Toxicity of Ionic Liquids and Organic Solvents towards
Escherichia coli and Pseudomonas putida

A thesis submitted to The University of Manchester for
the degree of Doctor of Philosophy in the Faculty of
Engineering and Physical Science

2011

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Abstract

The University of Manchester

Nicola Wood

Degree of Doctor of Philosophy

Toxicity of Ionic Liquids and Organic Solvents towards Escherichia coli and Pseudomonas putida

Funded by BBSRC

2011

By turning to biological catalysts such as whole cell microorganisms it is possible to both improve the specificity of a (bio) chemical transformation with the added benefit of reducing the energy demands of a number of synthetic processes which currently use chemical catalysts.

The replacement of chemical catalysts with whole cell biological catalysts is often limited by the toxicity of the substrate, side products or end products of a catalytic reaction. The exposure of microorganisms to these products or substrates is usually reflected in phenotypic alterations in membrane of the cell. The techniques of growth data, FT-IR spectroscopy and cluster analysis have been successfully used to establish the phenotypic changes occurring within a microbial culture.

The use of microorganisms as replacements for chemical catalysts in synthetic processes may be further increased by the replacement of conventional organic solvents, with a different class of solvents known as ionic liquids.

Ionic liquids have been widely reported as ‘green’ solvents due to their negligible vapour pressure; however reported toxicity testing has demonstrated that many ionic liquid structures have poor toxicity profiles when tested against a range of microorganisms.

Due to the large number of ionic liquids which are currently available it is desirable to have a fast and reliable method for initial toxicity screening of many ionic liquids against a wide range of microorganisms. To this end, a simple and cost effective method for ionic liquid toxicity testing using agar diffusion plates and specific growth rates, has been developed and employed to test a wide range of ionic liquid structures against a number of test bacteria.

Additionally, the phenotypic changes associated with the exposure of a number of test organisms to a set of both water miscible and water immiscible ionic liquids, have been successfully assessed by FT-IR spectroscopy, cluster analysis, growth data and viable count information, suggesting that this combination of techniques has great potential for future work involving the assessment of phenotypic alterations within microbial cultures.
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Dedicated to all of my family for their unwavering love and support and especially to Jess for keeping me company.
Literature Review

1.1. Ionic Liquids

1.1.1. Introduction and Early Development

Probably at no time in human history has the impact of our actions on the natural world been more closely scrutinized. Increasing public concern over the impact of industrial processes is pushing industry, government and academic research to rethink the use of industrial processes which are harmful to the environment and to find alternative, safe, environmentally benign alternatives. One such example of this is the renewal of interest in a group of chemicals known as ionic liquids. Although first reported in scientific literature in 1914 (Walden, 1914) it is only over the last twenty years that sustained interest in these chemicals has been shown.

The early literature concerning ionic liquid toxicity focused mainly, although not exclusively on the investigation of quaternary ammonium salts with long alkyl chains for use as antiseptic and germicidal agents (Shelton et al., 1946c), (Shelton et al., 1946b), (Shelton et al., 1946a), (Hoogerheide, 1945). In these early studies it was noted that ammonium salts which contained at least one long chained alkyl substituent were highly toxic to a range of micro-organisms, with C\textsubscript{16} being the optimum alkyl chain length for maximum germicidal activity. In this work it was also noted that the quaternary alkyl ammonium salts displayed an \textit{in vitro} toxicity, to a range of test organisms which were comparable or better than the bactericidal properties of antiseptics in frequent use at the time of the research, including iodine-based and mercury-based compounds (Hoogerheide, 1945). Alongside the investigation of quaternary ammonium salts the toxicity of the ionic liquid cetyl pyridinium chloride was also investigated, again the ionic liquid was reported to be toxic towards a range of test organisms including both Gram positive and Gram negative bacteria (Quisno and Foter, 1946).

The focus of this early toxicity work was the identification of compounds for use as bactericidal and biocidal compounds to use as replacements for the phenol and
mercury containing compounds which were in common use at the time of these publications.

Applications for ammonium salts, as well as aluminium chloride and 1-ethylbutylypyridinium bromide in electrodissposition were investigated (Hurley and Wier, 1951) as was the suggestion that ionic liquids could be used as electrolytes in batteries (Wilkes, 2004). Up until the 1970s the usefulness of ionic liquids in industrial processes had been severely limited by the viscosity of the ionic liquids as well as by their sensitivity to water (Plechkova and Seddon, 2008). However, the synthesis of a new class of ionic liquid using the imidazolium cation overcame both of these problems firstly by reducing the viscosity of the solvent (Wilkes et al., 1982) and secondly by synthesising ionic liquids which where not as sensitive to the presence of water (Wilkes and Zaworotko, 1992). The synthesis of ionic liquids that were both less viscous and less water sensitive allowed for a greater range of applications for these solvents to be considered. Previously ionic liquids could be used only in an inert moisture free environment, limiting their usefulness. These improvements in ionic liquid properties as well as the wider range of ionic liquids which were starting to be synthesised; i.e. combination of imidazolium cations with a wide range of different anions, led to an increased interest in ionic liquids and their potential.

1.1.2. New Applications

It was not until the 1990s that the potential for the application of ionic liquids to replace conventional organic solvents and to play a role in biocatalytic processes began to be appreciated (Seddon, 1997) and ionic liquids began to be considered as environmentally benign replacements for conventional solvents.

Whilst the development of air and water stable ionic liquids had undoubtedly increased the interest in them in both academic and industrial circles it does not wholly explain the unprecedented interest that these solvents have generated. The emergence of ionic liquids as potential replacements for conventional organic solvents has coincided with an increased awareness across industry, academia and
the wider public of the potentially devastating impact of man-made chemicals on
the environment and natural world, both in terms of the effect of the chemicals and
chemical processes themselves and in terms of the high energy demands i.e. energy
required to generate the high temperatures and pressures required for many
conventional catalytic processes. In response to this increasing public concern
industry and academia have been pushed towards finding environmentally benign
alternatives to conventional chemicals as some of the most harmful chemicals are
phased out of manufacturing and out of use following agreements such as the
Montreal Protocol in 1989 (Vijayendran et al., 2008). Moreover, other chemicals
and chemical processes have come under stricter regulation, for example the
introduction of European Union regulation REACh (Registration, Evaluation and
Authorisation of Chemicals), which will be applicable to all chemicals manufactured
in quantities of greater than 1 tonne per annum and will thus require the
manufacturer to provide extensive safety data with regard to the impact of the
chemical on both human health and the environment (Wood and Stephens, 2010).

To understand why ionic liquids have been seen as such an attractive alternative to
conventional solvents it is necessary to consider some of the general properties of
ionic liquids. Ionic liquids, or room temperature ionic liquids, are, as the name
suggests liquids which are composed entirely of ions at room temperature (Seddon,
1997). However, the name room temperature ionic liquids can be misleading,
suggesting that these salts are only liquid within a very narrow temperature range
i.e. at room temperature. This is far from the truth with most ionic liquids having a
wide liquidus range i.e. the range of temperatures between the normal freezing
point and boiling point of the liquid (Seddon et al., 2000). This is a particularly
attractive property for use in catalysis applications in which a wide range of
temperatures may be required.

