism may determine its specific ion metabolism.

S. ruber was found to have the ability to accumulate high levels of the non-physiological cations Li^+ and Rb^+. *S. ruber* Rb^+ and Cs^+ concentrations cannot be compared as *S. ruber* cells grown in the presence of Cs^+ were not analysed (due to insufficient biomass). However, *S. ruber* accumulated higher levels of Rb^+ than Li^+, which could be due to the similar size of Rb^+ with K^+, hence its greater ability to enter the cell (refer to section 4.3). The closest previous study to the current study is a smaller-scale analysis carried out by Jensen *et al.*, whereby ICP-MS was used to analyse the intracellular concentrations of ions within the halophile *H. marismortui* when exposed to the same alkali cations as were used in the present study (at much lower concentrations than used in the present study). It was found that the larger cations, Rb^+ and Cs^+ were less readily transported into the cell than smaller cations, which they postulated may be due to the large size of these ions [10], which may be the opposite from what was currently found regarding *S. ruber*. Other than the Jensen *et al.* study, there have been no other studies to analyse the effects on growth of a halophile of ions present in the environment in trace amounts, such as Li^+, Rb^+ and Cs^+.

Halophilic cation accumulation may follow a modified Hofmeister series, whereas non-halophilic cation accumulation follows a more direct Hofmeister series

From the data presented in the current study, it would appear that *S. ruber* preferentially accumulates the higher charge density Na^+ at the expense of lower

charge density cations, whereas *E. coli* and *E. vietnamensis* show the opposite trend, accumulating lower charge density cations over higher charge density cations. These differences could be explained based on the Hofmeister series, where stabilising cations (regarding intracellular proteins) may vary between halophiles and non-halophiles, based upon the higher level of acidic residues present on the surfaces of halophilic proteins [206] and their consequential increased affinity for smaller cations. The organisms were found to accumulate cations in the following order (from most highly accumulated to least):

E. coli: $Cs^+ \sim Rb^+ \sim K^+ > Na^+ > Li^+$
E. vietnamensis: $Cs^+ \sim Rb^+ \sim K^+ > Na^+ > Li^+$
S. ruber: $Na^+ > K^+ > Rb^+ > Li^+$

The traditional Hofmeister series for the cations used in this study is: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ [213]. Therefore, it would appear that the order for *S. ruber* ion accumulation is slightly different, with a preference for higher (but not the highest) charge density cations, whereas *E. vietnamensis* ion accumulation follows a direct Hofmeister series – *E. coli* ion accumulation is similar to that of *E. vietnamensis*. Based on the above series created for each organism, these are consistent with the theory of halophiles having a general preference for higher over lower charge density cations, and vice versa for non-halophiles – which may be based on the fact that cations of higher charge density are able to form contact ion pairs with carboxylic acid groups (i.e. on acidic residues) [227], whereas lower charge density cations cannot and will hence be favoured for non-halophiles, due to their less disruptive behaviour towards cellular proteins. Therefore, the specific ion preferences of an organism may be related to the degree of effects it produces on the intracellular proteins, with halophilic organisms displaying accumulation based on a modified Hofmeister series – this will be explored further in Chapter 6.

Rb^+ can be efficiently transported via K^+ transporters but may be an ineffective K^+ replacement

The fact that *E. coli* incubated in the presence of RbCl contained such high Rb^+ levels suggests that this accumulation may be the cause of the lack of growth, since *E. coli* was found to contain higher levels of Rb^+ than *E. vietnamensis*. It has been found that Rb^+ can be transported via the TrkA protein [495]. This is present in *E. coli* but not in *E. vietnamensis*, (refer to section 3.2.4), which may explain this discrepancy between the levels of Rb^+ in the two organisms. Moreover, the fact that the Rb^+ concentrations inside both *E. coli* and *E. vietnamensis* are a lot lower in the presence of Na^++ Rb^+ than in pure RbCl suggests that NaCl may help to lower Rb^+ accumulation, which may consequentially stimulate *E. coli* growth, which is similar to what has been found in a previous study, regarding the addition of K^+/Na^+ to Cs^+ [128]. The Rb^+ concentrations inside *E. vietnamensis* were only high when pure RbCl was used, suggesting that it utilises this cation only when no others are available. It was found for the halotolerant bacterium *Brevibacterium sp.*, that when Rb^+ was added to the medium, the Rb^+ concentration within the cell increased by a hundred-fold, suggesting a potential 'halotolerant' ability for the accumulation of non-physiological ions [478].

The fact that *S. ruber* was found to contain substantial levels of Rb^+, suggests that the adaptation of the *S. ruber* proteome may be the reason why this organism is able to grow within the presence of this cation, whereas *E. coli* is not. *S. ruber* proteins contain higher levels of COO^- groups on their surfaces, due to the higher level of acidic amino acids – as a consequence Rb^+ may be less likely to be adsorbed to the surfaces of these proteins. This could be explained by the law of matching water affinity, as Rb^+ may be more likely to be adsorbed to the surface of *E. coli* proteins due to the higher polarisibility of Rb^+ and hence may interact with more neutral groups (such as COOH) on the protein surface, which may be more predominant in *E. coli* than *S. ruber* proteins.

It has been found previously that Rb^+ is able to block outward K^+ channels in squid axonal cells, which suggests that Rb^+ may have the potential to block K^+ efflux channels, once inside the cell, which could potentially be another explanation relating the observed *E. coli* growth inhibition and Rb^+ accumulation levels [449]. In addition to this, Avery *et al* (1991) reported that this cation being added to the medium of *Synechosystis* lead to most of the internal K^+ being lost [109]. However, there appears to be no clear correlation in the present study suggesting that Rb^+ presence affects the K^+ concentration within the cells. Additionally, Meury *et al* (1985) found that when *E. coli* cells took up Rb^+ via the Trk transporter, no efflux was observed, as the osmodependent efflux pathway is not able to transport Rb^+ (in place of K^+) [107]. This may give an additional insight into the high Rb^+ concentrations found in the *E. coli* cells in this study: it could be the case that they can enter the cells but then have no way of effluxing and so remain trapped inside. This could further explain why *E. coli* has a greater internal Rb^+ concentration than *E. vietnamensis*.

It would be expected that Rb^+ concentrations will generally be higher in the bacterial cells than Cs^+ concentrations, due to the fact that both Trk and kup can transport Rb^+ [136,445]. However, Cs^+ concentrations in *E. coli* are often higher than Rb^+ concentrations (at comparable external salinities), which suggests that these ions may have different affinities for membrane transporters or have different effects once inside the cells. This effect is more pronounced for *E. coli* than *E. vietnamensis*, possibly due to absence of these transporters in *E. vietnamensis* (refer to section 3.2.4).

Cs^+ toxicity is consequence of specific transporters and cellular accumulation

The concentrations of Cs^+ found within *E. coli* are higher than that found in *E. vietnamensis*, which suggests that the main mechanism of toxicity may be due to the level of accumulation of Cs^+ - due to the fact that *E. vietnamensis* was able to grow in the presence of Cs^+, it would appear that these Cs^+ effects may be specific to *E. coli*. This indicates a role of a K^+ transporter only found in *E. coli*, which

might explain these higher cellular Cs^+ levels. As mentioned previously (Chapter 4), the kup K^+ transporter (not present in *E. vietnamensis* or *S. ruber*) has been found to be able to transport both Rb^+ and Cs^+, and in addition, Cs^+ has also been found to be able to block the Trk transporters [445]. This Cs^+ effect could also involve a block of efflux transport as in the experiments in the present study using equimolar KCl:CsCl (Tables 5.35 and 5.36), the K^+ concentrations are higher than they are in any other experiment, regarding *E. coli*. It has been found that both Cs^+ and Rb^+ can block the outward current in the squid giant axon, so a role of Cs^+/Rb^+ preventing cation efflux is not unreasonable to speculate [449]. It is crucial to note that the highest ion concentrations measured for *E. coli* were for Rb^+ and Cs^+ - 0.35g/L Cs^+ in KCl:CsCl and 0.36g/L Rb^+ in RbCl (Tables 5.36 and 5.26, respectively). Clearly, the presence of high levels of Cs^+ is associated with cellular toxicity, as has been found in previous studies [496], and this seems like the most likely cause of the growth effects on *E. coli*, as E*. vietnamensis* was not inhibited by Cs^+. This toxicity of Cs^+ (and sometimes also Rb^+) has been mirrored by other studies, and includes adverse effects on Arabidopsis, liver cells and heart cells [435,443,497]. In addition, Avery *et al* (1991) found that Cs^+ was accumulated to high levels in *Synechocystis* and this was correlated to decreased growth rates [109].

As an alternative explanation for the *E. coli*-specific Cs^+ toxicity, and as was mentioned in Chapter 4, is the finding that Cs^+ has been previously reported to be a K^+ channel blocker, in various cells types, including: glioma cells; cerebellar granule neurons, squid axons, cancerous cells and plant cells [435,448,449,452,498]. Therefore, *E. coli* may find Cs^+ toxic due to the blockage of a specific transporter within its membrane, such as kup. Hampton (2004) also states that Cs^+ toxicity can be 'over-ridden' by the addition of K^+, which suggests that a potential explanation of Cs^+ toxicity could be a decrease in K^+ concentration inside the cells [435]. However, *E. coli* cells grown in the presence of Cs^+ did not contain lower K^+ concentrations. It would appear that, in contrast to the theory of K^+-depletion caused by Cs^+ accumulation, K^+ concentrations in *E. coli* grown in the presence of Cs^+ actually may be higher than those grown in the presence of other cations (see Table 5.35), suggesting a potential K^+-efflux

blockage by Cs^+, supported by the fact that Cs^+ has previously been found to be a K^+-channel blocker [109,448].

The fact that *E. vietnamensis* accumulates Cs^+ suggests that other transporters must also be involved in Cs^+ uptake in different organisms, other than the kup transporter, previously reported to transport Cs^+ [445]. Zhang *et al* found a different system capable of Cs^+ transport in *Rhodococcus* bacteria [499]. However, the higher Cs^+ concentrations within *E. coli* as compared with *E. vietnamensis* are most likely due to a transporter than is lacking in *E. vietnamensis*, such as kup.

Since K^+ has a smaller ionic radius than Rb^+ and Cs^+, these may not be as good at stabilising proteins, hence meaning that enzyme function within the cells could be affected [128]. It has been previously found that Cs^+ is not able to stabilise protein structures in the effective way that K^+ does (regarding low concentrations) and so leads to a reduction in protein activity [435]. Cs^+ has been found to dissociate the 30s and 50s ribosomes of *E. coli*, in an irreversible manner [500]. Therefore, Cs^+ accumulation is clearly the cause of the *E. coli* toxicity, which may be due to the presence of the (specific to *E. coli* in the current study) kup transporter and may be caused by intracellular effects such as protein destabilisation or ion channel block (potentially affecting membrane potential and energy generation), which does not occur in *E. vietnamensis* as a consequence of its lower levels of this cation.

Effects of Li^+ may be due to levels of cellular accumulation

Regarding pure LiCl, the fact that *E. coli* contains the same levels of Li^+ at both 0.5M and 1M implies that it does not actively accumulate this cation in response to osmotic stress. This could be due to the fact that *E. coli* contains NhaA and NhaB (refer to section 3.2.4), which may act to extrude the Li^+ from the cell [419]. In contrast, since *E. vietnamensis* seems to accumulate more Li^+ with increasing LiCl concentration (Li^+ concentrations increase linearly with increasing external salinity), this suggests that Li^+ may have a role for the

osmotic balance of this organism. Li^+ could be acting as a K^+ 'replacement' for *E. vietnamensis*, but cannot act in this way for *E. coli*, due to the inability of Li^+ to pass through K^+ channels [501]. *E. vietnamensis* perhaps may be more versatile regarding the cations it can utilize for osmotic balance. Generally, *E. vietnamensis* tends to adapt its ionic composition dependent on the external salts available, i.e. it appears to be more adaptable to different ions and can utilize a wider range (hence grow in a wider range) of cations, than the other two organisms.

S. ruber contained a considerably higher Li^+ concentration than *E. coli* and *E. vietnamensis* (see Table 5.19). The bioinformatics analysis carried out in section 3.2.4 found that *S. ruber* does not contain the NhaA ion transporter ($Na^+(Li^+)/H^+$ antiporter), whereas both *E. coli* and *E. vietnamensis* do, which may explain the higher accumulation of Li^+ found in *S. ruber* cells and may consequentially lead to the growth inhibition seen from the *S. ruber* growth experiments in LiCl (section 4.2.5). The absence of this transporter has been reported to be associated with Li^+ accumulation and cellular toxicity, as has been found previously in *E. coli* Nha-knockouts [502]. The presence of the NhaA antiporter (Na^+/H^+) can provide a cell with Li^+ resistance, as Li^+ can replace Na^+ through this channel [143].

As *S. ruber* was able to grow in the presence of equimolar KCl:LiCl but not in either alone, and also accumulated substantial levels of Li^+, it could be the case that the addition of Li^+ could stimulate the accumulation of K^+, suggesting that Li^+ may be able to act as a Na^+ replacement, at least regarding Na^+/K^+ antiport, as has been suggested previously [109]. *S. ruber* contains a very high level of K^+ in this particular salt combination, so it could be the case that when Li^+ is present within the cell it cannot regulate its K^+ concentration, due to being unable to extrude Li^+. Additionally, it has also been found that Li^+ toxicity may occur due to the similar reactivity of Li^+ to Mg^{2+} and Ca^{2+}, and this may hence affect processes such as ribosome stability [411]. Therefore, the higher level of accumulation of Li^+ within *S. ruber* may ultimately lead to its lack of growth when other cations are not present (as in pure LiCl). It is postulated that this growth defect in the presence of pure LiCl may be due to the stronger interaction of the small Li^+ ion with the COO^- groups on protein surfaces, but when other cations are present

these effects are 'diluted' somewhat. Alternatively, the lack of Li^+ extrusion from the cell may result in an alteration of membrane potential and hence decrease growth rate, via this mechanism.

E. vietnamensis may have the ability to switch osmotic adaptation strategy

E. coli and *E. vietnamensis* generally accumulated similar ratios of Na^+:K^+ at lower salinities. However, it becomes evident with increasing salt concentration that *E. vietnamensis* has a greater ability to accumulate cations, most likely assisting in its halotolerant nature. The fact that the level of salt tolerance of a bacterium may be related to its ability to accumulate (mostly) K^+ was first documented by Christian and Waltho in 1961 [163,503], and would appear that this may be the case, from the data presented in the current study. The general preference of both of these non-halophilic organisms to accumulate greater levels of K^+ than Na^+, even when both cations are freely available in the medium (Figures 5.10 – 5.12), suggests that this is a general preference for non-halophilic as well as more salt-tolerant bacteria, as has been found in previous studies [504].

This larger cation accumulation capacity of *E. vietnamensis* may be an important insight into halotolerance, as this organism may have an overall better ability for ion accumulation. Moreover, *E. vietnamensis* may use the compatible solutes and excluding salt strategy at lower and medium salt concentrations, whereas it may switch to the accumulation of inorganic ions and salt-in strategy at higher salt concentrations, as has also been found for the halophilic bacterium (a 'traditional' salt out organism) *H. halophila* [190]. At high salt concentrations *H. halophila* has been found to accumulate high levels of K^+, but at lower salt concentrations this organism removes excess K^+ from its cytoplasm and contains an internal ion concentration similar to a non-halophile. However, this strategy may be unusual for halotolerant organisms, as generally they have been found to exclude Na^+ and use K^+ for initial osmotic balance, before accumulating compatible solutes [478,479].

This theory of *E. vietnamensis* utilising a hybrid osmotic adaptation strategy is due to the fact that at lower salt concentrations, *E. vietnamensis* only contains low levels of cations, whereas as the salt concentration of the medium increases it accumulates gradually larger levels of cations, especially once the external salinity reaches 2 – 3M. In addition, the Na^+:K^+ ratio within the *E. vietnamensis* cells increases with increasing salt concentration, which could be significant as K^+ has been found to stimulate compatible solute accumulation [505] - perhaps Na^+ is advantageous for the salt-in strategy, such as was found for *S. ruber*. The finding of *E. vietnamensis* using a hybrid osmotic adaptation strategy may be the first report of a halotolerant organism using this method of osmotic adaptation.

While the *E. vietnamensis* Na^+ concentration was relatively high in the presence of KCl and KBr, the Na^+ concentrations were lower than the K^+ concentrations, whereas they were higher in Na^+-containing media than in K^+-containing media. This may be a halotolerant characteristic as the halotolerant bacterium *Brevibacterium sp.* has been found to accumulate more Na^+ in proportion to the Na^+ concentration of the medium [506], i.e. when no Na^+ is added then the internal Na^+ concentration is low but when NaCl is added the Na^+ concentration inside the cell increases linearly with increasing NaCl concentration. Most bacterial cells use K^+ as the main inorganic ion to provide initial osmotic balance [507], and it appears as though *E. vietnamensis* may be more versatile in terms of the ions it can utilise for osmotic balance, which could be a characteristic common to halotolerant organisms in general.

Role of the anion may be significant

It was found that *E. coli* had a larger Na^+:K^+ ratio (more Na^+ than K^+) in NaBr than it did in NaCl (Figures 5.2 and 5.3). This could be a consequence of the presence of Br^- as a replacement of Cl^-. It has been found previously that various non-halophiles (including *E. coli*) require Cl^- for growth in the presence of moderate Na^+ concentrations [442]. Therefore, the role of the anion may be significant, at least regarding growth at higher Na^+ concentrations. In addition, when grown in

KBr:KCl, *E. coli* contains lower K^+ levels than when in KCl – this is suggestive of a crucial function of Cl^- for K^+ accumulation, like has previously been found for growth at high Na^+ concentrations [442].

At 0.5M NaBr:KBr *E. coli* contains a 3-fold greater Na^+ concentration than K^+, but this is not the case at 1M or for *E. vietnamensis*. This could be indicative of Cl^- dependence for removing Na^+ from the cells, especially at lower concentrations. Roebler *et al* reported that (in their study of a range of non-halophilic organisms, all showing similar results) for *E. coli* grown in NaCl, Na_2SO_4 and Na-gluconate, growth was significantly decreased in the absence of Cl^-, suggesting that Cl^- is essential for growth in the presence of Na^+ [442]. These authors suggested that Cl^- may be essential for efficient osmosensing and also for adequate Na^+ efflux, due to simultaneous export of Cl^- from the cell – and in the case of a lack of Cl^-, these could not occur. This could explain the effects observed in the present study, where *E. coli* cannot remove Na^+ and accumulate K^+ as effectively when Br^- replaces Cl^-. This suggests that *E. coli* may remove Na^+ more effectively and accumulate K^+ more effectively when Cl^- is present (i.e. it is essential for growth at lower salinities), which would assist in its growth.

Further remarks and next steps

It should be noted that experiments using equimolar LiCl + KBr were not performed, as organisms could not grow in the presence of this combination. Unlike the lack of growth of *E. coli* in the presence of RbCl and CsCl, where cells did not grow but could be harvested and proteins obtained, no significant biomass was able to be harvested from KBr:LiCl grown cells. This highlights the significance of the growth inhibition of KBr:LiCl. Additionally, *S. ruber* biomass for the majority of these experiments was inadequate – reflecting its fastidious nature.

In order to fully understand the levels of accumulated cations and their significance, especially in terms of understanding intracellular effects, the next step was to analyse the effects of specific salts in terms of their degree of

stabilisation on intracellular proteins – most easily determined by carrying out experiments to analyse the activity of an enzyme from each organism at various salt concentrations (previously determined to be MDH – refer to Chapter 3).

Chapter 6: Malate Dehydrogenase Enzyme Activity

6.1: Introduction

6.1.1: Enzymes and specific ion effects

Malate dehydrogenase (MDH) is an essential enzyme that functions within central metabolism [508]. The MDH from the extreme halophile *H. marismortui* (HmMDH) is the most extensively studied halophilic protein, which was discussed in section 1.4. Dym *et al* (1995) found that this protein contains more acidic residues and salt bridges than its non-halophilic counterparts and has been found to dissociate into its monomers and unfold at salt concentrations below 2M [345]. This has also been found for other halophilic proteins, which have been found to become inactive when the salt concentration reaches below a certain level, often around 2M [189]. This is in contrast to non-halophilic proteins, which become unstable as the salt concentration is increased [206].

Regarding the stabilisation of halophilic proteins, the particular salt used can have a major influence regarding the stabilisation of these enzymes, due to the effects that different salts have on the protein water interactions [189]. It has been found for HmMDH that the degree of enzyme stabilisation provided by various salts was of the order predicted by the Hofmeister series – i.e. higher charge density cations stabilised the protein at lower concentrations [191]. Moreover, the MDH from *H. marismortui* has been found to be stabilized more efficiently in the presence of NaCl as compared to KCl [208]. This is thought to be due to higher levels of hydration of the Na^+ ion, hence allowing for more effictive protein stabilisation. This increased stabilisation of the protein in NaCl as compared to KCl meant that it was able to remain active in lower concentrations of NaCl than KCl [191]. It has also been found that the MDH from *H. salinarum*

has better activity in the presence of sodium gluconate than sodium chloride, hence also suggesting a role for the anion in the stabilisation of halophilic proteins [96]. Higher charge density cations, even though they are generally destabilizing in non-halophilic proteins, can have a role in the stabilisation of halophilic proteins, due to their increased association with carboxylic acids on the surface of the protein, which is according to the Hofmeister series [240]. Therefore, the level of stabilisation/destabilisation that a particular ion gives to a protein may be due to the strength of the interaction between the ion, protein and the surrounding water.

This differential stabilisation of halophilic proteins that is dependent on the specific salt used is largely due to kosmotropic cations being stabilising towards halophilic proteins at low concentrations, but may lead to destabilisation at higher concentrations, such as was found for HmMDH, since lower concentratrations of NaCl as compared with KCl are needed to stabilise the enzyme, but NaCl is more likely to result in enzyme denaturation (than KCl) at higher concentratrations [191,509]. It has been found that carboxylic acid groups on protein surfaces have a two times greater affinity for Na^+ over K^+ [221]. This is of relevance due to the increased level of these groups present on the surfaces of halophilic proteins [197]. Research also suggests that the activities of two halophilic glutamate dehydrogenases were differentially stimulated by salt, i.e. one enzyme had the same activity in both NaCl and KCl, whereas the other enzyme had higher activity in NaCl than KCl [510]. Therefore, this indicates that the halophilic adaptation of proteins may be more complex and diverse than originally assumed to be.

Considering enzymes in general, chaotropic cations are considered to be more stabilising towards proteins, whereas chaotropic anions are considered to be more destabilising, which is largely due to their water affinities. For example, chaotropic cations do not interact with COO^- groups but chaotropic anions may directly interact with NH_4^+ groups on protein surfaces [511]. Moreover, smaller cations have been found to have more adverse effects towards (non-halophilic) enzyme function than larger cations, due to their interactions with the COO^-

groups on proteins [234]. For example, it has been found for a protease from HIV-1 that the presence of K^+ resulted in this enzyme showing a lower K_m and higher K_{cat} than when Na^+ was used, thus suggesting that K^+ is less inhibitory towards the activity of the enzyme than Na^+ is [512]. This decreased K_m in the presence of K^+ in comparison to Na^+ was thought to be due to stronger association of Na^+ to carboxylate groups on the protein [237]. In addition, some enzymes have been found to require lower charge density cations, mostly K^+, for optimal activity [513].

In addition, the modification of the anion of a salt, i.e. the replacement of Cl^- with Br^-, can result in alterations in enzyme activity. A previous study found that replacing Cl^- (from NaCl) with other anions, such as NO_3^-, resulted in a decrease in the affinity of the enzyme for its substrate, which was thought to be due to a change in the electrostatic interactions between the enzyme and its substrate at the active site, caused by the anion [514]. Br^- is more 'chaotropic' than Cl^-, i.e. it has a lower charge density and is larger in size, hence interacts more weakly with the hydration water around a protein [219]. Chaotropic anions may bind directly to the protein at the amide groups of the amino acids, since they generally are not strong water structure organisers, hence they can lead to protein destabilisation [439]. Therefore, Br^- may be more of a protein destabiliser than Cl^-, as can be predicted from the Hofmeister series [515,516].

6.1.2: MDH function

MDH is an essential enzyme to many aerobic organisms [517] as it functions within the tricarboxylic acid cycle (TCA) of central metabolism [508]. After the initial stage of cellular respiration (glycolysis), which only releases about 25% of the energy from the glucose, the pyruvate produced is converted into acetyl coenzyme A [518]. The TCA, aka Krebs/citric acid cycle, consists of 8 reactions, starting with the introduction of acetyl-coA into the 'cascade' [519]. The TCA cycle forms a major step in central metabolism, involved in the generation of ATP by aerobic respiration [517]. It involves the conversion (via 8 cascade reactions)

of pyruvate to CO_2, in order to generate ATP [520]. Figure 6.1 shows the reactions in the TCA cycle.

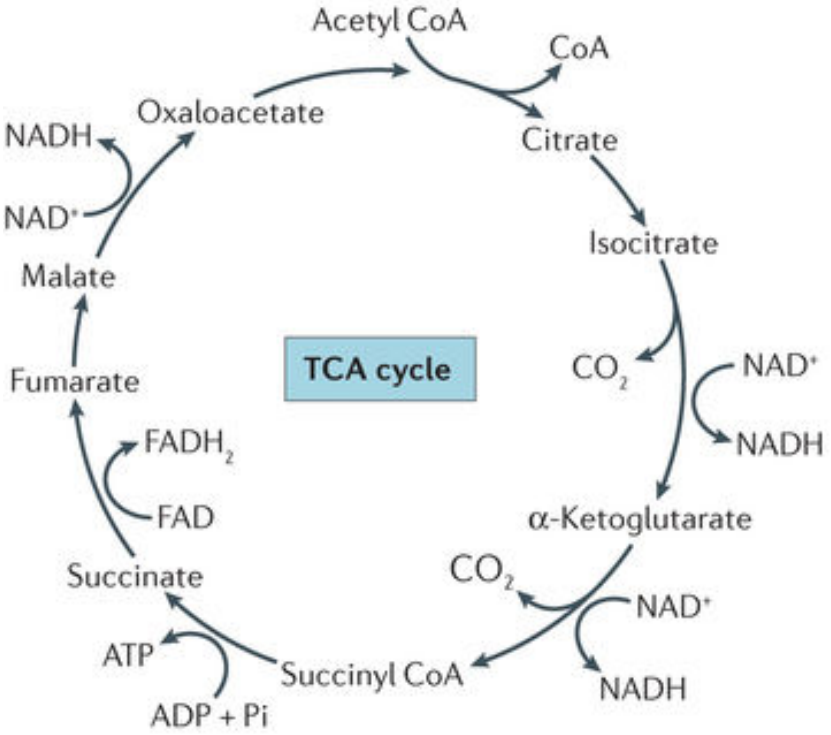

Figure 6.1. The tricarboxylic acid cycle. The 8 reactions of the TCA cycle are initiated by the interaction of acetyl-coA with oxaloacetate, which forms citrate and this is eventually degraded back down to oxaloacetate (catalysed by MDH), via the 8 reaction cascade. Two molecules of CO_2 are released, as well as 1 molecule of ATP, 3 NADH and 1 $FADH_2$, for each cycle that is carried out. Adapted from O'Neill, 2016.

The enzyme MDH converts malate to oxaloacetate, utilising the NAD^+/NADH shuttle, which is a reversible reaction [521]. The enzymatic reaction of MDH involves the oxidation (dehydrogenation) of malate to form oxaloacetate (the removed hydrogen is transferred to NAD^+ to produce NADH), and the reverse reaction of the reduction of oxaloacetate (adding a hydrogen) to form malate, brought about by the oxidation and reduction of NADH/NAD [522]. This is shown in Figure 6.2 [518]. The active site of MDH is composed of a hydrophobic vacuole that has both substrate and co-enzyme binding sites [521]. The histidine/aspartate pair within the active site form a 'charge-relay', which donates the proton to NAD^+

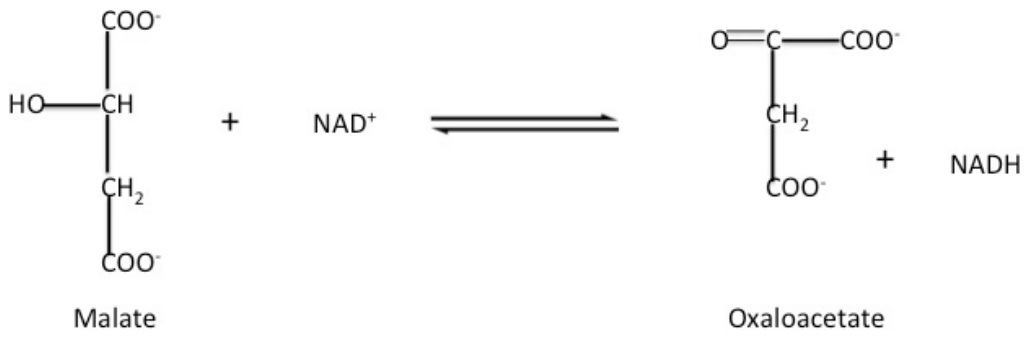

Figure 6.2. MDH reaction. Malate Dehydrogenase converts malate to oxaloacetate. Malate is dehydrogenated (oxidised) and this proton is transferred to NAD^+, to produce NADH + oxaloacetate.

6.1.3: Mechanism of MDH activity assay

Enzymatic assays are used in order to follow the progress of an enzyme catalysed reaction [523]. Most enzymatic activity assays take advantage of changes in absorbance of substrates or products, in order to directly measure the rate of the reaction, via spectroscopy [524]. In the case of the MDH catalysed reaction, the co-enzymes NAD^+ and NADH absorb differently at 340nm - NAD^+ does not absorb strongly at this wavelength whereas NADH absorbs very strongly [521,525]. Figure 6.3 shows a schematic of this absorption difference between NAD^+ and NADH. For the MDH reaction, NAD^+ is converted to NADH in proportion to product (oxaloacetate), and this change in absorbance can be utilised to follow the progress of this reaction, giving a direct measurement of the speed of the reaction [322]. In fact, this change in absorbance is so significant that NAD^+/NADH are often coupled to other enzyme reactions in order to follow their progress via this absorption change [526,527].

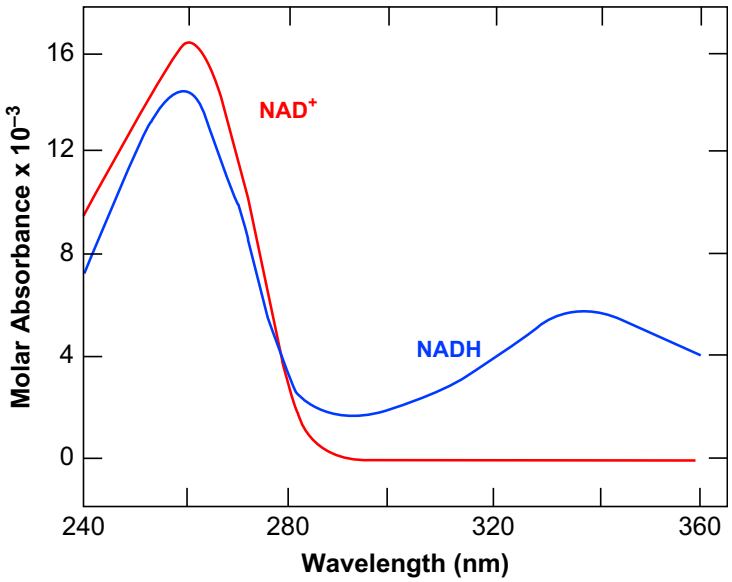

Figure 6.3. Absorbance spectrum of NAD⁺ and NADH. At 340nm NADH absorbs much more strongly than NAD⁺. Adapted from Purich, 2010.

Many studies have utilised the same basic MDH assay for the study of various MDHs, in both the forward (malate oxidation) and reverse (oxaloacetate reduction) reactions, e.g. Deuch (2013), Luo *et al.*, Plancarte *et al.* and Wynne *et al.* [297,522,528,529]. The reverse reaction involves observing the decrease in absorbance associated with the formation of NAD⁺ (and malate), whereas the forward reaction involves an increase in absorbance with the formation of NADH (and oxaloacetate) [519,530]. This assay is a relatively simple and effective method of measuring the reaction rate [297]. Therefore, the measurement of MDH activity via NAD⁺/NADH absorbance changes is a simple and convenient method of measuring the enzyme rate.

6.1.4: Enzyme Kinetics

When the progress of an enzymatic reaction is recorded by observing changes in absorbance via spectroscopy, this will result in a plot of the change in absorbance over the time course of the experiment [531]. However, in order to

compare different conditions and to compare different enzymes, it is vital to be able to measure the rate of the reaction. The measurements of enzyme rates and their analysis are most commonly performed by Michaelis-Menten kinetics [532].

Michaelis and Menten realised that if only the initial rate was used then other factors such as product inhibition and the reverse reaction would not need to be taken into account. The equation of product formation which they formulated is shown in Equation 6.1: it states that an enzyme substrate complex will form before any product is produced, with the enzyme-substrate complex dissociating to result in product formation [533,534]. The initial rate of a enzyme-catalysed reaction is proportional to the concentration of the enzyme-substrate complex (proportional to the initial enzyme and/or substrate concentration) [535].

$$E + S \rightleftharpoons ES \dashrightarrow E + P$$

Equation 6.1 Michaelis-Menten-type reaction equation. E is the enzyme, S is the substrate, and P is the product. An enzyme substrate complex (ES) will form before this dissociates to form the product.

The initial rate (V_0) of an enzymatic reaction can be defined as the rate at the start of the reaction, where less than 5% of the substrate has been depleted [322]. When the enzyme rate is plotted as absorbance change (Y axis) vs time (X axis), this initial rate is the initial linear slope of the resultant curve [534]. The gradient of this initial slope is equal to V_0.

When performing MM-kinetics, the initial rate of the reaction (V_0) is plotted against a range of substrate concentrations. This plot allows for the calculation of two kinetic parameters: K_m and V_{max}, shown in Figure 6.4 [534]. These are useful ways of expressing the speed of a particular reaction. V_{max} can be defined as the maximum rate of that enzyme reaction, given in units of mM min^{-1} (or µM min^{-1}), whereas the K_m is the substrate concentration at half of the V_{max} [322]. A low K_m will mean that the enzyme will be saturated with substrate relatively quickly and

hence the V_{max} can occur at lower substrate concentrations, whereas a large K_m means that the enzyme will need a lot of substrate to become saturated and therefore the V_{max} will occur at higher substrate concentrations [531]. Essentially, a low K_m shows high substrate affinity of the enzyme whereas a high K_m shows a low substrate affinity.

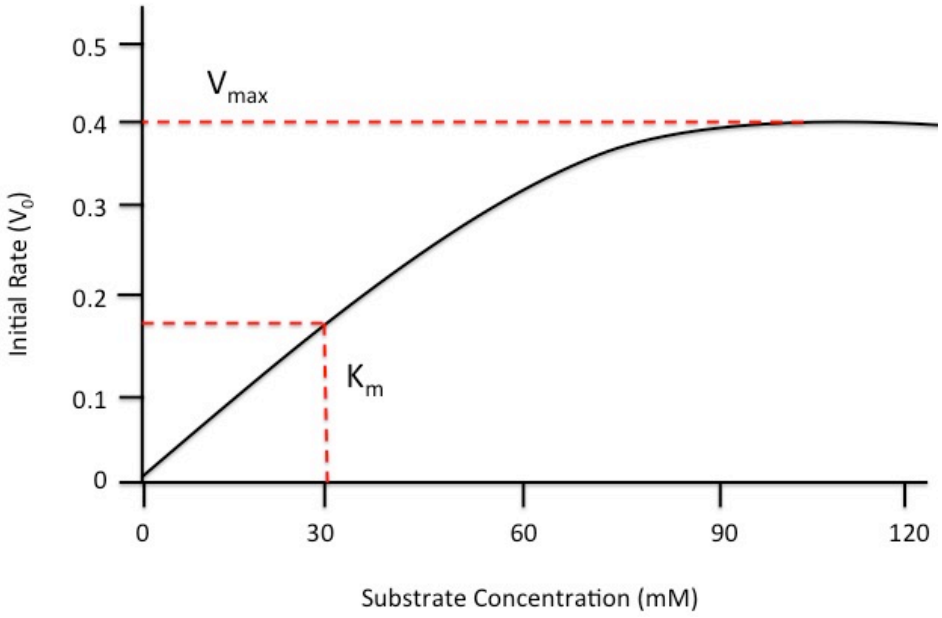

Figure 6.4. Schematic of an enzyme reaction. Data is plotted as the initial velocity (V_0) against substrate concentration (mM). V_{max} is the maximum rate and K_m is the substrate concentration at half of the V_{max}.