Additionally, ionic liquids have negligible vapour pressure under ambient conditions
making them non-flammable compared to conventional organic solvents (Petkovic
et al., 2011), (Welton, 2004). It is this property of negligible vapour pressure that
has largely led to ionic liquids being christened as ‘green’ solvents. The negligible
vapour pressure of ionic liquids means that the evaporation of an ionic liquid to the
atmosphere would be substantially less than evaporation of a conventional solvent. This has the double benefit of both effectively eliminating the possibility of air pollution as well as ensuring extra protection for individuals working with ionic liquids (Plechkova and Seddon, 2008).

Perhaps the most interesting and desirable property of ionic liquids is the realisation that it is possible to design an ionic liquid for use in a particular application. As ionic liquids are composed of both a cation and an anion it is possible to alter one or the other of these components thereby generating a unique ionic liquid which will have its own physical, chemical and toxicological profile (Sheldon et al., 2002), (van Rantwijk et al., 2003). This property of ionic liquids has led to their being described as ‘designer solvents’ (Freemantle, 1998).

The advantage of this property of ionic liquids is readily apparent. If, for example, an ionic liquid is required as a solvent in a whole cell biocatalyst reaction but the ionic liquid chosen is found to be toxic to the biocatalyst, the composition of the ionic liquid can be varied. This is achieved; either by substitution of the cation or anion or by alteration of the structure of one or other of the components; e.g., altering the length of associated alkyl chains or incorporating ether linkages into the structure. By varying the composition of the ionic liquid it should therefore be possible to find a combination of cation and anion which will result in an ionic liquid with the properties desirable for the application being developed. In contrast there are only a limited number of organic solvents available, if it is not possible to find a solvent which will fit the requirements of an application then it would be necessary to rethink the design of the application which would be a potentially costly and time consuming process as modification of the solvent or its properties would not be a possibility.

That the designer nature of ionic liquids is advantageous when it comes to the selection of a solvent specifically suited to a particular application is unquestionable; however this also generates a set of problems which are not encountered when dealing with conventional solvents. As there are a finite number of conventional solvents available, the majority of which have been well
characterized and their applications widely reported in the literature, this means that the pool of potential solvents can be quickly narrowed down to a handful of possibly suitable candidates which can then be considered and tested for suitability.

For ionic liquids the situation is very different, the possibility of combining any cation with any anion to produce an entirely unique ionic liquid means that the number of potential solvents is vast. It has been estimated that there are at least a million simple ionic liquids all of which would have their own unique properties (Plechkova and Seddon, 2008). Clearly with this many potential solvents to choose from the task of selecting a single solvent which will give optimal results for a given application is far more challenging than it would be with conventional organic solvents.

For example, the selection of an ionic liquid for use as a solvent in a whole cell biocatalytic reaction would need to examine several properties of the ionic liquids under consideration, including the solubility of the starting materials and end products in the solvent, the physical state, the ease of handling of the ionic liquid as well as the cost and ease of its synthesis. However, for systems in which an ionic liquid will be paired with a whole cell biocatalyst one of the most important considerations must be the toxicity of the ionic liquid towards the catalyst.

1.1.3. Ionic Liquids and Toxicity

An understanding of the toxicity of different ionic liquids towards living organisms is important both in terms of selecting appropriate ionic liquids for biological catalyst based reactions, as well as allowing us to understand the impact of the accidental release of these solvents into the environment.

As ionic liquids become more readily understood the number of applications in which they can be used increases. Currently ionic liquids have been employed in a number of different industrial processes and products including a process for the dissolution of cellulose, use as paint additives and as pharmaceutical intermediaries (Plechkova and Seddon, 2008). In addition, the possible use of ionic liquids has been investigated in a number of diverse areas. Some of these potential applications
include use of some phosphonium and ammonium based ionic liquids in the
treatment of cancer (Kumar and Malhotra, 2009), use as insect detergents (Hough-
Troutman et al., 2009), use as blood and tissue preservatives (Pernak et al., 2007),
phenol degradation (Baumann et al., 2005) and use as wood preservatives
(Zabielska-Matejk et al., 2010). However, increasing the application of ionic liquids
increases the possibility of accidental release of these solvents into the
environment. This possibility means that an understanding of the toxicity of
different classes of ionic liquids towards all taxa of life cannot be overestimated, as
it gives an understanding of how an accidental release of solvent will affect the
environment, as well as giving information to enable the synthesis of less toxic ionic
liquid structures.

The importance of understanding the toxicity and biodegradability of ionic liquids
has been well understood by the research community and the number of published
papers which have dealt with either the toxicity or the biodegradability of ionic
liquids has been huge making it impossible to discuss all of the reported findings
(Petkovic et al., 2011), (Zhu et al., 2009), (Zhao et al., 2007), (Frade and Afonso,
2010).

In general toxicity and biodegradation studies have focused on the most common
ionic liquids families, namely those containing imidazolium, pyridinium,
piperidinium and quaternary ammonium cations. The number of papers in which
toxicity of these cation families was considered huge and a complete review of all
toxicity papers would be beyond the scope of this literature review, therefore only
the main findings and results of the toxicity work which has been performed to
elucidate the relationship between ionic liquid structure and resulting toxicity will
be discussed.

By examining this small sub set of possible cation structures and testing them
against a range of organisms, - including Gram positive and Gram negative bacteria,
fungi, algae, zebra fish and cell lines, it became possible to elucidate a number of
toxicity to structure relationships. Most widely reported was the impact of
increasing the length of the alkyl chain associated with the cation having a linear
relationship with increasing toxicity of the ionic liquid, within a range of cation chain lengths from approximately 1 to 12 carbons in length (Pernak et al., 2003), (Docherty and Kulpa, 2005), (Wells and Coombe, 2006), (Ranke et al., 2007b), (Latala et al., 2005). The elucidation of the lipophillic nature of an ionic liquid correlating with its toxicity has led to an understanding of the mechanism of toxicity for ionic liquids. The lipophilic nature of the ionic liquid allows it to permeate the phospholipid bilayer of the cell disrupting the membrane structure. The more lipophillic the ionic liquid the further it is able to permeate into the membrane structure (Pernak et al., 2003), (Pernak et al., 2004), (Cornnell et al., 2008b).

The effect of the anion on the toxicity of the ionic liquid has also been investigated. Although the choice of anion appears to play a lesser role in the determination of the overall toxicity of an ionic liquid than does the choice of cation it has been reported that the choice of anion can impact the overall toxicity of the ionic liquid, with the anions [NTf₂] and [(CF₃SO₂)₂N] reported as significantly increasing the toxicity of the ionic liquid (Latala et al., 2009), (Stolte et al., 2006), (Salminen et al., 2007), (Kumar et al., 2009).