In order to calculate K_m and V_{max}, the Michaelis-Menten equation is used, which is shown in Equation 6.2 [536].

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Equation 6.2. MM equation. V_0: initial velocity; V_{max}: maximum velocity; K_m: Michaelis constant; S: substrate.

When an enzyme is not in a purified form (e.g. from a crude cell extract) it is often convenient to express its activity as the specific activity, since the

enzymatic activity will vary depending on the purity (i.e. different levels of the enzyme may be present in the samples) [537]. The specific activity (SA) can be found by dividing the rate of the reaction (V_0) by the protein concentration used for the assay [524]. Specific activity is the rate of the reaction per mg of total protein: SA = [V_0 x (volume of protein sample)]/[mass of protein in sample]

6.1.5: Rationale for current study

Non-halophilic enzymes have generally been found to have a low tolerance to NaCl [538]. In addition, Na^+ has been found to destabilise proteins to a greater extent than K^+ [539]. This is due to its lower charge density and hence less disruptive effect towards protein structure [219]. For example, the MDH from pea seeds was found to decrease in activity at NaCl concentrations above 0.02M [540]. Conversely, an enzyme from the halotolerant organism *S. aureus* was found to be stimulated by 0.17M NaCl [541]. This suggests that the level of salt tolerance of an organism may be proportional to the extent of salt tolerance of individual enzymes from that organism.

It has been previously found that the MDH from *S. ruber* (SrMDH) appears to be totally stable when no salt is present, although the enzyme could still retain some activity at high salt concentrations, which is not the case for many non-halophilic proteins [206]. However, a major flaw with the Madern and Zaccai study is that they only tested the effects of KCl on SrMDH activity and it could be the case that other salts may have more stabilising effects on this protein.

It was hoped that these enzymatic studies would give an insight into the effects that different salts have on the enzymes within the cells, specifically, the effects on the enzymatic activity. This was essential in order to better understand the mechanisms behind the observed growth effects and cellular accumulation levels – as well as to understand specific cation effects in relation to protein surface charges, as was calculated from Chapter 3.

The three organisms were grown to mid-exponential phase in their optimal media, harvested and lysed, with cell debris removed and the cellular extracts obtained (i.e. supernatant) were used for the enzymatic assays (refer to section 2.4). In order to understand fully how specific salts affect the activities of the enzymes, it is important to understand the general rates of the enzymes in 'normal' conditions. Different enzymes will have different rates of reaction, including the same enzymes from different organisms [542]. Therefore, in order to be able to accurately compare the enzymes, the general rates of each enzyme was analysed initially. The rate of a enzyme catalysed reaction will not only vary with substrate concentration, but will also vary depending on the kinetic parameters of that specific enzyme, as even small differences in the protein sequence can result in different levels of enzyme activity [536,543,544].

The MDH activity assays were used in order to measure the activity of the MDH from the three organisms in the presence of a range of salts (NaCl, KCl, NaBr and KBr) and at a range of salt concentrations (0 – 4M).

6.2: Results

6.2.1: Initial characterisation of the MDHs

MDH activity assays were performed in order to calculate the K_m and V_{max} of each of the enzymes. These experiments were carried out in a range (0mM – 320mM) of malate (substrate) concentrations. For each substrate concentration, the initial velocity (V_0) was calculated, according to the protocol described previously (see section 2.4). The data for these experiments are shown in Figure 6.5 and in Table 6.1.

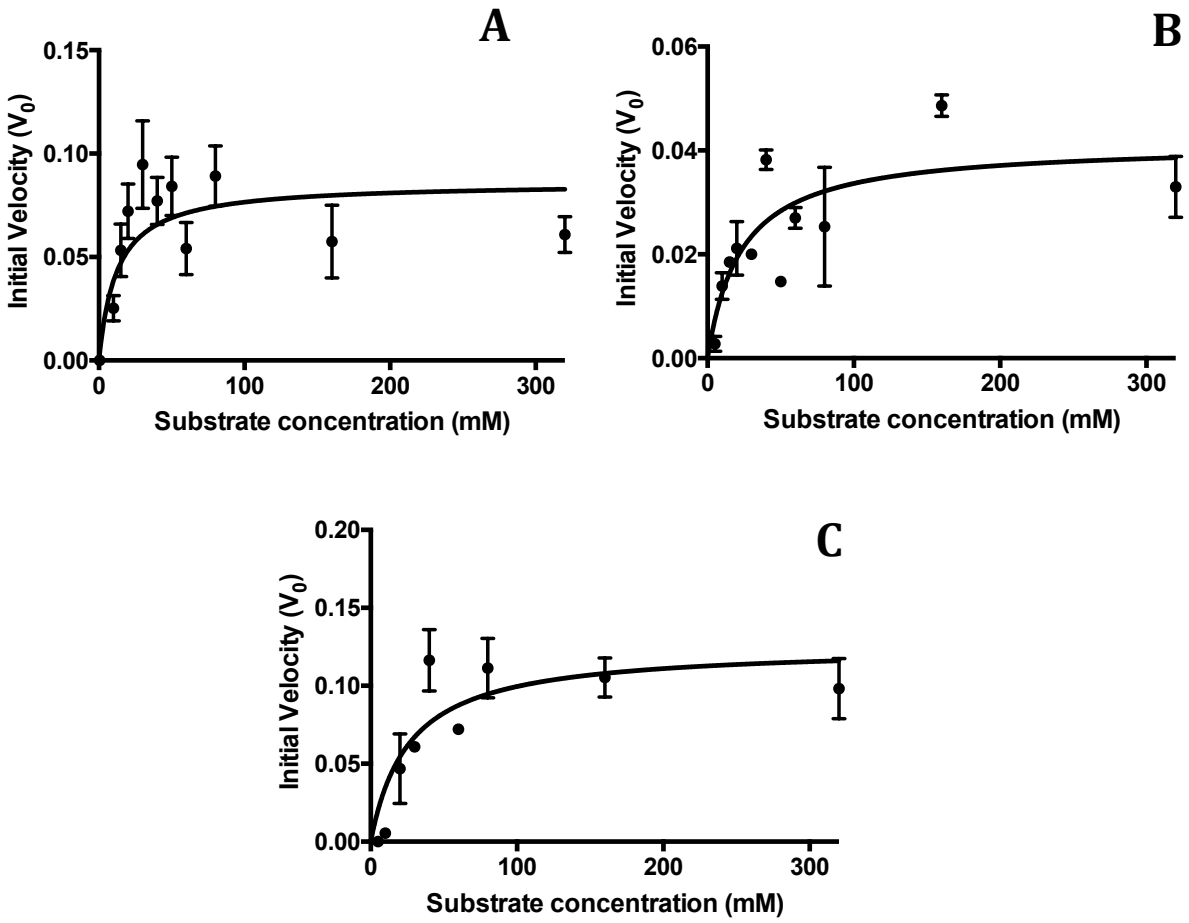

Figure 6.5. Initial velocities (V_0) of the MDHs in response to increasing substrate concentration. EcMDH (A), EvMDH (B) and SrMDH (C) activity was measured in a range of malate concentrations (0mM – 320mM), and MM-kinetics was used in order to determine the K_m and V_{max} of the enzymes. Data are plotted as V_0 (Y axis) against substrate concentration (mM) (X axis). Error bars represent the standard deviation between individual experiments (5 replicates per condition).

Table 6.1. K_m and V_{max} values of the MDHs. Data were calculated from the Michalis-Menten equation, using GraphPad Prism software. K_m units are in mM and V_{max} units are in mM min^{-1}.

	K_m	V_{max}
E. coli	12.34	0.08
E. vietnamensis	22.92	0.04
S. ruber	21.57	0.11

6.2.2: Effect of sodium chloride on MDH activity

The activities of the MDH from *E. coli*, *E. vietnamensis* and *S. ruber* were measured in the presence of a range of NaCl concentrations (0M – 3M), and in a range (30mM – 640mM) of malate concentrations. The specific activities are shown in Figure 6.6. These are also displayed below as the residual activity, shown in Figure 6.7. Additionally, K_m and V_{max} values for the enzymes treated with the salt are given in Table 6.2.

For all 3 enzymes, there is a significant decrease in activity with the addition of NaCl. However, SrMDH activity remains higher than the other two enzymes at the lower substrate concentrations (30mM and 80mM malate), up until 0.75M NaCl, and at 30mM malate the activity of SrMDH starts to increase again after 1M NaCl. At 30mM and 640mM malate, EvMDH remains more active than EcMDH.

SrMDH has optimal activity in the absence of salt but retains some activity with increasing NaCl concentration

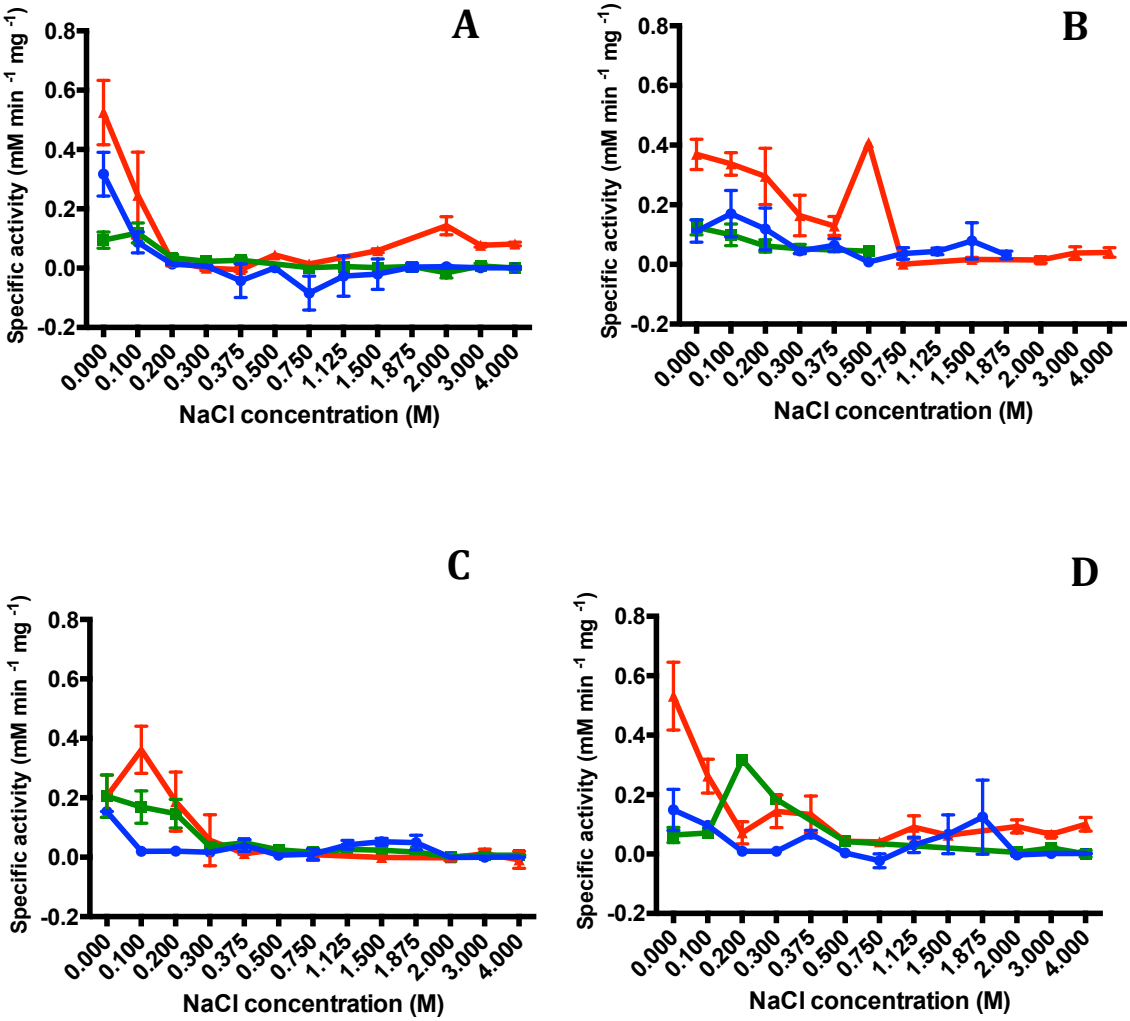

Figure 6.6. Specific activities of the MDHs in the presence of a range of NaCl concentrations. A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. Data are plotted as the specific activity (Y axis) against NaCl concentration (X axis). EcMDH: blue lines/circles; EvMDH: green lines/squares; SrMDH: red lines/triangles. Error bars refer to the standard deviation between individual experiments (3 replicates per condition).

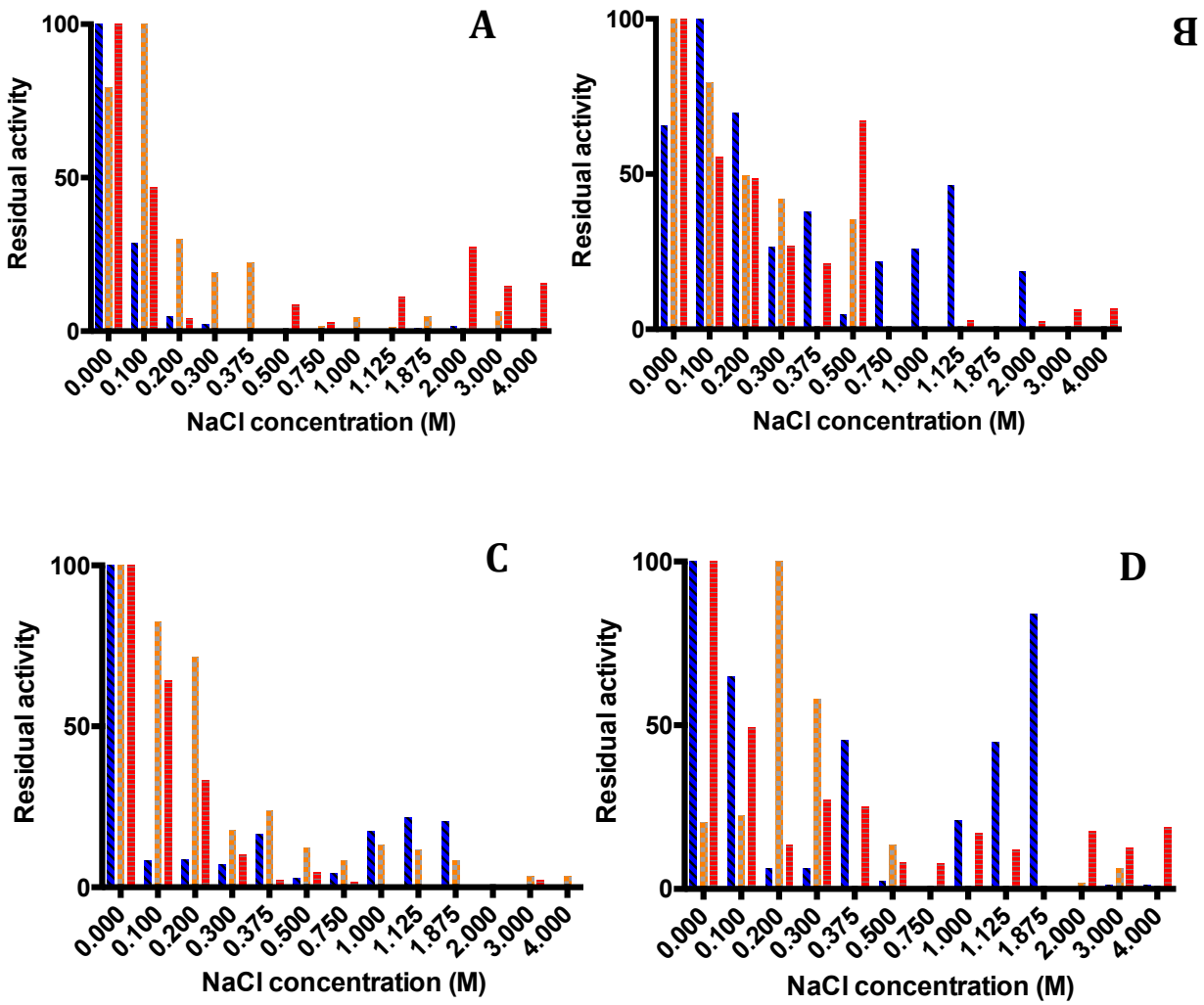

Figure 6.7. Residual activity of the MDHs in the presence of a range of NaCl concentrations.
A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. EcMDH: blue bars (diagonal stripes); EvMDH: orange bars (checkered); SrMDH: red bars (horizontal stripes). Data are displayed as the % activity that remains, in terms of the enzyme specific activity, in reference to the optimal salt concentration (shown on the graphs as 100% activity). Data are presented as residual activity (Y axis) against NaCl concentration (X axis).

Table 6.2. K_m and V_{max} of the enzymes in the presence of NaCl. K_m and V_{max} values are shown of EcMDH, EvMDH and SrMDH, in the presence of 0M – 4M NaCl. Data were calculated from the averaged curves using Michaelis-Menten kinetics, with GraphPad Prism. K_m units are mM, V_{max} units are mM min^{-1} mg^{-1} and the R^2 value refers to the fit of the MM-curve to the data. Blank: could not be reliably measured; N/A: no activity.

NaCl concentration (M)	EcMDH			EvMDH			SrMDH		
	K_m	V_{max}	R^2	K_m	V_{max}	R^2	K_m	V_{max}	R^2
0	12.34	0.09	0.60	22.92	0.04	0.77	26.07	0.13	0.68
0.1	-	-	-	6.44	0.20	0.88	28.09	0.36	1.00
0.2	-	-	-	38.82	0.11	0.87	44.05	0.15	0.96
0.3	-	-	-	58.14	0.07	0.95	218.5	0.35	0.77
0.375	46.70	0.06	0.99	40.03	0.07	0.96	88.15	0.24	0.84
0.5	77.44	0.01	0.81	35.11	0.04	0.74	-	-	-
0.75	303.5	0.14	0.90	1097	0.100	0.99	840.70	0.10	0.92
1.125	98.44	0.11	0.87	8.5E+16	1.7E+13	0.79	N/A	N/A	N/A
1.5	1366	0.62	0.95	4.8E+14	1.1E+11	0.94	N/A	N/A	N/A
1.875	2300	1.14	0.99	5.0E+20	5.8E+16	0.97	N/A	N/A	N/A
2	N/A	N/A	N/A	N/A	N/A	N/A	3585	0.35	0.99
3	N/A	N/A	N/A	N/A	N/A	N/A	238.40	0.02	0.97
4	N/A	N/A	N/A	N/A	N/A	N/A	-	-	-

6.2.3: Effect of sodium bromide on MDH activity

MDH activity assays were performed on the 3 MDHs in the presence of 0M – 3M NaBr, at 30mM – 640mM malate concentrations. Figure 6.8 shows the specific activities of the three enzymes and the residual activities are shown in Figure 6.9. Additionally, K_m and V_{max} values for the enzymes treated with the salt are given in Table 6.3.

At low substrate concentrations (30mM malate) the activities of all 3 enzymes decreases rapidly with increasing NaBr concentration. At higher malate concentrations (160mM and 640mM) SrMDH remains more active than the other two enzymes up until 3M NaBr. Except at the lowest substrate concentration (30mM) EcMDH and EvMDH remain active up until 0.5M NaBr, which is

particularly evident for EcMDH at 80mM malate and for EvMDH at 640mM malate.

SrMDH requires high substrate concentrations to remain active at high NaBr concentrations

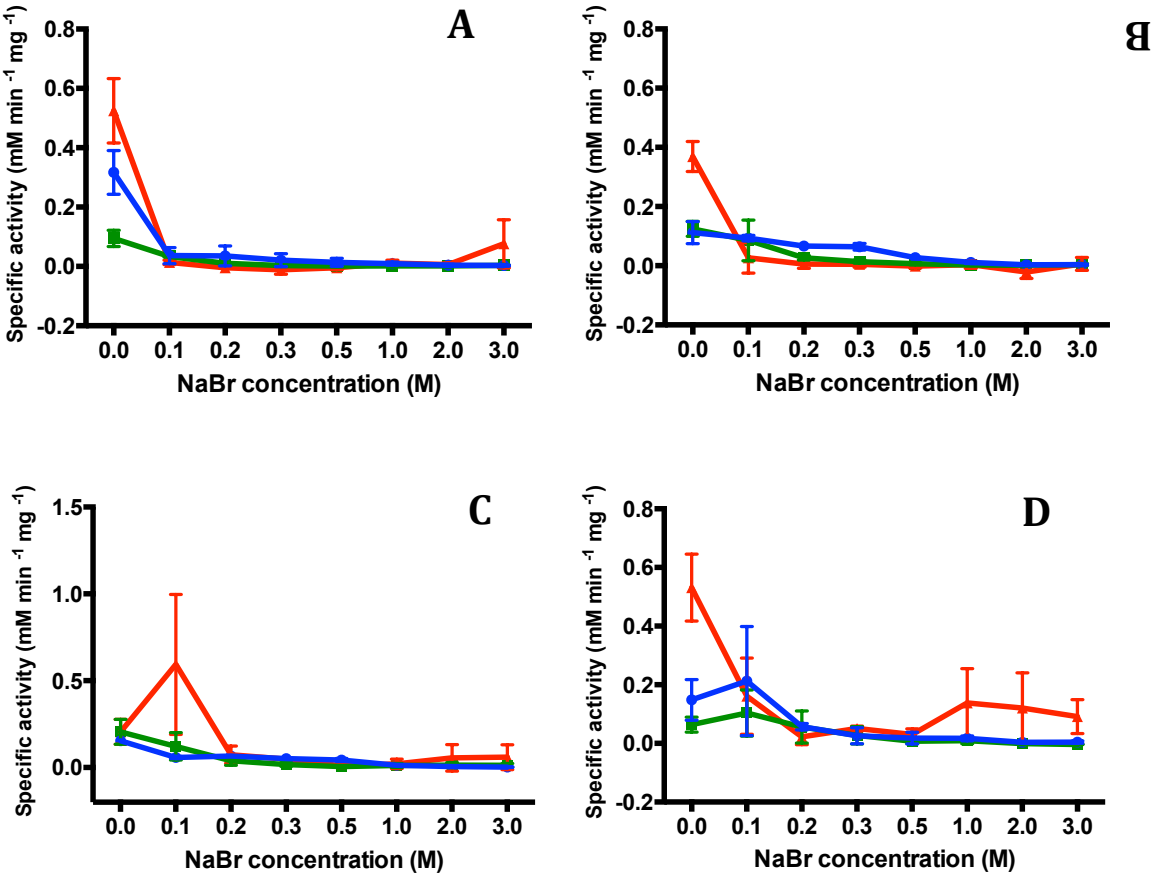

Figure 6.8. Specific activities of the MDHs in the presence of a range of NaBr concentrations. A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. Data are plotted as the specific activity (Y axis) against NaBr concentration (X axis). EcMDH: blue lines/circles; E.vMDH: green lines/squares; SrMDH: red lines/triangles. Error bars refer to the standard deviation between individual experiments (3 replicates per condition).

A

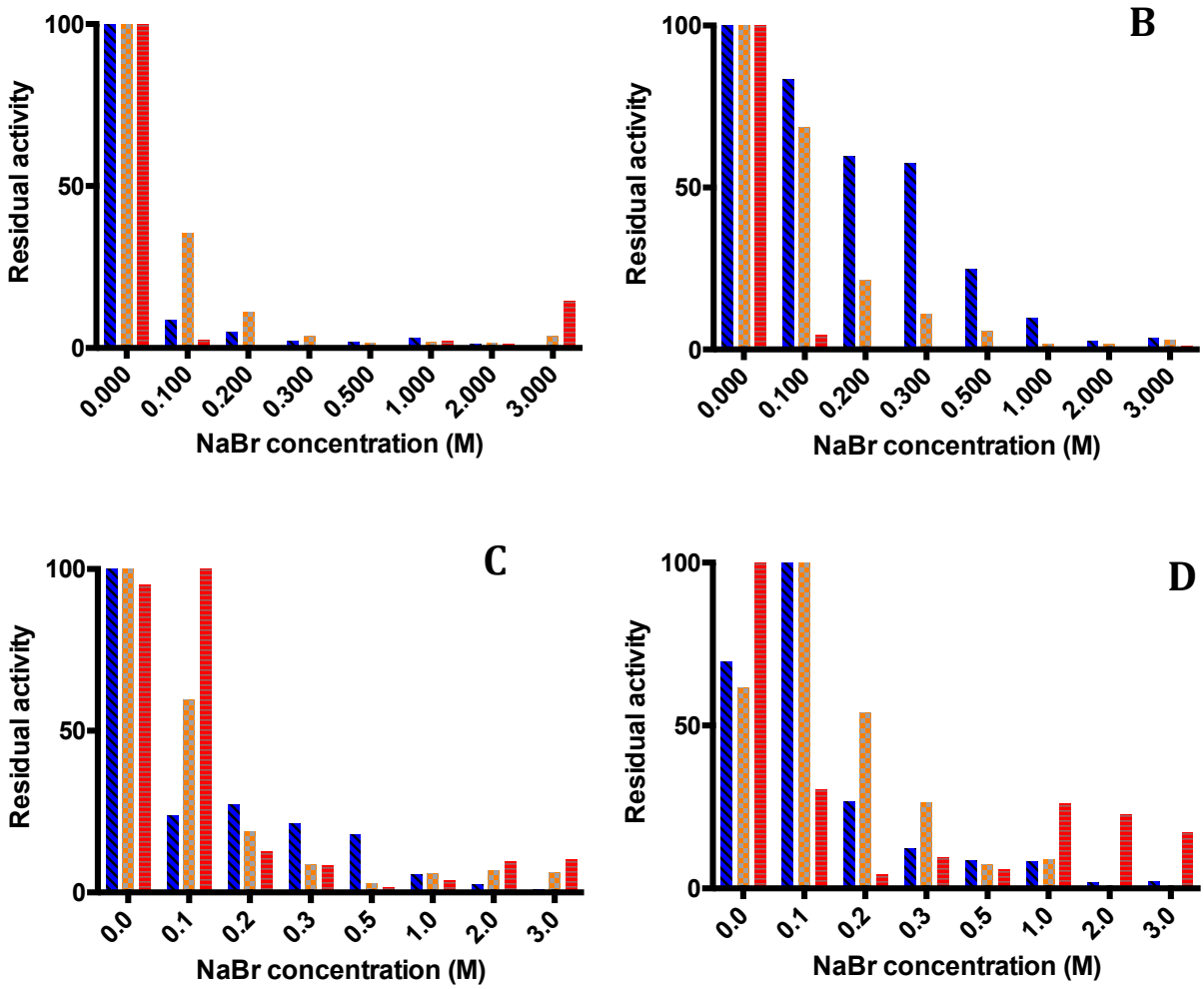

Figure 6.9. Residual activity of the MDHs in the presence of a range of NaBr concentrations. A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. EcMDH: blue bars (diagonal stripes); EvMDH: orange bars (checkered); SrMDH: red bars (horizontal stripes). Data are displayed as the % activity that remains, in terms of the enzyme specific activity, in reference to the optimal salt concentration (shown on the graphs as 100% activity). Data are presented as residual activity (Y axis) against NaBr concentration (X axis).

Table 6.3. K_m and V_{max} of the enzymes in the presence of NaBr. K_m and V_{max} values are shown of EcMDH, EvMDH and SrMDH, in the presence of 0M – 3M NaBr. Data were calculated from the averaged curves using Michaelis-Menten kinetics, with GraphPad Prism. K_m units are mM, V_{max} units are mM min^{-1} mg^{-1} and the R^2 value refers to the fit of the MM-curve to the data. Blank: could not be reliably measured; N/A: no activity.

NaBr concentration (M)	EcMDH			EvMDH			SrMDH		
	K_m	V_{max}	R^2	K_m	V_{max}	R^2	K_m	V_{max}	R^2
0	12.34	0.09	0.60	22.92	0.04	0.77	26.07	0.13	0.68
0.1	36.52	0.09	0.84	55.68	0.23	0.88	30.77	0.11	0.30
0.2	58.29	0.08	0.82	260.4	0.16	0.99	330.7	0.07	0.94
0.3	68.15	0.06	0.97	334.10	0.09	0.99	182.10	0.08	0.88
0.5	65.02	0.04	0.96	100	0.02	0.92	1298	0.15	0.99
1	183.30	0.03	0.72	136.50	0.03	0.73	2.57E+15	1.00E+12	0.99
2	N/A	N/A	N/A	620.30	N/A	0.73	309.20	0.36	0.99
3	N/A	N/A	N/A	N/A	N/A	N/A	225.20	0.21	0.79

6.2.4: Effect of potassium chloride on MDH activity

MDH activity assays were performed on *E. coli*, E*. vietnamensis* and *S. ruber* MDH, in a range of KCl concentrations, and at a range of substrate concentrations (30mM – 640mM). This is shown in Figure 6.10 as the specific activity and in Figure 6.11 as the residual activity. Additionally, K_m and V_{max} values for the enzymes treated with the salt are given in Table 6.4.

Especially at high substrate concentrations (160mM and 640mM malate), SrMDH retains more activity than the other two enzymes with increasing KCl concentration, up to moderate KCl concentrations (0.5M KCl). EcMDH retains activity up until 3M at 80mM and 160mM malate. EvMDH activity is low in all conditions, except for 0M (and 0.1M at 640mM malate).

EcMDH can retain a high level of activity in the presence of KCl

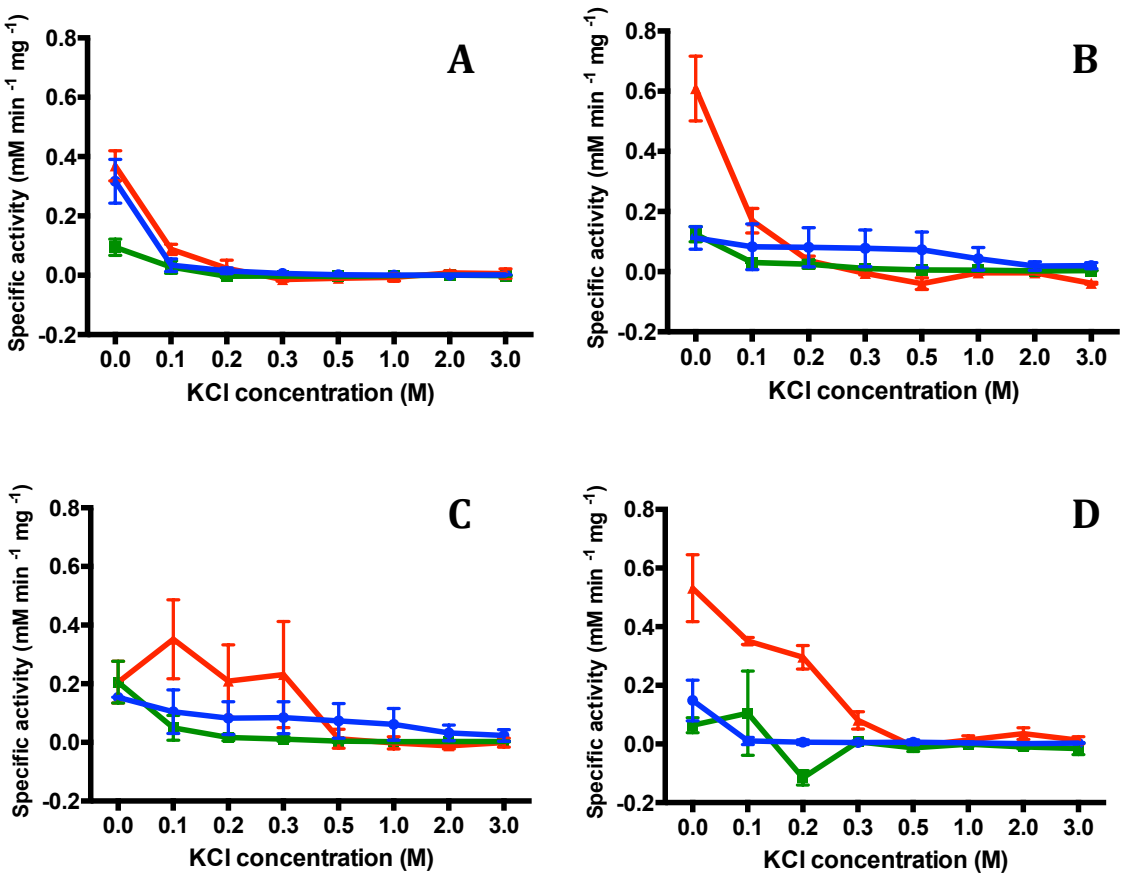

Figure 6.10. Specific activities of the MDHs in the presence of a range of KCl concentrations. A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. Data are plotted as the specific activity (Y axis) against KCl concentration (X axis). EcMDH: blue lines/circles; EvMDH: green lines/squares; SrMDH: red lines/triangles. Error bars refer to the standard deviation between individual experiments (3 replicates per condition).

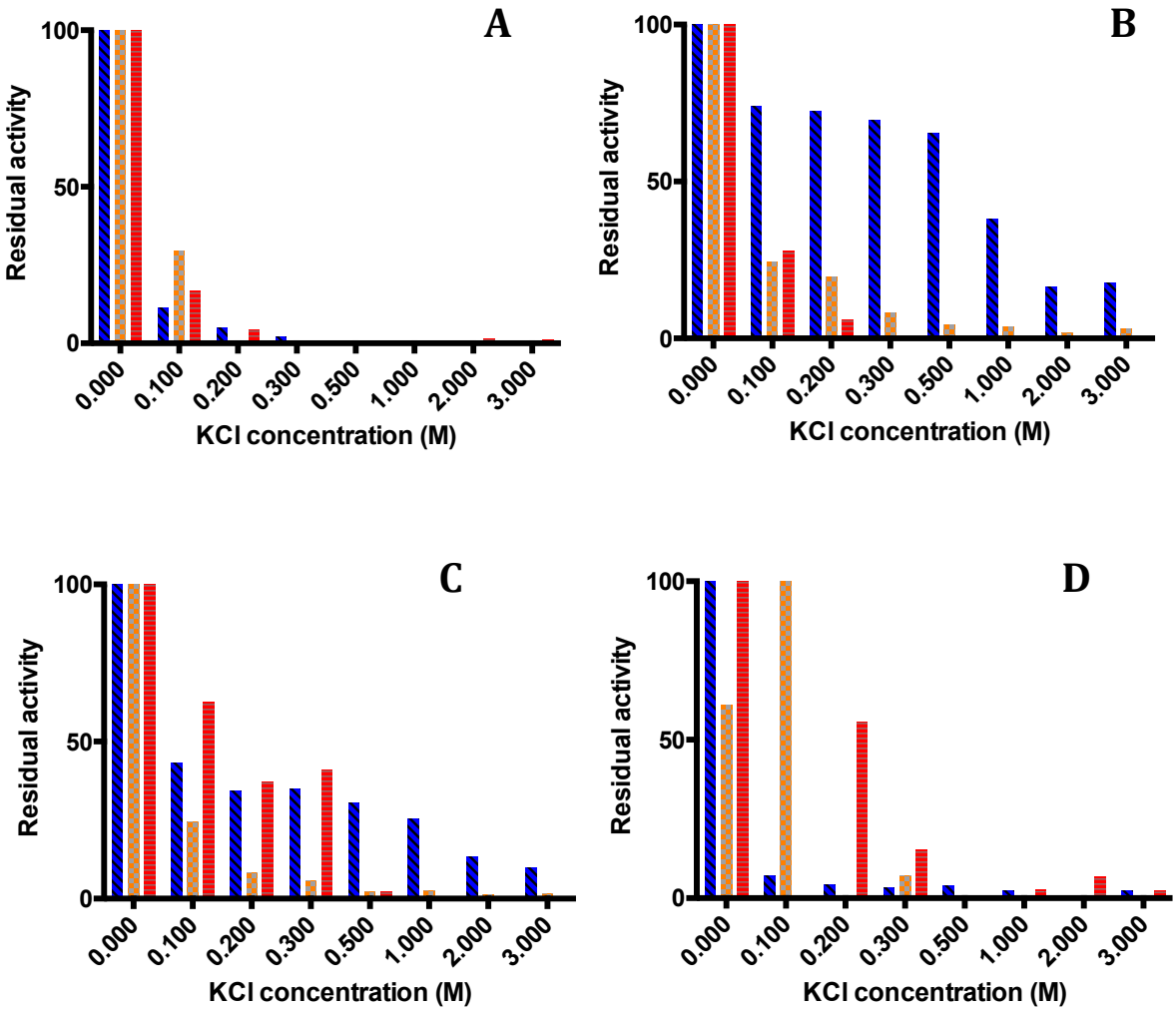

Figure 6.11. Residual activity of the MDHs in the presence of a range of KCl concentrations. A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. EcMDH: blue bars (diagonal stripes); EvMDH: orange bars (checkered); SrMDH: red bars (horizontal stripes). Data are displayed as the % activity that remains, in terms of the enzyme specific activity, in reference to the optimal salt concentration (shown on the graphs as 100% activity). Data are shown as residual activity (Y axis) against KCl concentration (X axis).