The effects of altering the structure of the cation alkyl chain have also been investigated with the introduction of ester linkages producing more biocompatible cations (Gathergood et al., 2006), (Morrissey et al., 2009).

The work reported so far on the relationship between structure and toxicity has been instrumental in elucidating ‘design rules’, allowing either for structures to be discarded or included for consideration for use in particular studies and to guide the synthesis of new ionic liquids. However the structures which have been so far investigated represent only a small fraction of the potential number of ionic liquids.
1.1.4. Assessment of High Throughput Screening Systems

Portions of this section (1.1.4) have previously been published in Green Chemistry, the full text of the article is shown in Appendix 2 - Published Literature Review.

Currently, the industry standard tests for the measurement of ecotoxicity include EC\textsubscript{50} and LC\textsubscript{50} measurements, using both unicellular and complex, multicellular organisms (OECD). An EC\textsubscript{50} value is the concentration of ionic liquid at which 50% of the test organisms are either immobilized or killed, an LC\textsubscript{50} value is the concentration of ionic liquid required to kill half of the test organisms following a specified exposure time (Studzinska and Buszewski, 2009), (Kulacki and Lamberti, 2008), (Bernot et al., 2005a), (Bernot et al., 2005b), (Cho et al., 2007), (Cho et al., 2008), (Docherty and Kulpa, 2005), (Garcia et al., 2004), (Garcia-Lorenzo et al., 2008), (Matzke et al., 2007), (Nockemann et al., 2007), (Pretti et al., 2006), (Pretti et al., 2009), (Li et al., 2009). Further detailed studies such as histological effects, mutagenicity and metabolic fate (Pretti et al., 2006), (Sipes et al., 2008) may also be required.

As different organisms will respond differently to each ionic liquid it is necessary to repeat these tests with a number of different organisms to gain a true understanding of the environmental impact of a given ionic liquid.

For this reason a detailed battery of tests for ionic liquid ecotoxicity testing has been developed (Jastorff et al., 2003). This test battery begins with an initial high throughput screen, if the results from the initial screen are promising then further testing is done using a hierarchical system of tests, with increasing biological complexity. The test battery assesses ionic liquid toxicity using multidimensional risk analysis by analysing five ecotoxicological risk factors – Release, Spatiotemporal Range, Bioaccumulation, Biological Activity and Uncertainty (Jastorff et al., 2003).

These tests provide accurate and reliable information which is essential for defining the toxicity of an ionic liquid which may be used in industry, in consumer products or is in some other way at risk of accidental environmental release. However, the tests described are time consuming, costly and require considerable training and
experience on the part of the operator. The prohibitive cost of these tests means that few universities or industrial labs would have the resources to test large numbers of structures using this test battery.

Therefore the importance of selecting an initial high throughput screening system is of crucial importance. The system must be reliable enough to identify the most toxic ionic liquids and yet rapid enough that large numbers of structures can be tested. In addition, it would be desirable that the test is simple enough to be performed by an operator who is not an expert in toxicological testing, such that it would be suitable for use by a research chemist, allowing initial toxicology data to be reported alongside chemical characterization data for a newly synthesised ionic liquid.

At present a range of screening methods are available to test the toxicity of ionic liquids. Due to financial considerations the vast majority of these methods utilise micro-organisms, rather than complex multicellular organisms, since microbiological media components are relatively cheap and the equipment required such as Bunsen burners and incubators are available in most laboratories.

Commonly used toxicity tests include measurements of minimum inhibitory concentrations (MICs) or minimum biocidal concentrations (MBCs). In order to measure MICs, the test organism is grown with the test chemical at a wide range of concentrations, either in broth or as agar cultures. The MIC is the lowest concentration of the test chemical which produces no visible growth. MBCs are measured by exposing the organism to the test chemical at different concentrations and then determining the lowest concentration at which there are no survivors, as measured by the absence of colony formation on agar plates. The tests can be combined and can be used as effective measures of ionic liquid toxicity (Pernak et al., 2004), (Ganske and Bornscheuer, 2006), (Cybulski et al., 2008), (Skrzypczak et al., 1997). However, considerable microbiological expertise is required to prepare the test cultures, the media and the dilution series of the test chemical under sterile conditions.
Further quantitative information about toxicity can be obtained by measuring inhibition of microbial growth rates in the presence of the test chemical. This allows rapid determination of EC$_{50}$ values. Growth rates can be measured either in a high throughput screening system such as a plate reader (Rebros et al., 2009), (Cornmell et al., 2008a) or in flask cultures (Ganske and Bornscheuer, 2006), (Dipeolu et al., 2009), (Hussain et al., 2007). The main drawbacks with these methods are that plate readers are expensive, whereas aseptic sampling is crucial to measure growth in flasks. Furthermore, flask cultures generally require significant quantities of ionic liquid for testing. Both methods require careful preparation of inocula and culture media and therefore require significant experience in microbiology.

Further information regarding ionic liquid toxicity can be gleaned through the measurement of cell viability. The traditional method is to measure viable counts (Hussain et al., 2007), (Matsumoto et al., 2004a), (Matsumoto et al., 2004b). Viable counts are measured by growing a culture, making a number of (serial) dilutions, and then spreading small samples over the surface of agar plates. As for growth rate measurement this is also a fairly complex procedure and requires microbiology experience.

Alternatively commercially available cell viability test kits (e.g., LIVE/DEAD BacLight bacterial viability kits) are also available. These kits measure microbial cell viability by measuring membrane integrity or cellular ATP content (Lloyd and Hayes, 1995), (Comas and VivesRego, 1997), (Petty et al., 1995). Although these kits use smaller volumes of ionic liquid, they still require growth of cultures and preparation of samples for analysis. In addition, the kits and reagents are often expensive and specialised equipment is required to read the assay results. Although these test kits provide faster experimental throughput than viable counts, there has been a literature report to suggest that changes in cell morphology caused by exposure to ionic liquids can alter the readings from a cell membrane integrity assay (Pfruender et al., 2004).

Recently the Agar Diffusion Test (or Kirby-Bauer test) has been adopted to measure ionic liquid toxicity (Rebros et al., 2009). This method is widely used in clinical
laboratories to determine antibiotic susceptibility of microorganisms (Gavin, 1957), (Davis and Stout, 1971), (Vedel et al., 1996). The method is simple, inexpensive, requires no specialised equipment and uses only a small volume of ionic liquid. In this method, the ionic liquid is added to a sterile filter paper disc. The filter paper is then transferred, aseptically, to a prepared lawn of microbial cells and incubated overnight. If a clear inhibition zone is formed around the filter paper, this indicates that the ionic liquid is inhibitory. The size of the inhibition zone can be measured to give an indication of the degree of toxicity. Conversely, confluent growth around the filter paper indicates that the ionic liquid is not toxic to the test organism.