Table 6.4. K_m and V_{max} of the enzymes in the presence of KCl. K_m and V_{max} values are shown of EcMDH, EvMDH and SrMDH, in the presence of 0M – 3M KCl. Data were calculated from the averaged curves using Michaelis-Menten kinetics, with GraphPad Prism. K_m units are mM, V_{max} units are mM min^{-1} mg^{-1} and the R^2 value refers to the fit of the MM-curve to the data. Blank: could not be reliably measured; N/A: no activity.

KCl concentration (M)	EcMDH			EvMDH			SrMDH		
	K_m	V_{max}	R^2	K_m	V_{max}	R^2	K_m	V_{max}	R^2
0	12.34	0.09	0.60	22.92	0.04	0.77	26.07	0.13	0.68
0.1	-	-	-	-	-	-	55.93	0.45	0.76
0.2	105.20	0.25	0.85	N/A	N/A	N/A	157.70	0.37	0.64
0.3	173.60	0.32	0.82	149.6	0.01	0.77	520.30	0.10	0.55
0.5	183.00	0.31	0.81	3.60E +19	2.00E +15	0.99	N/A	N/A	N/A
1	729.60	0.04	0.95	N/A	N/A	N/A	N/A	N/A	N/A
2	4.37E+ 18	1.60E +15	0.95	N/A	N/A	N/A	7.40E+ 19	6.38E +15	0.99
3	881.80	0.29	0.93	N/A	N/A	N/A	N/A	N/A	N/A

6.2.5: Effect of potassium bromide on MDH activity

MDH activity assays were performed on the three MDHs in the presence of 0M – 3M KBr (at 30mM – 640mM malate). Figure 6.12 shows the specific activities and Figure 6.13 shows the residual activities. Additionally, K_m and V_{max} values for the enzymes treated with the salt are given in Table 6.5.

At higher malate concentrations, SrMDH activity remains higher than the other proteins at lower KBr concentrations, whereas EcMDH remains more active than the other two enzymes at higher KBr concentrations, especially at higher malate concentrations. EvMDH remains active at high KBr concentrations, but only at 640mM malate.

EvMDH has a high tolerance to KBr

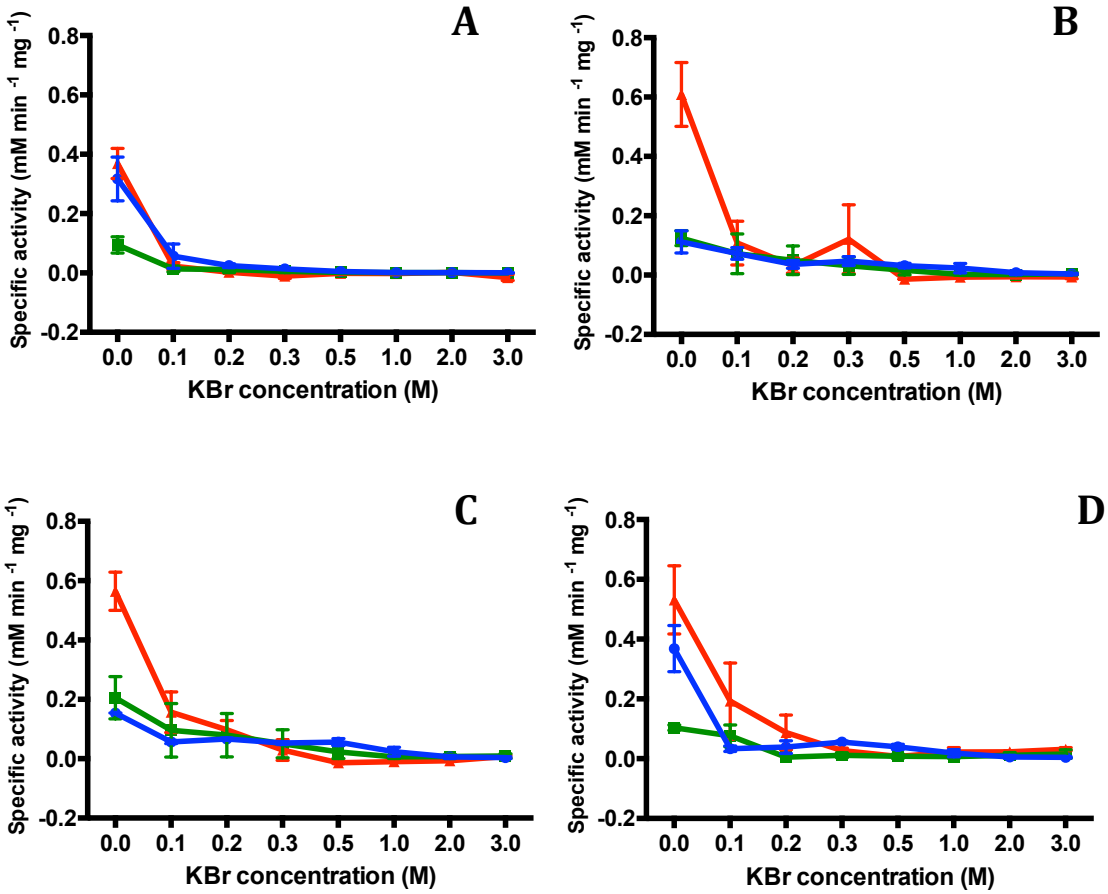

Figure 6.12. Specific activities of the MDHs in the presence of a range of KBr concentrations. A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. Data are plotted as the specific activity (Y axis) against KBr concentration (X axis). EcMDH: blue lines/cirlces; EvMDH: green lines/squares; SrMDH: red lines/triangles. Error bars refer to the standard deviation between individual experiments (3 replicates per condition).

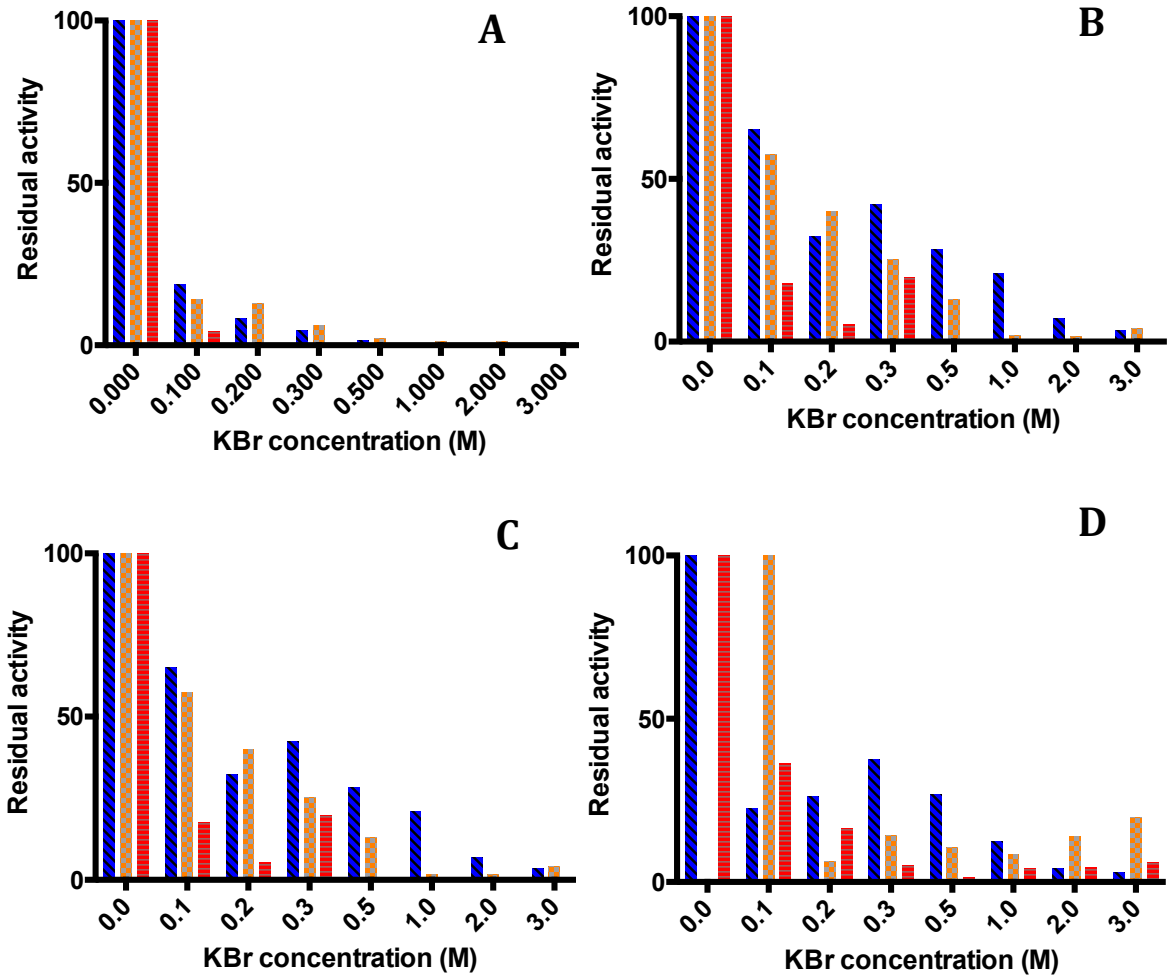

Figure 6.13. Residual activity of the MDHs in the presence of a range of KBr concentrations. A: 30mM malate; B: 80mM malate; C: 160mM malate; D: 640mM malate. EcMDH: blue bars (diagonal stripes); EvMDH: orange bars (checkered); SrMDH: red bars (horizontal stripes). Data are displayed as the % activity that remains, in terms of the enzyme specific activity, in reference to the optimal salt concentration (shown on the graphs as 100% activity). Data are shown as the residual activity (Y axis) against salt concentration (X axis).

Table 6.5. K_m and V_{max} of the enzymes in the presence of KBr. K_m and V_{max} values are shown of EcMDH, EvMDH and SrMDH, in the presence of 0M – 3M KBr. Data were calculated from the averaged curves using Michaelis-Menten kinetics, with GraphPad Prism. K_m units are mM, V_{max} units are mM min^{-1} mg^{-1} and the R^2 value refers to the fit of the MM-curve to the data. Blank: could not be reliably measured; N/A: no activity.

KBr concentration (M)	EcMDH			EvMDH			SrMDH		
	K_m	V_{max}	R^2	K_m	V_{max}	R^2	K_m	V_{max}	R^2
0	12.34	0.09	0.60	22.92	0.04	0.77	26.07	0.13	0.68
0.1	39.52	0.06	0.80	38.36	0.17	0.68	120.10	0.39	0.96
0.2	54.96	0.07	0.77	77.49	0.01	0.74	148.30	0.19	0.90
0.3	82.50	0.07	0.94	53.19	0.01	0.99	1.31E+02	7.03E+010	0.72
0.5	62.19	0.04	0.75	238.20	0.01	0.94	N/A	N/A	N/A
1	122.60	0.04	0.70	125.80	0.02	0.90	N/A	N/A	N/A
2	181.40	0.02	0.77	274.60	0.03	0.92	N/A	N/A	N/A
3	183.70	0.01	0.82	707.50	0.06	0.91	N/A	N/A	N/A

6.2.6: Summary

Figure 6.14 displays a summary of the data presented in this chapter, shown as the average remaining activity of each of the enzymes, in the presence of NaCl, NaBr, KCl and KBr.

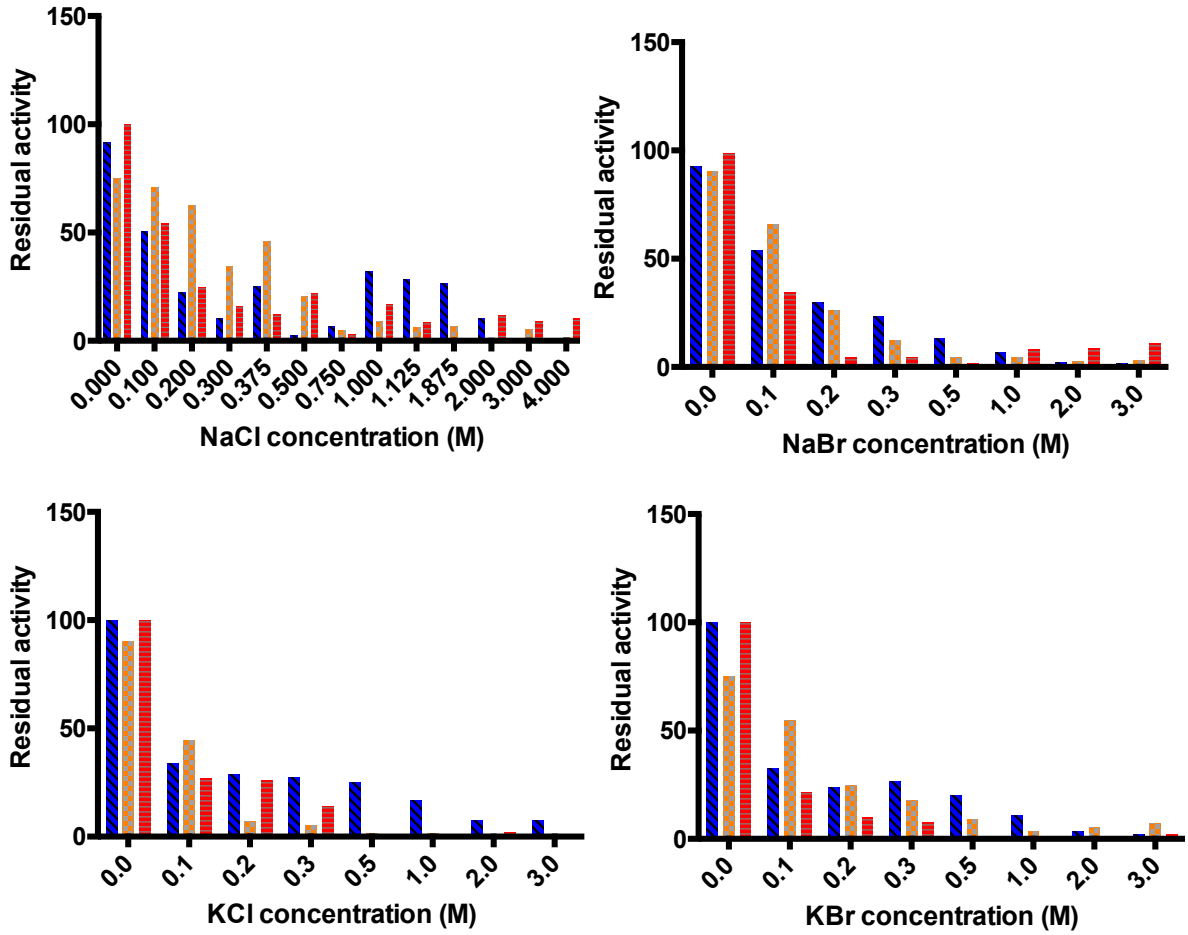

Figure 6.14. Activity remaining of EcMDH, EvMDH and SrMDH in the presence of NaCl, NaBr, KCl and KBr. Data are expressed as the average residual activity (%) of each enzyme, taken from the data for all 4 malate concentrations (Figures 6.7, 6.9, 6.11 and 6.13). EcMDH: blue bars (diagonal stripes); EvMDH: orange bars (checkered); SrMDH: red bars (horizontal stripes). Data are plotted as the % activity remaining (Y axis) against the salt concentration (x axis).

6.3: Discussion

EcMDH has the lowest K_m value out of the three enzymes (12.34mM), whereas SrMDH has the highest. As K_m defines the amount of substrate required for enzyme saturation [536]- with lower values meaning the enzyme is saturated at lower substrate concentrations - EcMDH has the highest affinity for the substrate out of the three enzymes and SrMDH has the lowest affinity. However, SrMDH has the highest V_{max} value (0.1148mM min^{-1}), meaning that this enzyme is able to achieve a higher reaction rate (Chen et al., 2010; Johnson and Goody, 2011), although the high K_m states that this maximal velocity is not easily achieved, as the enzyme has a lower affinity for its substrate [531,533]. EvMDH has the lowest V_{max} value (0.04136mM min^{-1}), suggesting that this enzyme is the slowest in terms of rate of the three, as it takes longer to reach its maximal velocity. Therefore, these varying K_m and V_{max} values suggest that the enzymes may naturally have different activities and therefore supports the use of specific activity for comparisons between the enzymes.

SrMDH is not a typical halophilic enzyme

Although SrMDH activity does not initially decrease as much as the non-halophilic enzymes with increasing salt concentration (especially for sodium salts), it does not appear to be typically halophilic as it functions best in the absence of salt. However, it does seem to have a higher tolerance of NaCl at lower substrate concentrations than the other two enzymes. However, in many of the experiments it was found that EcMDH actually retained significantly greater activity than SrMDH – this will be discussed further later on. *S. ruber* has an acidic proteome and the MDH has a pI in the acidic range (as was found in

Chapter 3), so this 'non-halophilic' character appears unexpected. It has been reported previously that SrMDH is less stable in KCl than in the absence of salt [209], and from the data presented here this may indeed be the case regarding salt in general, and not just for KCl. However, it has also been found previously that some proteins from halophilic organisms may decrease in stability with increasing salt concentrations [189], so what has been seen in the present study for SrMDH may not be entirely unusual. The enzyme may not require salt for activity but the fact it remains active in its presence may be indicative of non-optimal adaptation, i.e. it can cope with the environment the organism is exposed to but does not function optimally in these conditions [545]. Perhaps SrMDH is an 'intermediate' case of halophilic adaptation, i.e. lie somewhere between halophilic and non-halophilic proteins, in terms of salt-sensitivity. This could be a consequence of its evolutionary history, such that most known salt-in halophiles are archaea and *S. ruber* has evolved differently as it is a bacterium, and no other closely related organisms to *S. ruber* are extreme halophiles, therefore this case of convergent evolution may have lead to differences in the salt sensitivities of its enzymes [546].

It has been previously found that other *S. ruber* enzymes have varying activities in the presence of salt, with the NAD-dependent iso-citrate dehydrogenase having optimal activity between 0.5M – 2M KCl, and the NADP-dependent iso-citrate dehydrogenase having increasing activity with increasing KCl concentration. This, in addition to what was found in the present study, suggests that the responses of *S. ruber* enzymes towards salt may be variable and may depend on the specific enzyme as well as the specific salt, which has previously been found to be the case for HmMDH, as it is more easily stabilised in Na^+ than in K^+ [191]. In the current study, it was found that SrMDH functioned better at higher concentrations in the presence of Na^+ than K^+, and the fact that it has previously been found that activity of *S. ruber* hexokinase activity totally disappeared at 2.1M KCl and also at 2.8M NaCl supports the data in the current study, regarding SrMDH retaining more activity at higher NaCl than KCl concentrations. Clearly, the enzymes of *S. ruber* may have varying requirements for salt - activities vary depending on the specific salt present as well as

depending on the particular enzyme. As an example from another organism, the dihydrofolate reductase of *H. volcanii* can function in the presence of as low as 0.5M salt, suggesting that not all proteins from salt-in halophiles require salt according to the traditional salt-in halophilic view [34,88].

In addition, the fact that the specific activity of SrMDH is higher than the other two organisms in the presence of KCl but only at higher substrate concentrations (up to 0.3M) suggests that the enzyme must require high substrate concentrations in order to function effectively in the presence of KCl, thus implying that K^+ may increase the K_m of SrMDH more than Na^+. It has been found previously that different ions may have varying effects on the substrate affinity of enzymes [233]. Additionally, the fact that SrMDH functions better in the presence of higher NaBr concentrations than the other two enzymes, but only at higher substrate concentrations, also suggests that the anion has an effect on substrate binding, hence lowering its affinity and resulting in more substrate being required for activity (higher K_m). It has been found that chloride may compete with the MDH binding site (in pea seed MDH) for oxaloacetate, hence increasing the K_m of the enzyme, and it has been previously suggested that different anions may interact with enzyme binding sites, hence affecting the substrate affinity [540,547]. Therefore, since SrMDH functioned better in NaCl and needs higher substrate concentrations for activity in NaBr, Br^- may more adversely affect the substrate binding, as compared with Cl^-.

The increased activity of SrMDH in higher concentrations of Na^+ salts than K^+ salts relates to the growth data, where this organism requires Na^+. Therefore it would seem as though the Na^+ requirement for growth may also reflect a protein-based preference, i.e. Na^+ is preferable over K^+ for *S. ruber* ion metabolism. Furthermore, the MDH of *S. ruber* is sensitive to salt but still retains function within high salt, but with a diminished rate – at the optimal salt concentrations for growth of this organism. This offers an explanation for the lower growth rate of *S. ruber*: MDH is essential for metabolism [548] and so its lower enzymatic rate may result in less energy being available to the cell, hence slower growth.

Hofmeister effects contribute towards different ion preferences for halophilic vs non-halophilic enzymes

SrMDH is more active at lower substrate concentrations (30mM), as compared to the other two enzymes, in higher NaCl concentrations, which may be due to its 'halophilic' nature, potentially explained by the Hofmeister effect (i.e. the increased stabilisation of halophilic proteins by Na^+ over K^+) [191]. Although SrMDH activity does drop with increasing NaCl concentration, it retains more activity than the other two enzymes at higher NaCl concentrations, but only at low substrate concentrations. EcMDH activity was actually higher than SrMDH activity in many experiments – thus further suggesting the non-halophilic nature of SrMDH.

S. ruber was most active in NaCl, which are the highest charge density cation and anion used for the experiments in the present study. It has previously been found that HmMDH was stabilised most effectively by high charge density cations as well as high charge density anions, similar to what was found in the present study. Na^+ has a stronger effect on protein structure than K^+ [231]. Therefore, SrMDH may prefer Na^+ and Cl^- (over other ions) due to their superior water binding abilities, in comparison to K^+ and Br^- [219]. The fact that Na^+ binds to protein surfaces more strongly than K^+, may further suggest why the three enzymes have different ion preferences – i.e. SrMDH functions best in NaCl, due to its increased level of stabilisation in Na^+ as compared to K^+ [237]. Even if SrMDH is not a typical halophilic protein, it has an excess negative surface charge (refer to Figure 3.14) and so is presumably stabilised more effectively in Na^+ over K^+. Since EvMDH also functioned better in NaCl compared to KCl, this may suggest a degree of 'haloadaptation' of this enzyme, further shown by its increased surface charge as compared to EcMDH – i.e. the level of surface charge on a protein may be directly proportional to the salt tolerance of the organism (refer to section 3.2.2). However, it should be noted that SrMDH is clearly more active in the absence of salts, but the fact that it is more active in higher

concentrations of NaCl than KCl is not characteristic of halophilic proteins [345]. This could be rationalised based on the effects of the anion – Cl^- and Br^- are both chaotropic and therefore may be adsorbed to the surface of the protein, and so the strong interaction of Na^+ with the protein surface may help prevent destabilisation due to these interactions (due to providing increased rigidity as well as helping to maintain solvation), whereas K^+ is ineffective as it does not interact as strongly with the protein surface [237].

The activity of all 3 enzymes is lower in the presence of NaBr as compared to NaCl, therefore it appears that Br^- affects the activity of the enzymes, halophilic, halotolerant and non-halophilic, alike - more adversely than NaCl does. Anions generally have a stronger effect on protein stability than cations and it has been found, for example, that the glutamate dehydrogenase from a plant decreased in activity depending on the specific anion used, and this was according to the Hofmeister series, with Br^- causing a greater degree of deactivation than Cl^- [549]. However, for the current study it would appear that the cation has a more significant effect on SrMDH activity, but the anion has a more significant effect on EcMDH - EvMDH has no clear anion preference (specifically, it can tolerate more Br^- at higher malate concentrations). When considering the general effects observed for the replacement of Cl^- with Br^-, it has been reported that Br^- can be used in order to phase protein crystal structures [550]. The general finding of an increased loss of activity in the presence of Br^- containing salts is consistent with the larger polarisability of Br^- ions. This may be due to Br^- binding directly to the protein surface as it is less strongly hydrated and therefore will be more likely to interact with non-polar COOH groups [228,235] with the interaction of Br^- with these potentially leading to protein destabilisation. Of particular interest is a study by Bilaničová *et al.*, who found that the specific activity of a lipase from *Pseudomonas cepacia* was lower in the presence of NaBr than in NaCl , further suggesting that Br^- may more adversely affect enzyme activity due to Hofmeister-type effects [219,551], and further supporting the findings in the current study. Therefore, enzymes in general may be less stable in Br^- than in Cl^-. This is further supported by the fact that all 3 proteins have a pI lower than the pH of the buffer (7.4), and anions have been reported to obey a direct Hofmeister

series in this case – i.e. Br^- will be more destabilising towards protein structures than Cl^- [552]. However, the fact that EvMDH had no clear anion preference suggests that this organism and this particular enzyme should be further investigated in terms of haltolerance and anion specificity.

Moreover, SrMDH had lower activity in the presence of KBr than the other salts tested. This is consistent with the fact that it showed higher activity in the presence of NaCl – i.e. it has a preference for Na^+ and Cl^- over K^+ and Br^-: indicating a preference for higher charge density ions in general. Since Na^+ interacts with COO^- groups more strongly than K^+ [237], it may be more effective to neutralise the excess negative charges on this protein, as well as being more effective at compensating for destabilisation caused by chaotropic cations. Conversely, since EcMDH had better activity in KBr over NaBr but had better activity in KCl over KBr, this emphises the less disruptive effect on non-halophilic proteins of both K^+ and Cl^-, as compared with Na^+ and Br^-, which can be rationalised based on the Hofmeister series.

A high substrate affinity may assist in enzyme stability at high salt concentrations – due to effect of salt on K_m

The fact that EcMDH retains higher activity in KCl at lower substrate concentrations and functions better in NaCl at higher substrate concentrations could perhaps be explained by the fact that Na^+ has been found to increase the K_m of enzymes more than K^+, hence increasing the amount of substrate needed for activity [512]. Wondrak *et al.* found that the addition of KCl resulted in a lower K_m than the addition of KBr or LiCl [239], thus suggesting that the particular ionic species present can have a significant effect on the substrate affinity of the enzyme – which may be a consequence of the kosmotropic/chaotropic nature of the salt. More importantly, the K_m of this same protein (HIV-1 protease) has been found to be increased more considerably in the presence of NaCl than KCl [512], which supports what was found in the current study regarding EcMDH. Pokorna *et al.* postulated a theory that the

higher affinity for Na^+ as compared to K^+ to a protein surface may be behind this effect, especially if aspartic acid or glutamic acid residues are close to the active site of the protein.

Since higher salt concentrations may cause a decrease in the substrate binding affinity of an enzyme (resulting in a K_m increase), this may explain why all 3 enzymes functioned best at higher substrate concentrations when in the presence of higher salt concetrations – i.e. the enzymes need higher levels of substrate in order to retain activity at high salt concentrations. This may have been more pronounced for EcMDH due to its higher natural K_m - meaning that it can naturally bind more strongly to its substrate at potentially destabilising salt concentrations (more so than the other two proteins), hence its increased ability to retain activity at high salt and high substrate concentrations. Since EcMDH was more active than SrMDH in the presence of salt, this may reflect the higher intrinsic affinity of EcMDH for its substrate over that of SrMDH.

Low salt concentrations may prove stimulatory towards EvMDH activity – potentially suggesting a slightly haloadapted protein

It is possible that low concentrations of salt may prove stimulatory towards EvMDH, as the optimal activity was often seen in the presence of 0.1-0.2M salt. This enzyme had optimal activity at 0.1M NaBr/KCl/KBr and 0.2M NaCl at 640mM malate, and also at 0.1M NaCl in 30mM malate. This may reflect the halotolerant nature of this protein – further seen by a slightly negative pI and surface charge (refer to Chapter 3, Figures 3.10 and 3.14). It has been found in a previous a study using the MDH from pea seeds that very low concentrations of NaCl (0.02M) could be stimulatory towards enzyme activity [540] – however, this is significantly lower than the concentrations used in the current study. This salt-stimulation has has also been found within other studies, for low (under 0.1M) salt concentrations [538].

Halotolerant enzymes have not been investigated to an as great extent as halophilic enzymes. However, an enzyme of a halotolerant *Bacillus* has

previously been shown to have activity with increasing salt concentration that was proportional to its growth at those salt concentrations [553]. This may be the case for *E. vietnamensis*, due to its wide range of growth with increasing salt concentration (refer to Chapter 4). The fact that EvMDH was able to retain even low levels of activity to high salt concentrations (often up to 3M) correlates with the growth rates of *E. vietnamensis* – it can tolerate high concentrations but with decreased growth rates, and this decrease in growth rate could potentially be due to its decreased enzymatic activity (all enzymes) at these concentrations. However, EvMDH functioned best in KBr and yet this salt was not well tolerated by *E. vietnamensis* – producing generally lower levels of growth than NaCl, KCl and NaBr. Since *E. vietnamensis* was found to have a slightly acidic proteome and the surface of EvMDH was found to be less neutral than that of EcMDH, containing an increased negative surface charge as well as an overall increased level of acidic residues (Chapter 3), this 'salt stimulation' may indicate a slightly 'halo-adapted' protein.

EvMDH was able to retain more activity within a wider range of KBr concentrations as compared to the other salts. It was able to retain a significant level of activity up to 3M but only when the substrate concentration was high (640mM malate). The fact that EvMDH has no clear a preference of Br^- or Cl^- suggests that it may have a more complex relationship with specific ions than the other two organisms so this should be investigated further in the future.

Further remarks

It should be noted that the error associated with the measurements of SrMDH activity is generally higher than for the other two enzymes – this could be a natural trait of this enzyme as it shows variable activities within the presence of salt, i.e. this may reflect the general instability of this particular enzyme.

In addition, the data shows that *E. coli* MDH activity does not directly correspond to the salt concentrations this organism grows best at (i.e. the MDH can retain activity at higher concentrations than *E. coli* is able to grow at). This is most

likely to due to the catalytic efficiency of this enzyme – exemplified by the fact that it was able to retain higher activity in salt than the halophilic SrMDH.

The data presented above suggest that the extent of specific ion effects experienced by a protein could perhaps be a direct consequence of the salt tolerance of the organism. However, it is also suggested that haloadaptation needs to be re-considered as clearly a full proteome wide adaptation (in terms of salt stability) is not the case, as the non-halophilic EcMDH functioned more efficiently within these conditions than the haloadapted SrMDH.

Chapter 7: General Discussion and Conclusions

Specific ion preferences of an organism may be related to its level of salt tolerance

S. ruber may be differently adapted from other salt-in halophiles

It was found from the growth experiments (Chapter 4) that *S. ruber* required Na^+ for growth (except when Li^+ and either of K^+/Cs^+/Rb^+ were present), as well the elemental analysis data showing that it generally accumulated higher levels of Na^+ (Chapter 5) and its MDH remained more stable in higher concentrations of Na^+ over K^+ (Chapter 6). This is in contrast to *E. coli* and *E. vietnamensis*, which had a preference for K^+ over Na^+, in terms of growth and cellular accumulation. Observing the growth of halophiles in response to specific cations has been poorly studied [10]. However, salt-in halophiles have generally been found to accumulate K^+ in excess of Na^+, even when Na^+ is the predominant cation in the external medium – such as has been found for the extreme halophiles *H. marismortui* and *Haloanaerobium prevalens* [494,554,555]. Similarly to these organisms, S*. ruber* was found to contain higher K^+ than Na^+ concentrations in Salinibacter medium (3.3M NaCl present within this medium) as well as at 2.5M NaCl:LiCl and 3M – 4M NaCl (Chapter 5). Crucially, Oren *et al* also found that when *S. ruber* was grown in the presence of 3.3M NaCl, the intracellular K^+ concentration exceeded that of Na^+ [181]. Therefore, it would appear that at medium salt concentrations this organism may accumulate more K^+ but at lower and higher concentrations Na^+ may be accumulated more highly. This discrepency may be rationalised based on Na^+ being preferred at lower external salinities due to the greater protein stabilising power of Na^+, but K^+ is more effective in higher concentrations due to the potentially destabilising effects of Na^+ when accumulated in higher concentrations. However, *S. ruber* does have a clear cation preference for Na^+ over K^+ (in terms of accumulation as well as for

growth), but may vary its accumulation of these depending on the level of osmotic stress it is under, i.e. in different salinities.

Salt tolerance directly influences cation preferences

Continuing from the discussion above, the data presented in the current study have shown that the halophilic organism *S. ruber* cannot grow when Na^+ is replaced by K^+ and also often accumulates higher levels of Na^+ as compared to K^+ in its cytoplasm. Its MDH also appears to be more stable in the presence of higher concentrations of Na^+ than K^+, whereas the two non-halophiles, *E. coli* and *E. vietnamensis*, grow better in the presence of KCl than NaCl and also accumulate higher levels of K^+ in their cytoplasms, as compared to Na^+. This is more pronounced for *E. coli*, however, and the complexities of *E. vietnamensis* ion metabolism will be discussed further later.

At the whole organism level as well as the protein level, these effects can be rationalised based on the Hofmeister series. *S. ruber* prefers more high charge density cations for growth (Na^+) as well as for ion accumulation and enzymatic activity, whereas *E. coli* and *E. vietnamensis* both show a preference for K^+ - although *E. vietnamensis* shows no clear ion preference for the activity of its MDH. These ion preferences may be related to proteome acidity, as Na^+ may be better suited towards the stabilisation of acidic proteomes (i.e. *S. ruber*), whereas this may be destabilising for non-acidic proteomes.

From a perspective of protein stability, it seems unexpected that halophilic organisms have been reported to exclude Na^+ and accumulate K^+, since the malate dehydrogenase from the halophile *H. marismortui* has been found to be more stable in the presence of NaCl than in KCl. It has been reported, based on the Hofmeister series, that high charge density cations are the best at stabilising halophilic proteins, due to their higher affinity for the increased level of carboxylic acid groups on these surfaces [208,240]. It should therefore be asked why it is reported that most halophiles prefer K^+ as the 'osmolyte', if Na^+ would provide better protein stabilisation. This may be explained by osmotic reasons –

since Na^+ is more stabilising at lower concentrations (i.e. lower concentrations as compared to K^+ are required for halophilic protein stabilisation) and since organisms living in hypersaline environments will require to accumulate very high levels of a particular cation for osmotic balance (to keep a cytoplasm at least iso-osmotic with the environment), it may not feasible to accumulate such high Na^+ levels as would be required for organisms living in the conditions optimal for the extreme archaeal halophiles [78], as even halophilic proteins may be unstable at these internal Na^+ concentrations. However, *S. ruber* appears to have a slightly lower optimal salt concentration for growth than is typical for salt-in halophiles, as has been found in the current study (refer to Chapter 4). Therefore, *S. ruber* may generally accumulate more Na^+ than K^+ since Na^+ provides more efficient protein stabilisation, as is seen from the enzyme assay data (Chapter 6), without adverse consequences of having to counterbalance its cytoplasm with a more highly concentrated environment. Madern and Zaccai found an association between the level of hydration of a cation with the minimum concentration required for enzymatic stabilisation of a halophilic protein, i.e. the higher the charge density of the cation the lower concentration required for stabilisation, as was also found by Ebel *et al.* for NaCl vs KCl and D_2O vs H_2O [191,437]. Therefore, this 'minimum' concentration may be of relevance to *S. ruber* as its optimal salt concentration has been found to be somewhat lower than for many the salt-in halophiles that have been found to accumulate K^+. Na^+ may therefore provide more efficient stabilisation of internal proteins at lower concentrations and due to the fact that *S. ruber* does not grow in as high salt concentrations as many other salt-in halophiles [4], it is better suited to utilise Na^+ for osmotic balance.

It was found in the current study that as the growth rate of *E. coli* and *E. vietnamensis* decreased with increasing salt concentration, the Na^+:K^+ ratio increased, but this was not the case for *S. ruber*. This suggests that there is a greater influx of Na^+ and lower levels of efflux with increasing salt concentration, which may be a consequence of osmotic stress and less energy available to pump out Na^+ - which further reduces the growth rate of the organisms due to a lack of Na^+/H^+ antiport [506]. This was not the case for *S. ruber* however, perhaps

reflecting its differential cation preferences (i.e. Na^+ presence intracellularly is less inhibitory) – as well as reflecting the fact that *S. ruber* may compensate for the altered membrane potential by utilising other sources of energy generation, such as that provided by rhodopsins [204].