The great advantage of this test is that the state of the inoculum is not critical, so it would be possible to use commercially available cultures of live bacteria (e.g. from molecular biology suppliers). Similarly, pre-sterilized, poured agar plates are also available, leaving only the filter paper discs in need of sterilization. The other apparatus can either be bought pre-sterilized or can be sterilized by a Bunsen burner. Thus, the test is simple, cheap, requires little preparation and only basic microbiology skills are required.

The agar diffusion method has previously been used to screen a range of ionic liquids against both Gram positive and Gram negative bacteria (Rebros et al., 2009), (Saadeh et al., 2009). Although the test can be used reliably to identify highly inhibitory ionic liquids, it is more difficult to rank the least inhibitory structures (Rebros et al., 2009). Therefore the method is intended only as preliminary screening tool to identify ionic liquids suitable for further in depth ecotoxicological testing. There are a number of potential problems with the microbial toxicity tests and the agar diffusion method in particular. These problems must be carefully considered when reviewing the data generated from these tests.

Of most concern when assessing the results of any test for ionic liquid toxicity is the lack of understanding of the way in which the ionic liquid interacts with the culture media and any other test reagents. Ionic liquids are salts and may, therefore react with the ionic components of the culture medium (Dipeolu et al., 2009), resulting in pH changes, formation of precipitates etc. Such interactions would affect
measurements of microbial growth rates, and in extreme cases may affect the integrity of the tests. Similarly, these interactions may affect the agar diffusion test, since it relies on diffusion of water miscible ionic liquids through the medium to create a concentration gradient. Interactions between medium components and the ionic liquid may influence the diffusion rate or may affect the stability of the resulting concentration gradient. The formation of a stable concentration gradient also assumes a uniform diffusion through the agar for all ionic liquids, regardless of size or other properties, this assumption may not be correct for all ionic liquids (Petkovic et al., 2011).

A further problem with screening ionic liquids in microbial systems is that the ionic liquids themselves may be chemically reactive or may act catalytically to accelerate changes in the composition of the culture medium or materials used to prepare the assays. For example, the interaction between certain ionic liquids and cellulose has been widely reported (Barthel and Heinze, 2005), (Swatloski et al., 2002) and this may have an influence on the outcome of the agar diffusion test, in which cellulose filter papers are used.

Agar itself is a carbohydrate, so it is possible that some ionic liquids may interact with the agar plates used in MIC/MBC, viable count and agar diffusion tests. In addition the phase behaviour of ionic liquids may interfere with the analysis of results when using spectrophotometric methods to measure cell growth or viability (e.g. in biphasic systems, or when emulsions, gels or pastes are formed).

Therefore, the need to inspect cultures and test assays visually for any signs of colour change, precipitate formation or changes to physical state is of great importance in helping to assess the reliability of the collected results. Also greatly desirable is a measurement of the culture pH, as this would give an indication of any possible interactions between the ionic liquid and the culture media components.

Due to these possible limitations no single test system should be used in isolation to assess the toxicity of an ionic liquid. To get the most accurate picture of an ionic liquid’s toxicity it is preferable to consider the data collected from a range of
different ecotoxicological tests. However the combination of data from a disparate range of test organisms, using different testing methods and presenting data in varying formats is challenging.

At the BATIL2 meeting (Dechema, Frankfurt 2009) it was suggested by Tom Welton that a scoring system for toxicity should be adopted. In this system the data from each test is given a score (e.g. 1-3 with 1 being non-toxic and 3 being highly toxic) so scores from different tests can in this way be combined to give an overall toxicity score for each ionic liquid. A further initiative to come from the BATIL2 meeting is the development of a database which will incorporate toxicity test information for different ionic liquids screened against various test organisms (UFT/Merck).

As well as providing information regarding the specific toxicity of a tested ionic liquid the data generated from toxicity screens can also be used to develop computational models (quantitative structure – activity relationships QSAR) which will allow ionic liquid toxicity to be predicted so that the number of ionic liquids which need to be synthesised and tested can be reduced. QSAR works on the principle that the structure of a chemical can be directly linked to a particular property by the development of a predictive model. Models are developed by using ‘training data’ this is data relating to the particular property under investigation generated from a number of different chemical structures.

As for the toxicity testing assays themselves there is no definitive method for the production of an ionic liquid QSAR program and a number of different mathematical models have been proposed and reported in the literature. These models include the separate consideration of the toxicity profiles of the cation, the anion and the side chains (Irabien et al., 2009), (Luis et al., 2007), multilayer perceptron (Torrecilla et al., 2009) and the use of the electronic structure of a molecule assessed by an artificial neural network (Torrecilla et al., 2010).
The Future for Ionic Liquids

The potential usefulness of ionic liquids in developing more environmentally benign chemical processes can not be overstated. However, in the rush to replace conventional solvents with ionic liquids it is important to ensure that one set of problems is not simply being replaced by another.

That ionic liquids offer a safer alternative than conventional solvents in terms of their low volatility and negligible vapour pressure, thereby reducing environmental losses and exposure of workers, is well reported. However, the toxicity and biodegradation profiles for some ionic liquids are extremely poor.

As further work is performed with ionic liquids and new applications uncovered the possibility of either deliberate or accidental release of ionic liquid into the environment also increases. An understanding of the toxicity, bioaccumulation and persistence of different ionic liquids to a range of organisms, in different environmental settings is therefore desirable.

Due to the vast numbers of potential ionic liquids in-depth toxicity testing of every possible cation and anion combination is simply not feasible. Therefore it is desirable to develop high throughput screening assays which can identify highly toxic ionic liquids and exclude them from further work, or which can identify biocompatible ionic liquids, which may be interesting targets for in-depth ecotoxicological testing.

A number of screening methods are currently in use, each method has both positive and negative aspects as well as possible problems. It is therefore desirable to combine information from a number of different screening assays using different organisms to give a clearer indication of the toxicity profile of a particular ionic liquid.

To this end efforts are already underway to combine toxicity testing results in a clear and comprehensive way which is freely accessible. In this way not only can the toxicity profile of currently synthesised ionic liquids be better understood but
toxicity information from those ionic liquids can be used to inform the design of new solvents.

1.2. Response of Gram negative microorganisms to salt and solvent stress

1.2.1. Introduction

When assessing the toxicity of a new ionic liquid or solvent there are two components to consider, firstly the toxicity of the test solvent, as discussed above certain cation and anion structures are reported to be more damaging to living organisms than others; secondly, the response of the test organism towards the solvent must also be assessed.

A solvent which is toxic for one microorganism at very low concentrations may be tolerated at much higher concentrations by a different microorganism. The first part of the microorganism to come into contact with a solvent will be the cell wall, as such several response and adaption mechanisms of the cell wall have been investigated and reported in the literature and will be briefly discussed here.

1.2.2. Structure of the Gram negative cell wall

The structure of the bacterial cell wall is what determines whether a microorganism is classed as Gram positive or Gram negative. This determination is made by use of the Gram stain, in which Gram positive microorganisms are stained purple, whilst Gram negative microorganisms are stained red to pink (Madigan, 2006).