Li^+ and K^+ charge densities may be sub-optimal for *S. ruber*

The Nha transport proteins are Na^+/H^+ antiporters, exporting Na^+ as well as Li^+ and importing H^+ [143,556]. The fact that the cation transporter analysis (Chapter 3, Table 3.8) found that *S. ruber* does not contain any of these transporters suggests that if Li^+ enters the cell then it will not be able to be exported out of the cell. *S. ruber* does contain relatively high levels of Li^+ (Chapter 5 – Tables 5.16, 5.18, 5.19), so this explanation is plausible. It could be the build-up of Li^+ within *S. ruber* cells, when grown in LiCl alone, that is responsible for growth inhibition, as was found in section 4.2.5. When no additional cations are also present (i.e. Na^+ or K^+) the effects of Li^+ being 'stuck' inside the cells may result in protein destabilization or interference of enzymatic activities due to the previously described effects of Li^+ and its similarity to Mg^{2+} [411], leading to competition with Na^+ or K^+ for binding to protein surfaces, resulting in a lack of *S. ruber* growth (due to Li^+ being more destabilizing than Na^+ or K^+), whereas the other two organisms are able to control the internal concentration of Li^+ more effectively (as they possess the Nha transporters) and can hence grow in its presence. Growth may be able to occur when another cation is added in equimolar proportions with Li^+ due to competition with Li^+ for protein binding, resulting in lower levels of destabilization – i.e. a limited level of Li^+ binding to the proteins can provide the appropriate level of stabilisation, due to 'dilution' with more weakly hydrated cations. Nha genes have previously been reported as being required for Li^+ detoxification, with high intracellular Li^+ levels being correlated to Li^+ toxicity [144,419,491]. Therefore, the Li^+ toxicity observed within *S. ruber* is most likely due to Li^+ build-up within the cytoplasm, which is a consequence of the absence of the Nha-transporters within this organism. Crucially, the results may suggest that Na^+ provides optimal

stabilisation of *S. ruber* proteins – as opposed to the more destabilizing Li^+ and the less effective K^+.

SrMDH may act like a non-halophilic protein due to the presence of a small level of positively charged amino acids on its surface

The enzyme assay results for SrMDH were unexpected. From its highly negative surface charge as well as the large level of acidic amino acids on its surface (section 3.2.2), indicate that it is a typical halophiic enzyme and would suggest that the presence of salt should in fact stabilise this enzyme and increase its activity, in comparison to when no salt is present, as has been found for other halophilic enzymes [88,170,189,206]. However, the fact that this enzyme is less active in the presence of salt, as compared to in the absence of salt, suggests there are other factors that need to be taken into account rather than merely an increased level of acidic residues on the protein surface. Figure 3.11 shows that SrMDH contains slightly less acidic residues than HmMDH, HvMDH and HsMDH (the other salt-in halophiles), with SrMDH containing 51 acidic residues whereas HmMDH, HvMDH and HsMDH contain 62, 58 and 58, respectively. In addition, Figure 3.10 shows that SrMDH also contains a slightly higher pI than the MDHs from these other salt-in halophiles (SrMDH pI is 4.42, in contrast to 4.2, 4.2 and 4.12 for HmMDH, HvMDH and HsMDH, respectively) – this small difference may account for a dramatic difference in salt stability. More significantly, this result can perhaps be explained by the presence of positively charged residues on the SrMDH protein surface, more obvious from the monomeric surface charge prediction (Figures 3.13 and 3.14). Although SrMDH has less basic residues on its surface than both EcMDH and EvMDH, it also contains more basic residues than the well characterised halophilic enzyme HmMDH, which is stable at high salt concentrations and unstable at low salt concentrations – the electrostatic potential surface of this protein is shown in Appendix Figure A. HmMDH contains no visible positive surface charges, which may correlate to its increased stability at high salt concentrations [191,197,345]. The much lower tolerance of SrMDH towards salt as compared with other halophilic proteins may be explained by the

fact that lower levels of positively charged residues (particularly lysine) on halophilic proteins is associated with their stability in the presence of high salt concentrations, due to allowing increased flexibility within these conditions [348,557]. Therefore, the most likely explanation of the instability of SrMDH at high salt concentrations is the presence of positively charged amino acids on its surface, which may result in the protein becoming too rigid in the presence of high salt concentrations, hence decreasing its reaction rate.

The ICP-MS analysis found that *S. ruber* is able to maintain a cytoplasm with high concentrations of salt (for example, refer to Tables 5.1, 5.3 and 5.5). However this result does not correlate to the instability of SrMDH in the presence of salt – thus suggesting that the MDH of *S. ruber* functions sub-optimally within the physiological conditions of this organism. However, the fact that *S. ruber* was found to accumulate more Na^+ than K^+ and SrMDH was slightly more stable in the presence of higher concentrations of Na^+ than K^+ suggests that the effect of specific ions on enzymatic activity may be correlated with ion accumulation. The fact that the MDH of *S. ruber* is sensitive to salt but still retains function within high salt concentrations, but with a diminished rate, could potentially explain the lower growth rate of *S. ruber*: MDH is essential for metabolism [558] and therefore its lower rate (at the physiological conditions of *S. ruber*) may result in less energy being available to the cell, and hence slower growth. It has previously been found that *E. coli* deficient in the MDH gene had severely decreased growth rates [559].

The fact that *S. ruber* is naturally found in hypersaline environments and yet its MDH does not function optimally within these conditions could result in it being outcompeted by faster growing species (at an evolutionary level), due to less energy being available to the cell (from central metabolism) and hence competition for nutrients may have been detrimental to the organism [560]. However, if nutrients in the environment were not scarce then this may not be the case – *S. ruber* has a natural slow rate of growth and is still abundant in these environments, therefore suggesting that it was not outcompeted by 'stronger' strains. Additionally, it could be the evolutionary history of *S. ruber* that results

in the decreased salt stability of its MDH, in comparison to other halophilic MDHs. For example, HmMDH is from an archaeon [494,546] and has been subject to different evolutionary pressures from that of the bacterium *S. ruber* – i.e. *S. ruber* is more closely related to non-salt-in organisms, whereas *H. marismortui* is closely related to many 'salt-in' halophiles.

Although the general cation preferences of an organism regarding growth, accumulation and enzymatic function correlate (i.e. cations which promote growth and accumulate in moderate concentrations generally result in greater enzymatic activity), the range of growth of an organism in a specific salt does not appear to correlate directly to the salt concentrations where the MDH functions optimally. For example, at higher substrate concentrations EcMDH retains activity within salt concentrations that *E. coli* is unable to grow at, and with the exception of KBr, *E. vietnamensis* has a larger growth range (with respect to salt concentration) than the range that its MDH is able to function within. This suggests that the decrease in MDH function alone cannot be the only factor responsible for growth inhibition and that an analysis of the activities of different proteins in these conditions will be vital to determine the correlation between specific ion effects on proteins and its relation to the growth and metabolism of an organism.

Cation transport is essential towards tolerance to specific ions

Presence of kup (TrkD) may determine level of toxicity of Rb^+/Cs^+

E. coli contains the kup (TrkD) K^+ transporter, which has previously been shown to also be able to transport Cs^+ [445]. However, analysis of cation transport proteins present in the three organisms (Table 3.8) found that this transport system was lacking in *E. vietnamensis* and *S. ruber*. The ICP-MS data (section 5.2.6) showed that *E. coli* generally accumulated larger levels of Rb^+/Cs^+ than *E. vietnamensis* (at the same concentrations), presumably due to the presence of this kup (TrkD) transport protein. In addition, *E. coli* also contains the TrkG and

TrkH K^+ transport proteins, *E. vietnamensis* does not and *S. ruber* only contains TrkH. TrkG and TrkH have been previously found to be able to transport Rb^+ [499,561,562], which may explain the higher concentrations of this ion in *E. coli* than *E. vietnamensis*. In addition, *S. ruber* was found to accumulate significant levels of Rb^+, which may be explained by the presence of TrkH, whereas *E. vietnamensis* Rb^+ accumulation may be lower on account of the lack of either of these proteins. Even though kdp cannot transport Cs^+/Rb^+, the fact that it is only present in *E. coli* suggests that it is possible that these larger cations could be blocking the kdp transporter (from the outside), as Cs^+ ions have been found previously to be able to block other K^+ transporters [130,450,451]. Alternative transport systems that are able to transport Cs^+ have been found in other bacteria, such as *Rhodococcus sp.*, so this Cs^+ transporting ability is not unique to *E. coli* [499]. It cannot be conclusively stated that other membrane transporters with the ability to transport Rb^+/Cs^+ do not exist in either *E. vietnamensis* and *S. ruber*. However, the specific membrane transporters present in these two organisms that have previously been characterised in other organisms do not have the ability to transport these non-physiological ions (other than TrkH of *S. ruber*).

Blockage of K^+ efflux may be the cause of the toxicity seen in *E. coli*. It has previously been found that Cs^+ can block outward K^+ currents (i.e. K^+ efflux) in the squid giant axon [449], therefore Cs^+ (once inside the cell) may potentially block the Kef K^+ efflux transporters in *E. coli*, as KefB, KefC KefF and KefG are not present in either *E. vietnamensis* or *S. ruber*, hence having specific toxicity for *E. coli* (refer to Table 3.8). Blockage of these proteins could result in an increased K^+ concentration inside the cells – although this does not appear to be the case from the data presented in Chapter 5. In addition, no literature could be found on the block of Kef-channels by larger cations [140,563]. An alternative explanation involving channel block is the blockage of the *E. coli* kup transporter from the cytoplasmic side. However, this would presumably result in lower levels of K^+ inside the cells, which did not appear to be the case. Therefore, the *E. coli* specific K^+ toxicity is most likely due to cellular entry via the kup transporter and due to four potential explanations: blockage of Kef K^+ efflux transporters, hence

preventing K^+ efflux; blockage of kup K^+ channels, hence preventing the influx of K^+; alterations of membrane potential caused by Cs^+/Rb^+ presence, hence meaning lower energy generation; and molecular effects on protein function, due to higher polarisability of Cs^+/Rb^+ as compared with K^+. It has previously been found that high Cs^+ concentrations can cause the dissociation of the 50S and 30S ribosomes of *E. coli*, and this is irreversible [126]. Cs^+ is the largest cation used in the current study and hence is the most polarisable and may have more adverse affects on protein structure [213]. Therefore, the effects on *E. coli* growth may be due to a combination of specific transporters causing the entry and/or channel block and this accumulation then leads to specific intracellular effects of Rb^+/Cs^+ on intracellular proteins, leading to growth inhibition.

Salt-tolerant organisms contain 'specialised' cation transporters – which may be essential for halotolerance

The finding that *E. vietnamensis* contained NhaD, as well as *S. ruber* containing MnhB and MnhG cation transporters (section 3.4) provides additional insight into mechanisms of bacterial salt tolerance. Both of these proteins have previously been described to confer Na^+ tolerance, with NhaD only (so far) being characterised in salt-tolerant organisms and a deficiency of Mnh (and paralogues) being reported to lead to an increased salt sensitivity [378–380]. Additionally, since NhaD has been reported to import Na^+ into the cell (as opposed to exporting it) this may explain the mechanism of the increase in Na^+:K^+ ratios of *E. vietnamensis* with increasing external salinity - which may aid its tolerance of higher Na^+ concentrations within its environment. The fact that this cation transporter (NhaD) has been reported to confer Na^+ tolerance to various species (salt tolerant and halophilic organisms) and when expressed in *E. coli* was found to increase Na^+ tolerance, suggests that this system may be crucial for salt tolerance in many organisms [564–566]. Therefore, these transporters merit further research in terms of their contributions towards the salt-tolerance of an organism – with potential applications for the development of salt tolerance towards organisms of commercial value.

In addition, the fact that both *E. vietnamensis* and *S. ruber* do not contain the K^+ efflux system proteins KefB, KefC, KefF and KefG may further suggest that this could be 'symptomatic' of salt tolerant organisms, as well as their much lower levels of mechanosensitive channels, as compared to *E. coli*. *E. vietnamensis* was also found to have a lower level of efflux pumps generally, perhaps reflecting its greater natural requirement for cation accumulation, due to the ionic composition of its natural niche [298]. Additionally, it should be noted that *S. ruber* also contains a cation transporter annotated as 'CPA2', which could suggest a potential salt-tolerance related protein, as CPA3 family proteins have previously been associated with an increase in salt resistance [381]. However, since kef transporters have been stated to be members of this family of proteins [567], the role of CPA2 in *S. ruber* salt tolerance is unclear and therefore should be further characterised, as well as Mnh and NhaD, in terms of their roles regarding salt tolerance.

LiCl and KBr when used together may have promise as an antimicrobial agent

The results from the growth experiments on the effects of equimolar LiCl:KBr were dramatic: growth of both *E. coli* and *E. vietnamensis* was totally inhibited. Since *E. vietnamensis* was able to grow in every other ion combination tested (21 different combinations), there is clearly a toxicity effect of this particular salt combination. It has previously been found that using chlorine together with bromine has a more effective antibacterial effect than using either element alone [460]. Chloride has also been found to be essential for growth at high NaCl concentrations for some organisms (including *E. coli*) [442] - perhaps the lower concentrations of Cl^- (replaced by Br^-) may mean that the organisms struggle more to grow at high salt concentrations as Br^- cannot adequately take the place of Cl^-. In addition, Br^- block of Cl^- channels within the cell membranes could lead to partial toxicity, as it has been shown in a previous study that Br^- can impair the passage of Cl^- through Cl^- channels and may potentially have channel block activity [568]. Therefore the presence of both anions could result in sub-optimal

Cl^- levels in the cell, hence resulting in toxicity – especially if Cl^- is required at high salt concentrations, for survival of the organisms, as has been previously reported for other organisms [569,570]. Furthermore, the presence of both Li^+ and K^+ may contribute – when Na^+ was used instead of of K^+ (NaBr:LiCl), growth was not inhibited to the same degree as in equimolar LiCl:KBr (although it was inhibited more than in NaCl:LiCl). It has been found that K^+ accumulation in *E. coli* is affected by Li^+ presence within the cells as it is unable to extrude Li^+ as efficiently as it can extrude Na^+, and due to this lower level of Li^+ extrusion this will mean that K^+ cannot enter the cells as effectively - due to the altered membrane potential caused by Li^+ presence in the cells. This could result in a reduction in the levels of K^+ within the cell as well as having an effect on energy generation, i.e. affecting growth [147]. This effect could be exaggerated by the lack of K^+ inside the cells - Li^+ may bind to proteins and ribosomes with no competition from K^+, and may result in their destabilisation. Since the mechanism of Li^+ for the treatment of bipolar disorder is thought to involve a modulation of the membrane potential of neurons [571], this further emphasises the effect that this cation may have on the membrane potential of a cell, which may affect energy generation and consequentially cell growth. This growth inhibitory effect may result in total toxicity when combined with the effect of Br^- on intracellular Cl^-.

The mechanisms of growth inhibition by this salt combination need to be further investigated. However, the fact that this salt combination appeared to totally inhibit bacterial growth suggests that it could have potential as an anti-septic agent, particularly given the increased resistance of bacteria to more traditional methods [572–574]. Due to the effectiveness of this salt combination, regarding bacterial growth inhibition, a patent application is pending, for the use of this particular salt combination as an antiseptic agent.

The halotolerant and extremely halophilic classification of *E. vietnamensis* and *S. ruber* should be reconsidered

E. vietnamensis was found from the current study to be able to grow within salt concentrations that would be considered to be optimal for many moderately halophilic organisms (for example refer to Figures 4.11, 4.24 and 4.26). There is no doubt that *E. vietnamensis* is a halotolerant organism, as opposed to a halophile, as it is indisputable that it grows better in the absence of salt or at lower salt concentrations. However, the fact that it can tolerate such high salt concentrations strongly suggests that it should be re-classified from a halotolerant organism to an extremely-halotolerant organism, according to the widely used classification system established by Don Kushner (refer tosection 1.1.3), whereby extremely halotolerant organisms have the ability to tolerate 2.5M salt or greater [84].

Additionally, the data presented in the current study suggest that *S. ruber* should be re-classified as a borderline-extreme halophile (refer to section 1.1.3 for details of halophile classification). *S. ruber*, since its initial characterisation, has been classified as an extremely halophilic bacterium [199]. *S. ruber* contains an acidic proteome (Chapter 3) and utilises the salt-in adaptation strategy (Chapter 5), traits that are consistent with extremely halophilic organisms [206]. However, the data presented in the current study suggest that this classification needs to be re-examined. The proteome of *S. ruber* was found to have an average pI of just over 6, which is significantly higher than that of the other extreme halophiles (refer to Figure 3.15 and Table 3.4) and is only slightly lower than that of the 'salt-out' halophiles (Figure 3.16 and Table 3.5). This, in addition to the behaviour of SrMDH within salt, i.e. it functions optimally in the absence of salt, although can still retain some activity at high NaCl concentrations (Figures 6.6 and 6.7), and the fact that its optimal growth in NaCl occurs between 2M – 3M NaCl (Figure 4.8), suggest that it should be re-classified as a borderline-extreme halophile [84].

E. vietnamensis may use a hybrid strategy of osmotic adaptation

Due to both its growth at high salt concentrations and its high level of cation accumulation (refer to Chapters 4 and 5), it is suggested that *E. vietnamensis* may use a hybrid osmotic adaptation strategy. At lower salt concentrations, the intracellular cation concentration of *E. vietnamensis* is low (similar to that of *E. coli*), and presumably accumulates compatible solutes (for osmotic balance). However, as the concentration of salt in the medium increases, this organism accumulates much higher concentrations of cations within its cytoplasm, suggesting that once the salt concentration reaches a certain level (2 - 3M) the osmotic adaptation strategy of this organism switches. This is perhaps similar to that of *H. halophila*, which is able to switch osmotic adaptation strategy dependent on the external salinity, i.e. it accumulates molar levels of K^+ at higher concentrations and compatible solutes at lower concentrations [190]. In addition, *H. halophila* has a slightly acidic proteome, which was also found in the current study for *E. vietnamensis*, as well as the finding that the MDH of *E. vietnamensis* contains an increased level of acidic amino acids in comparison to the MDH from *E. coli* (Figures 3.11 and 3.12). *H. halophila* has an average proteome pI of around 6.2 and *E. vietnamensis* has an average pI of around 6.6 (lower than that of the other halotolerant organisms analysed in this study), both slightly acidic. This further supports the theory of *E. vietnamensis* utilising this hybrid osmotic adaptation strategy, which has so far only been characterised in a few organisms, including *H. halophila* and *H. halophilus* (discussed in section 1.3.3) [97].