The cell wall structure of Gram positive microorganisms is simpler than that of Gram negative microorganisms. The cell wall of a Gram positive bacterium is composed of an inner cytoplasmic membrane, formed from a phospholipid bilayer and a thick layer of the cross-linked polysaccharide, peptidoglycan (Madigan, 2006).

A Gram negative bacterium, has in addition to the inner cytoplasmic membrane a much thinner peptidoglycan layer as compared to a Gram positive bacterium as well as possessing an outer membrane. The outer membrane, like the inner, is a
phospholipid bilayer. However unlike the inner membrane the outer membrane includes lipopolysaccharide molecules (Madigan, 2006).

A schematic diagram of a Gram negative cell wall is shown in Fig 1.1, the figure was re-drawn from information in Madigan and Martinko (Madigan, 2006) and Singleton (Singleton, 2004).

![Fig 1.1 – Schematic diagram of a Gram negative cell wall figure re-drawn from information in Madigan and Martinko (Madigan, 2006) and Singleton (Singleton, 2004)](image)

The inner membrane of the cell wall is composed of phospholipid and protein (Singleton, 2004). The phospholipid membrane has two main functions:- to isolate the cell components from the outside environment and to allow the selective transport of molecules into and out of the cell. The phospholipid bilayer is formed as a result of the structure of the phospholipid molecule. Each phospholipid molecule has a polar, hydrophilic head group attached to which are two non-polar hydrophobic fatty acid chains. Therefore the molecules align themselves so that the hydrophobic chains point inwards towards each other whilst the hydrophilic head groups are exposed on each edge of the bilayer (Yurkanis Brucie, 2007).
integrity of the phospholipid membrane is crucial to the survival of the cell, and damage to the inner membrane can result in cell death (Madigan, 2006).

The second component of the inner membrane is protein. The role of the membrane proteins within the phospholipid bilayer is the transport of molecules into and out of the cytoplasm. Membrane transport proteins are associated with one of three transport systems; simple transport, group translocation and the ABC system.

Simple transport involves the movement of a particular type of molecule through the phospholipid membrane, transport can occur in either direction, dependent on the type of transport protein, the process requires energy and the transported molecule is not chemically altered during the transport process (Madigan, 2006).

In group translocation the transported molecule is phosphorylated during transport into the cell. This transport system has been studied in both Escherichia coli and Salmonella typhimurium. In E. coli the carbohydrates glucose, fructose, mannose, hexitols and β-glucosides were phosphorylated during transport into the cytoplasm (Bramley and Kornberg, 1987).

The ABC system (ATP–binding cassette), requires energy from ATP and consists of a chain of three proteins, an ATP-hydrolyzing protein inside the cytoplasm, a membrane spanning transport protein and a periplasmic binding protein located within the periplasmic space. This transport system is used for both organic and inorganic molecules, the molecule to be transported is passed along the chain of the three proteins into the cytoplasm (Madigan, 2006).

Between the inner and outer membrane of the cell wall is the periplasmic space containing layers of peptidoglycan. Peptidoglycan is formed in sheets from the sugars N-acetylglucosamine and N-acetylmuramic acid as well as the amino acids L-alanine, D-alanine, D-glutamic acid and either lysine or diaminopimelic acid. The sheets of peptidoglycan are cross-linked by the bonds between the amino acids in neighbouring sheets. The function of the peptidoglycan layer is to provide mechanical strength to the cell (Madigan, 2006).
The outer membrane of the cell wall is again composed of a phospholipid bilayer, into which are embedded porins and lipopolysaccharides. Lipopolysaccharides contain an endotoxin component which can cause illness in mammals. LPS is essential for bacterial cell survival and as such has been the target of many antibiotics (Wang and Quinn, 2010).

Porins are transmembrane proteins involved in the transport of small hydrophilic molecules from outside the cell wall into the periplasmic space. Porins can be either specific or non-specific and unlike other membrane proteins allow passive diffusion rather than active transport of molecules (Madigan, 2006).

1.2.3. Effect of organic solvent exposure

As has been previously mentioned the exposure of cells to organic solvent can cause cell death through the disruption of the cell membrane. The damage that will be caused by an organic solvent can be estimated from the logP value of the solvent. The logP value is a measure of the lipophilicity of a solvent and is determined by the partitioning of the solvent between octanol and water. Organic solvents with a logP between 1.5 – 3 have been reported to be highly damaging to cell membranes (Segura et al., 2003), whilst solvents with a logP value greater than 5 have been reported to be biocompatible (Heipieper et al., 1994). Organic solvents with logP values within the toxic range of 1.5 – 3 have been shown to accumulate to high levels within the phospholipid membrane, thus disrupting the function and structure of the membrane (Weber et al., 1993), (Ramos et al., 2002). Microorganisms have developed a number of mechanisms to cope with the presence of toxic organic solvents, those that relate to E. coli and P. putida will be discussed.

1.2.3.1. Efflux Pumps

Efflux pumps can be classified as belonging to one of five families: the resistance cell nodulation family (RND), the small multidrug resistance family (SMR), the major facilitator superfamily (MFS), the multidrug and toxic compound extrusion family (MATE) and the ATP binding cassette family (ABC) (Fernandes et al., 2003).
For *E. coli* it has been reported that the efflux pump family most involved in solvent extrusion is the RND family (Ramos *et al.*, 2002). The AcrAB-TolC efflux pump reported to be responsible for the extrusion of toluene from *E. coli* is located in the inner membrane of the cell. The AcrB-TolC pump contains three proteins which together span from the inner membrane through to the outside of the cell, to form a continuous tunnel or pore through which toxic solvents can be removed from the cell. Expression of the genes which regulate these proteins is reportedly increased in a number of solvent tolerant mutants of *E. coli* (Asako *et al.*, 1997).

As with *E. coli* the main efflux pump associated with the extrusion of toxic organic solvents from *P. putida* KT2440 belongs to the RND family, the efflux pump protein TtgABC. Recent proteomic analysis has reported that in the presence of phenol the three proteins of the Ttg ABC efflux pump are expressed at higher levels whilst conversely a decrease in the proteins associated with porins in the outer membrane of the cell wall decreases (Roma-Rodrigues *et al.*, 2010).

For the microorganism *P. putida* DOT-T1E, three efflux pumps have been identified as being associated with solvent extrusion TtgABC, TtgDEF and TtgGHI (Ramos *et al.*, 2002). The efflux pump TtgABC is similar to that which has been described above for *P. putida* KT2440. The efflux pump TtgDEF is inducible by both toluene and styrene and is linked to the tod pathway for toluene degradation (Mosqueda and Ramos, 2000). The third efflux pump TtgGHI is encoded on a large self transmissible plasmid and has been shown to be involved in the extrusion of both toluene and other toxic organic solvents from the microorganism (Rodriguez-Herva *et al.*, 2007).

The combination of the three efflux pumps TtgABC, TtgDEF and TtgGHI gives *P. putida* DOT-T1E the greatest advantage of the three organisms considered when it comes to the extrusion of toxic organic solvents from the cell (Rojas *et al.*, 2001).

1.2.3.2. Changes to the membrane structure

In addition to the extrusion of organic solvents through efflux pumps, a number of other adaptation mechanisms relating to the alteration of the structure of the
phospholipid membrane have also been reported. These adaption mechanisms have primarily been reported for the *P. putida* DOT-T1E strain.

The first mechanism to be employed by the *P. putida* DOT-T1E cell on exposure to a toxic organic solvent is the *cis* to *trans* isomerisation of the fatty acids within the phospholipid bilayer. The *cis* fatty acid isomer is the only one which is naturally produced by cells. The amount of *trans* fatty acid present within the cell membrane increases proportionally with the amount of organic solvent which is accumulated within the cell membrane (Heipieper *et al.*, 1992). The *cis-trans* isomerisation reaction is not energy dependent and does not require the synthesis of new lipid molecules, this was proven by work in which the synthesis of new lipid was blocked by the addition of the lipid synthesis inhibitor ceralenin, then measuring the ratio of *cis-trans* fatty acid. The ratio of *trans* to *cis* fatty acid was shown to have increased following solvent exposure, thereby showing that existing lipid molecules must have been converted to the *trans* configuration (Diefenbach *et al.*, 1992).

The structure of the *cis* fatty acid chain contains a 30° ‘kink’ caused by the geometry of the double bond. This kink limits the number of phospholipids which can be packed together. By rotating the position of the double bond into the *trans* configuration, the kink is removed and denser packing of the phospholipid molecules is possible. The denser packing of the phospholipid molecules helps to counter the solubilising effect of the solvent on the phospholipid membrane (Heipieper, 2003).

The *cis-trans* isomerisation reaction is carried out by the *cis-trans* isomerise enzyme (Cti), located in the periplasm of the cell. The enzyme was identified by the creation of a mutant in which the cti enzyme was knocked out. The resulting mutant strain had a lower tolerance to a sudden toluene shock than the wild type strain (Junker and Ramos, 1999). The *cis-trans* adaption mechanism has primarily been reported in *P. putida* strains and has not been reported as occurring with *E. coli* MG1655, however *E. coli* mutants which were deficient in the enzyme required to produce *cis* fatty acid were shown to be more tolerant to ethanol exposure when supplemented with *trans* fatty acid than when supplemented with *cis* fatty acid.
The cis-trans isomerisation mechanism does not appear to have evolved solely for the purpose of solvent tolerance, cultures exposed to high temperatures have also shown an increase in the proportion of cis to trans fatty acid (Junker and Ramos, 1999), (Holtwick et al., 1999).

The main advantage of the cis-trans isomerisation reaction is that it allows the cell to respond immediately to the presence of a toxic solvent, without the need to synthesis new lipid molecules (Segura et al., 1999). That the process is energy independent is also a significant advantage. The accumulation of a toxic solvent in the cell membrane will disrupt the structure and function of that membrane, as one of the most important functions of the membrane is the generation of ATP through the proton motive force (pmf), it is beneficial to have an adaption mechanism which is not dependent upon energy produced from this source (Madigan, 2006).

The cis-trans isomerisation reaction is an immediate response to the presence of a toxic solvent, however there are other reported solvent adaption mechanisms which occur over a longer period of time. Following exposure to an organic solvent it has been reported that the proportion of saturated fatty acids present in the membrane increases. This increase in saturated fatty acid increases the transition temperature of the membrane and thereby reduces the overall fluidity of the membrane, which has been increased due to its exposure to organic solvent. The increase of the proportion of fatty acid in the membrane has been noted in several species including both E. coli and P. putida strains (Segura et al., 1999). Unlike the cis-trans mechanism which modifies existing cellular components, increasing the proportion of saturated fatty acid within the membrane requires the de novo synthesis of new lipid molecules (Segura et al., 1999).

In several studies it has been noted that the addition of the divalent cations Mg$^{2+}$ and/or Ca$^{2+}$ improved the survival of microorganisms when they were cultured in the presence of organic solvents (Ramos et al., 1995), (Weber and deBont, 1996), (Desmet et al., 1978). Based on the observations made in these studies it has been suggested that these divalent cation molecules reduce the charge repulsion between the neighbouring LPS molecules allowing the membrane to be packed.
more densely and thereby compensating for the increased membrane fluidity caused by the accumulation of organic solvent within the membrane (Segura et al., 1999), (Ramos et al., 2002).

The final mechanisms to have been reported regarding alteration to the structure of the membrane are the change in composition of the phospholipid head groups and in the protein to phospholipid ratio in the cell membrane. There are three main classes of phospholipid head groups, phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and diphosphatidylglycerol or cardiolipin (CL). In a study performed using the test organism P. putida S12 in which cells were not exposed to organic solvent the largest proportion of phospholipids in the membrane were made up of PE. However, following exposure to toluene it was noted that the amount of PE in the membrane decreased, whilst an increase was seen in the amount of both PG and CL. Increasing the CL content of the membrane is believed to help stabilize the cell by reducing the fluidity of the membrane. In addition the head group area of both CL and PG is larger than that of PE. By replacing the smaller PE head groups with larger PG and CL head groups it has been suggested that this compensates for the increase in lipid volume caused by the accumulation of the solvent in the bilayer (Weber and deBont, 1996).

A study of E. coli cultures which had been adapted to ethanol showed that the membranes of these cells were more rigid than the membranes of cells which had not been adapted to ethanol exposure. Further analysis revealed that the adapted cells had a higher protein to lipid ratio in the cell membrane than did the cells which had not been adapted to ethanol (Dombek and Ingram, 1984).

1.2.3.3. Metabolism of solvents

Some Pseudomonas species such as P. putida F1 have the ability to metabolise various organic solvents. However other strains which are not known to metabolise organic solvents also show similar levels of tolerance. A mutant strain of the P. putida DOT-T1E microorganism, which was unable to metabolise toluene was created; despite its inability to metabolise toluene, this mutant strain showed a tolerance to the solvent which was comparable to that of the wild type organism.
(Mosqueda et al., 1999). This suggests that although the ability to metabolise solvent may be of slight benefit it is not a major factor in the tolerance of a microorganism to organic solvent.

1.2.4. Conclusion

In order to understand the toxicity profile of a given solvent fully it is necessary to understand both the inherent toxicity of the solvent; i.e., toxicity which is the result of either the structure of the solvent as for ionic liquids or the properties of the solvent such as the lipophilic nature of toluene, as well as to understand the intrinsic resistance of the test organism toward the solvent.

In this section a number of adaption mechanisms displayed by Gram negative organisms following exposure to organic solvents have been discussed. By understanding these mechanisms and combining them with knowledge of the toxic nature of a test solvent it is possible to get a clearer picture of both the response of an organism when exposed to an organic solvent as well as predicting the impact of exposure to new, un-tested solvents.