Furthermore, the Na^+:K^+ ratio in *E. vietnamensis* increases with increasing external salinity (refer to Chapter 5). This is supportive of the theory that Na^+ is more effective at providing osmotic balance for the salt-in strategy (at moderate salinities) and K^+ is more effective for the salt-out strategy (low salinities), as K^+ has been found to stimulate accumulation of the compatible solute glutamate [505]. Therefore, it is suggested that *E. vietnamensis* utilises a hybrid osmotic

adaptation strategy – at low and moderate salinities it accumulates compatible solutes and at higher salinities it switches to accumulate inorganic ions, supported by its acidic proteome and high inorganic ion content.

Conclusions

The ion effects and mechanisms elucudated from the current study are shown in Table 7.1. This work has shown that specific cation preferences are directly related to the salt tolerance of a particular organism. This is based on the fact that *S. ruber* showed a general preference for Na^+ in terms of growth, cellular accumulation and enzymatic activity, whereas the non-halophilic organisms preferentially accumulated K^+, as well as showing better growth and (to some extent) better enzymatic activity in its presence, which was most profound for the non-halophile, *E. coli*. This preference of *S. ruber* for Na^+ is different to what has previously been found for salt-in halophiles, as well as being different to what has been found for non-halophiles. This can be rationalised based on the fact that large concentrations of Na^+ have been found to be destabilising even towards halophilic proteins, but since the data from the current study show that *S. ruber* has a lower optimal salt concentration than is typical for salt-in halophiles (i.e. the extremely halophilic archaea), Na^+ may actually provide a better degree of stabilisation of this organism's proteome than K^+, without the risk of destabilisation. This is justified based on its requirement for lower levels of intracellular cations than is required for the extremely halophilic archaea, which is due to *S. ruber* showing a a lower salt concentration growth range than these organisms - i.e. it does not need to balance its cytoplasm with an as highly concentrated environment as the salt-in archaea do. The fact that *S. ruber* was found to have a less acidic proteome than the other salt-in halophiles may also reflect its salt concentration growth range – i.e. proteome acidity may be a direct indicator of the degree of salt tolerance. The fact that *S. ruber* may be differently adapted than other salt in halophiles is reflected in the instability of its MDH at high salt concentrations. This typically haloadapted protein may be unstable at high salt concentrations due to the presence of a small amount of positively

charged residues on its surface – further indicating differences between *S. ruber* and the archaeal extreme halophiles.

This is further supported by the data presented for *E. vietnamensis*. This organism was found to have a slightly acidic proteome and contained significantly higher levels of cations than was found for *E. coli*. Consequently, it was postulated that this organism seems to use a hybrid osmotic adaptation strategy – resulting in a higher tolerance for cations than non-halophiles. This hybrid strategy (i.e. ability to accumulate high levels of cations when the external salt concentration is high) may be determined by proteome acidity, as the other halotolerant organisms analysed (in terms of proteome pIs) are not known to accumulate high levels of cations and hence did not contain acidic proteomes. Therefore, it would appear proteome acidity might be a major determining factor for the specific cation preferences of an organism – which hence determines its level of salt tolerance.

Regarding common mechanisms of salt tolerance, the data presented suggest that specific cation transporters within the cell membrane appear to be crucial. Firstly, the toxicity of large cations towards *E. coli* is likely to be due to the presence of a specific cation transporter (kup), not present in the salt-tolerant organisms - hence suggesting that salt tolerant organisms may have an overall increased tolerance to a wider range of cations. Additionally, it could be the case that an acidic proteome protects organisms from adverse effects by larger cations by decreased adsorption to their surfaces, a consequence of the increase in COO^- groups – i.e. a decreased level of the protonated form, which highly polarisible cations may be more likely to interact with. It was found that both *E. vietnamensis* and *S. ruber* contained cation transporters that were not present in *E. coli*, which are known to be associated with increased salt tolerance – MnhB and MnhG (cation proton antiporter activity) of *S. ruber* and NhaD (Na^+/H^+ antiporter – potentially involved in Na^+ import into the cell) of *E. vietnamensis*. These distinct proteins may be a major factor in the overall salt tolerance of these organisms – in addition to the acidic proteomes and hence cation preferences. The fact that *S. ruber* did not contain Nha transporters may also be

related to its cation preferences. These were found to be present in both *E. coli* and *E. vietnamensis* and have also been found to be present in salt-in extremely halophilic archaea – hence furthering support for the theory that *S. ruber* has different cation preferences to these organisms, i.e. it does not remove Na^+ from its cytoplasm to an as great extent as the other 'salt-in' halophiles.

Table 7.1. Ion effects and mechanisms. The ion effects found within the present study and their potential mechanisms are summarised.

Ion(s)	Effects	Mechanisms	Crucial factors for effects
Li^+	May be stabilising towards halophilic proteins at lower concentrations/destabilisaing towards halophilic proteins at higher concentrations.	Due to its kosmotropic nature, high charge density Li^+ interacts strongly with kosmotropic COO^- groups, which are more abundant on halophilic proteins – small level = stabilising, moderate levels = protein destabilisation and lack of cell growth. May have similar reactivity to that of Mg^{2+} May also affect growth by altering the membrane potential.	Presence of Nha transporters results in Li^+ being removed from cell and Li^+ tolerance. Absence of these results in a decreased ability to remove Li^+ from the cell and hence Li^+ build-up in cell can lead to the aforementioned protein destabilisation/altered membrane potentials. Proteome pI may also determine the extent of the effects, as does the intracellular concentration.
Na^+	Destabilising towards higher pI proteins. Moderate concentrations stabilise lower pI proteins more effectively than lower charge density cations - higher concentrations may be destabilising – even towards acidic proteins.	Can stabilise halophilic proteins at moderate concentrations by the increased association with kosmotropic COO^- groups. At higher concentrations may lead to destabilisation of even halophilic proteins by so called 'salting in'.	Proteome pI determines extent of effects and tolerance. External salt concentration also determines effects – if cell has to balance a more hypersaline environment then higher Na^+ concentrations accumulated may be destabilising towards cellular proteins, but lower accumulation may be stabilising (halophilic proteins).

K^+	Cation of preference for non-halophiles – low levels may be optimal for osmotic balance and enzymatic activities.	K^+ interacts less strongly with COO^- and therefore protein remains hydrated – due to chaotropic/kosmotropic nature of K^+/COO^-	Salinity determines level of accumulation – non-halophiles will prefer to accumulate K^+ due to its less disruptive effect on proteins and may be more beneficial for osmotic adaptation of halophiles growing at very high salinities – due to the level of cation accumulation required and its lower level of protein destabilisation (at higher concentrations) than Na+.
Rb^+ and Cs^+	May be destabilising towards proteins – especially non-halophilic proteins	Due to adsorption to protein surfaces, caused by the large polarisability of these cations – may be more pronounced for more neutral proteins. The high level of kosmotropic COO^- groups on salt adapted proteins may impact a degree of protection from this effect.	The presence of specific proteins within the membrane that can transport these cations – kup (TrkD) and TrkA/TrkH – leads to a higher level of accumulation and hence higher potential for toxicity. Proteome pI also may influence degree of protein destabilisation.
Cl^- and Br^-	Cl^- is less inhibitory towards bacteria of all salt tolerances than Br^-.	Cl^- may be required for Na^+ transport into (*S. ruber*) or out (*E. coli*) of the cell. Br^- may interact more strongly with protein surfaces than Cl^- (with NH_4^+) – according to the Hofmeister effect	External salinity may determine degree of effects due to extent of Cl^- requirement. May be less harmful towards lower pI proteins.

Next steps and future research

The effects of specific ions should be extended to include more organisms (of varying salt tolerances), in order to further determine the relation between proteome pI, salt concentration range and intracellular cation preferences, to

determine if the results found from the current study are universal throughout nature. This research has applications within astrobiology – if life can support the presence of non-physiological ions, especially at higher concentrations, then this may suggest that 'similar life could exist elsewhere on other planets, utilising these more 'unusual' ions within metabolism. Therefore, further explorations regarding the limits of life in terms of ion metabolism should give increased insight into these questions.

A thorough analysis of more *S. ruber* proteins in terms of surface charges and the relation of this towards enzymatic activity should be assessed, to determine to what extent its proteins are adapted to high salinity, given that the pI value of its proteome was higher than was found for the other salt-in halophiles (refer to Table 3.8). Additionally, a similar analysis of moderate halophiles may give increased insight into the full spectrum of salt tolerance.

The effect of the KBr + LiCl salt combination should be more thoroughly assessed as a next step for this research: lower concentrations should be tested on *E. coli* and *E. vietnamensis* to determine the minimum inhibitory concentrations (MICs) and this should then be extended to test a wider range of bacteria – particularly those of clinical relevance, such as *S. aureus*, which is a halotolerant bacterium [575,576] [575]. It is hoped that further research on this combination could eventually result in a novel salt-based antiseptic/disinfectant, which there is presently a demand for due to the rise in microbial resistance to currently used antiseptics [577]. Additionally, since overuse of traditional antibiotics is associated with an increased level of bacterial resistance [578], other methods of antibacterial treatment, such as the salt-based method presented here, could help to lower the rate at which antibiotics are administered, thus helping to reduce the spread of antibiotic resistance.

E. vietnamensis should be further characterised in terms of its salt concentration range of growth, as well as its tolerance to a wide array of cations, since these appear to be unique features of this organism. *E. vietnamensis* may also have applications within industry due to its ability to tolerate a wide range of

salinities. For example, it could have potential to be utilised for the biodesalination of seawater and hypersaline waters up to as high as 3M total salt concentrations – making it extremely versatile in terms of the environments it could be utilised within. Its potential for this application is enhanced by its relatively fast growth rates (especially in comparison to the halophile *S. ruber*) and the fact that the presence of certain salts was even found to stimulate growth – making it an especially useful candidate for this purpose.

Finally, the Mnh and especially the NhaD cation transporters should be analysed further, in terms of their overall contributions towards the salt tolerance of an organism (perhaps via knock-out experiments), in addition to the analysis of other salt tolerant organisms with the aim to detect additional cation transporters essential for salt tolerance. The finding that these proteins may be responsible for imparting salt tolerance to bacteria has potential to be utilised for the bioengineering of salt resistance to organisms of commercial value, specifically crops growing in hypersaline soils.

Concluding remark

The current study was carried out with the aim of investigating the specific effects of various cations on bacteria and the relation of this towards bacterial salt tolerance mechanisms, at the interface of biology, physics and chemistry. The extent of the salt tolerance of an organism was found to be a product of its proteome pI as well as the presence of specific membrane transporters. This in turn influences the optimal salt concentration for growth of that organism, which is a major factor influencing the specific cation preferences of that organism – which can be rationalised based on the Hofmeister effect. This study gained a range of important insights into into bacterial salt tolerance, as well as the finding of a potential novel antiseptic agent (patent application currently pending) and characterisation of a so far little studied organism (*E. vietnamensis*). The current study and its findings have a wide array of potential applications, including within astrobiology, medicine and industry.

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Appendix

Appendix A

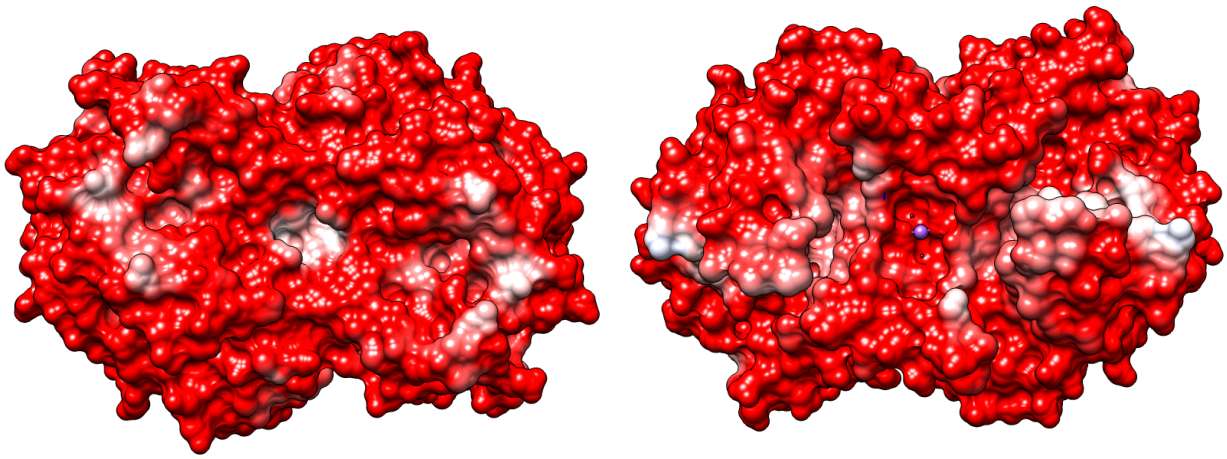

Figure A. Electrostatic potential of the surface of the tetrameric MDH from *H. marismortui.* Structures are coloured according to the surface electrostatic potential: red = negative; blue = positive; white = neutral. Structures were coloured via the Coulombic surface colouring tool in Chimera.

Appendix B

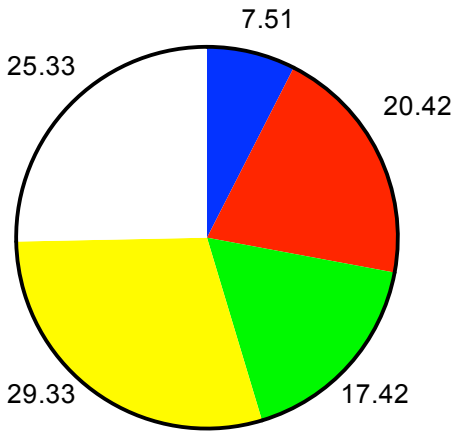

Figure B. Amino acid composition of HmMDH. Amino acid compositions were calculated via ProtParam. Blue = basic amino acids; red = acidic amino acids; green = polar amino acids; yellow = apolar amino acids; white = other amino acids.

Appendix C

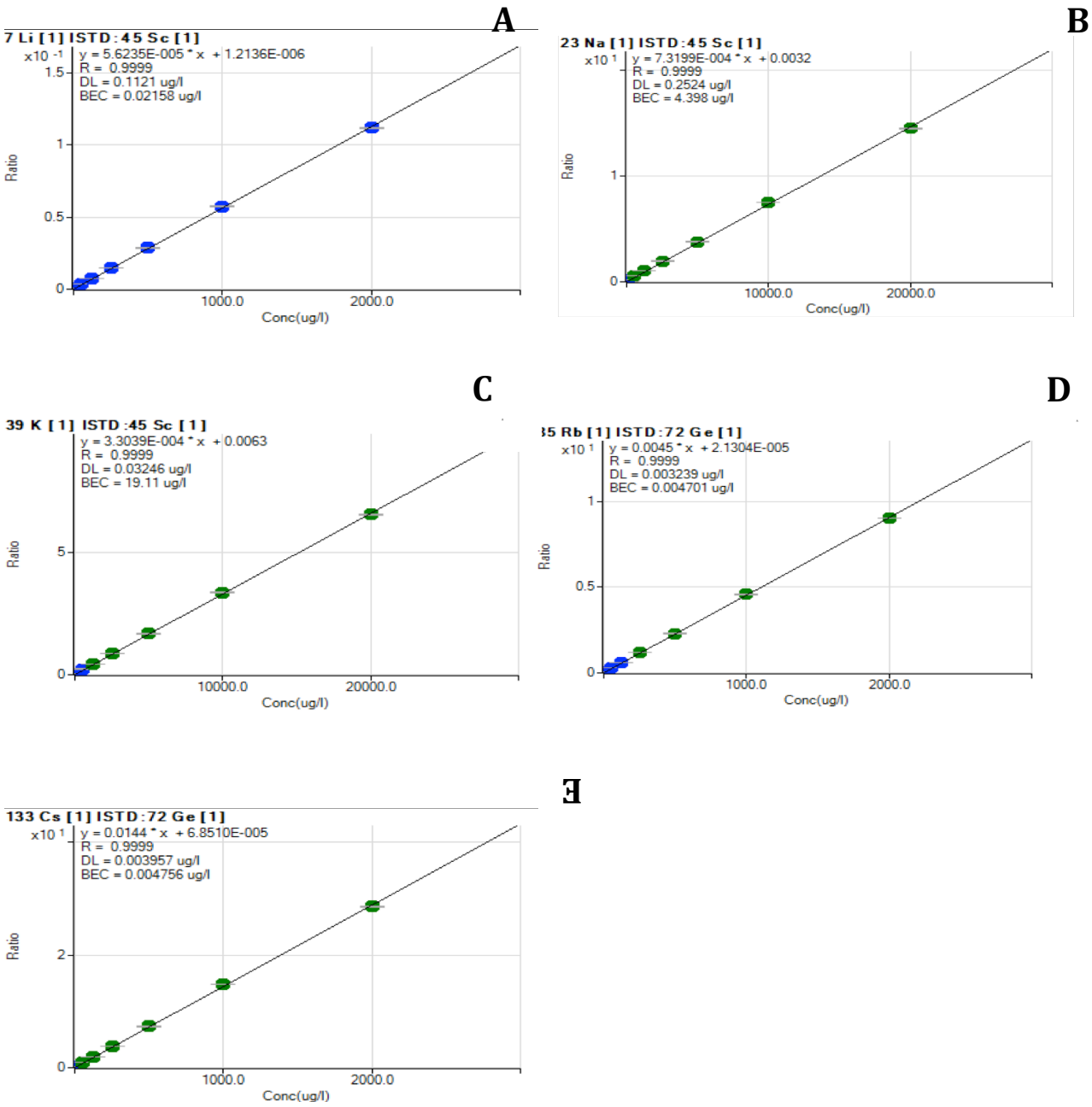

Figure C. Calibration curves for ICP-MS. A: Li^+, B: Na^+, C: K^+, D: Rb^+, E: Cs^+.