1.3. FT-IR Spectroscopy Applied to Monitoring of Phenotypic Changes in Cells

1.3.1. Introduction to FT-IR Spectroscopy

The exposure of bacterial cultures to either organic solvent or to ionic liquids may produce a number of alterations to the phenotypic profile of the cell. In order to assess whether a phenotypic change has occurred within a cell it possible to use Fourier transform infrared (FT-IR) spectroscopy to monitor the biochemical ‘fingerprint’ of the culture. In this case the fingerprint refers to the biomass sample, whilst the ‘footprint’ of the culture, i.e. the spent culture medium from which the biomass samples have been removed, can also be measured.

FT-IR spectroscopy is used to measure the vibrational modes of the functional groups of molecules within samples when they interact with infra red (IR) radiation (Winder et al., 2004). The generation of vibrational spectra is often likened to
having two masses at either end of a spring, in which the spring would represent the chemical bond whilst the masses would represent the atoms at either end of the bond. When the spring is pulled the masses will vibrate with a frequency which is characteristic of both the masses and that of the strength of the spring. This example can be related back to a molecule such that when the molecule is excited by interaction with IR radiation the molecule will vibrate at a frequency that is characteristic of both the atoms and the type of bond (Rubinson and Rubinson, 2000).

The metaphor of the spring and masses is applicable only to molecules which consist of two atoms. For most molecules which contain larger numbers of atoms the resulting vibrational spectra are more complex. For the simple system with two atoms described above there is only one way in which the bond can vibrate, i.e. pulling the spring or stretching the bond. Therefore this system has only one vibrational mode (Atkins, 2002).

For molecules which contain more than two atoms the number of vibrational modes is dependent upon whether or not the molecule is linear. For linear molecules the number of vibrational modes is determined by the equation $3N-5$, in which $N$ is the number of atoms in the molecule. For non-linear molecules the number of vibrational modes is determined by the equation $3N-6$.

Therefore when considering a simple molecule such as water, there would be three modes of vibration. The three modes of vibration for a water molecule can be seen in Fig 1.2 (redrawn from information in (Atkins, 2002), (Rubinson and Rubinson, 2000). However not all vibrational modes will be infra red active, i.e. these modes will not be detectable in an IR spectrum. In order to be IR active a vibrational mode must cause a change in the dipole moment of the molecule. In the example of water, the negative charge is pulled towards the oxygen atom, leaving the hydrogen atoms with slight positive charge; therefore water has a permanent dipole. The stretching and bending of the water molecule following absorption of IR radiation alters the dipole of the molecule thus making the resulting vibrations IR active, i.e. visible in an IR spectrum. Although the example of water has been used
it is not necessary for a molecule to have a permanent dipole moment in order to be IR active (Atkins, 2002).

All molecules (with the exception of enantiomers) will contain different atoms connected by different bonds, the vibrational spectra for each molecule will be unique. An IR spectrum can be said to compose of roughly three regions: - (1) a functional group region (4000-1300 cm⁻¹) which reports vibrations from particular functional groups such as amines, carbonyls and hydroxyls, the presence or absence
of peaks within the region can be used to discern structural information regarding the molecule under investigation, (2) a fingerprint region (1300-910 cm⁻¹), this region of the spectrum is usually highly complex and it is often difficult to conclusively identify bands belonging to specific vibrations within this region, (3) an aromatic region (910-650 cm⁻¹) area of the spectrum in which bands resulting from vibrations of aromatic rings often seen (Rubinson and Rubinson, 2000). Thus, for simple molecules it is possible to use the IR spectrum to elicit information regarding the composition and structure of the molecule.

However for more complex samples such as large molecules or samples of cultured cells it is often not possible to deconstruct the spectra to determine which peaks are caused by which vibrational mode. In this instance the fingerprint region of the spectrum can be of great assistance in assigning identity to unknown samples. By searching the generated spectrum against databases of spectra collected from known samples it may be possible to at least partially identify unknown samples.

For cell culture samples comparison of spectra collected under different culture conditions or across different exposure times can be compared against each other in order to give an indication of what if any phenotypic changes are occurring within the culture. Due to the complex nature of the collected spectra it is necessary to use some form of chemometric analysis in order to visualise changes within the collected spectra. One such example is the use of clustering analysis. Clustering analysis can be either unsupervised e.g., Principal Components Analysis (PCA) in which the combination of variables which represent the largest degree of variance within the data set are selected and can be plotted against each other, the arrangement of the variables within the plot space can then be used to infer differences or similarities between different groups. Alternatively, supervised clustering analysis e.g., discriminant function analysis (DFA), can be used. In this instance the model is given a priori information e.g., identity of biological replicates, this information is used to minimise variation within groups whilst maximising the variation between groups (Manly, 2005).
1.3.2. FT-IR Instrument

An FT-IR instrument can be broken down into four main components: the source, the interferometer, the sample compartment and the detector. A schematic overview of an FT-IR instrument can be seen in Fig 1.3. Figure re-drawn using information from Atkins and de Paula (Atkins, 2002) and www.thermo Nicolet.com.

Fig 1.3– Schematic overview of FT-IR instrument, figure re-drawn using information from Atkins and de Paula and www.thermo Nicolet.com

The source for an FT-IR instrument is typically a black body radiation source. For spectrometers operating in the mid infra red region of the spectrum (200-4000 cm\(^{-1}\)) the commonly used sources are either a Nerst filament or a globar made of SiC, both of which require heating to high temperatures (Atkins, 2002).

The interferometer contains a beam splitter, when radiation from the source passes into the interferometer it is split into two beams of radiation, by doing so the path lengths of the two beams is varied. Therefore, when the two paths are recombined
in the detector there is a phase difference between the two paths. The advantage of splitting the source beam into two separate paths is that the sensitivity of the instrument is increased as all IR frequencies are interrogated at the same time, in comparison with instruments in which a monochromator is used in place of a beam splitter (Atkins, 2002).

In the sample compartment, the sample is introduced into the instrument and into the IR beam. The sample compartment is generally fitted with a motorised stage to allow sample plates to be moved by the instrument. The presence of the movable mirror and a sample compensator ensure that the two paths from the beams split in the interferometer pass through the same thickness of material, i.e. pass through the sample at the same point (Atkins, 2002).

The detector covertsthe radiation into a signal which can be converted into a spectrum by the instrument’s computer. There are several types of detector available however the two most commonly used are the mercury cadmium telluride (MCT) detector and the deuterated triglycine sulphate (DTGS) detector. Whilst the MCT detector has better sensitivity than the DTGS detector, the MCT requires immersion in liquid nitrogen during operation whilst the DTGS can be operated at room temperature (Hsu, 1997).

Once the signal from the two different path lengths has left the detector it must be broken down in a way that will give the original vibrational frequencies of the sample, this process is described as Fourier transformation. The application of Fourier transformation to IR data allows for the use of a polychromatic source, increasing the sensitivity of the instrument as it allows all of the different wavelengths from the source to be recorded simultaneously (Atkins, 2002).

1.3.3. Applications of FT-IR Spectroscopy in Analysis of Phenotypic Changes

As has been mentioned above it is possible to use FT-IR spectroscopy to investigate phenotypic changes within a bacterial culture by comparison of the FT-IR spectra collected either from bacteria cultured under different conditions or from bacteria analysed at different time points over an exposure experiment.
Several examples of these uses of FT-IR spectroscopy have been reported in the literature. The identification by FT-IR spectroscopy of 59 bacteria involved in urinary tract infections (UTIs) in a clinical setting has been reported (Goodacre et al., 1998). In this instance it was possible to distinguish between 5 groups of microorganisms which are the most common causative agents of urinary tract infection (Escherichia coli, Pseudomonas aeruginosa, Klebsiella species, Proteus mirabilis and Enterococcus species).

As has been previously mentioned direct visual comparison of spectra collected from complex samples such as bacterial cultures is not possible, due to the large number of overlapping spectral features present in such a spectrum. Therefore it is necessary to employ some type of multivariate data analysis to analyse the resulting spectra. In this example FT-IR spectra from isolates known to belong to the five bacterial groups of interest were used to train an artificial neural network, after which this was used to identify correctly unknown UTI bacterial samples (Goodacre et al., 1998).

The use of whole cell fingerprinting has also been reported as being used to highlight phenotypic differences between cells growing as part of a biofilm and those growing in a liquid or on solid culture medium. The microorganism Bordetella pertussis, the causative agent of whooping cough, was analysed using FT-IR spectroscopy. Differences between the spectra collected from microorganisms growing in liquid or solid culture and between microorganisms growing as a part of a biofilm were identified by the assignment of spectral peaks (Bosch et al., 2000).

The use of FT-IR spectroscopy as a whole organism fingerprinting approach was again reported in the discrimination of poorly characterized Acinetobacter down to the sub-species level. Although in this instance there was not complete correlation between the collected FT-IR spectra and the identifications made by 16-23S rDNA intergenic spacer region (ISR) analysis there was a good correlation in the dendogram produced from the FT-IR spectra with results which had been previously published (Winder et al., 2004).
The accumulation of ionic liquids in the membranes of *E. coli* MG1655 cultures has also been observed through the collection of FT-IR spectra and through the use of principal components analysis (PCA) and discriminant function analysis (DFA) it has been possible to show that there are significant changes in the phenotype of the culture following exposure to ionic liquid (Cornnell *et al.*, 2008b).

FT-IR spectroscopy in combination with multivariate statistics has also been used to monitor the ability of microbial communities to degrade the toxic solvent phenol (Wharfe *et al.*, 2011), as well as to assess the phenotypic response of whole cell biocatalysts when exposed to the toxic solvent toluene as part of a biotransformation reaction (Winder *et al.*, 2011).

### 1.3.4. Conclusion

FT-IR spectroscopy is a rapid, non destructive means of assessing the phenotypic changes occurring within a microorganism culture. This combined technique of whole organism fingerprinting in conjunction with multivariate data analysis methods can be employed to monitor the phenotypic changes occurring within a microorganism culture under a range of growth conditions. This reported work suggests that FT-IR spectroscopy is a suitable technique for further use in the investigation of phenotypic changes to microorganisms resulting from exposure to either organic solvent or ionic liquid.

### 1.4. Research Aims

The aims of the research will be three fold

- Investigate the use of FT-IR spectroscopy to detect phenotypic alterations in bacterial cultures exposed to conventional solvents
- Investigate the use of rapid screening methods to identify potentially biocompatible ionic liquid structures as well as highly toxic structures
- Investigate the use of FT-IR spectroscopy to detect phenotypic alterations in bacterial cultures exposed to ionic liquids in flask cultures.
2. Methods and Materials

2.1. Culture Maintenance

2.1.1. Lysogeny Broth (LB) and Agar Preparation

A 400mL volume of LB broth was prepared by dissolving 4g tryptone (Fluka, UK), 4g NaCl (Fisher, UK) and 2g of yeast extract (Fisher, UK) in approximately 300mL of distilled water. The pH was adjusted to pH 7 using 1M KOH (Fisher, UK). The resulting solution was then made up to 400mL using distilled water. For preparation of agar plates 6g of Agar No 2 (LabM, UK) was added. The mixture was autoclaved for 20 min at 121°C. After autoclaving the mixture was allowed to cool before pouring into sterile Petri dishes. Agar plates were allowed to set before use.

All cultures were routinely maintained on LB agar plates (recipe above), antibiotics were added to the LB plates in the concentrations listed in Table 2.1

<table>
<thead>
<tr>
<th>Organism</th>
<th>Rifampicin 20µg/mL</th>
<th>Kanamycin 50µg/mL</th>
<th>Streptomycin 300µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> MG1655</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> KT2440</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> DOT-T1E</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> DOT-T1E 18</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> DOT-T1E PS28</td>
<td>✓</td>
<td>X</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 2.1 – Antibiotic concentrations used for culture maintenance
2.1.2. MSX Broth Preparation

Prepare four solutions A, B, C and D

*Solution A*

Dissolve 6g KH$_2$PO$_4$ (Sigma, UK) and 2mL of Vishniac solution (recipe below) in approximately 800mL of distilled H$_2$O. Adjust the pH to 7.2 using 1M KOH. Make up to a final volume of 970mL using distilled water. Autoclave solution at 121°C for 20 min.

*Solution B*

Dissolve 2g MgSO$_4$.7 H$_2$O (Fisher, UK) and 15g NH$_4$Cl (Sigma, UK) in 100mL of distilled water. Autoclave solution at 121°C for 20 min.

*Solution C*

Dissolve 20g glucose (BDH, UK) in 100mL of distilled H$_2$O. Autoclave solution at 121°C for 20 min.

*Solution D*

Prepare thiamine (Sigma, UK) stock solution of 10mg/mL.

Combine solutions in following volumes:

<table>
<thead>
<tr>
<th></th>
<th>For 1000mL of MSX medium</th>
<th>For 25mL of MSX medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A</strong></td>
<td>970mL</td>
<td>24.25mL</td>
</tr>
<tr>
<td><strong>Solution B</strong></td>
<td>20mL</td>
<td>0.5mL</td>
</tr>
<tr>
<td><strong>Solution C</strong></td>
<td>10mL</td>
<td>0.25mL</td>
</tr>
<tr>
<td><strong>Solution D</strong></td>
<td>0.5mL</td>
<td>12.5µL</td>
</tr>
</tbody>
</table>
2.1.2.1. Vishnic Trace Element Solution

Each of the following components was added to approximately 80mL of distilled H$_2$O in the given order, ensuring that each component was fully dissolved before adding the next.

EDTA (Sigma, UK) – 5g
Solid KOH added until EDTA completely dissolved
ZnSO$_4$.7 H$_2$O (Fisher, UK) – 0.34g
CaCl$_2$ (Fisher, UK) – 0.554g
MnCl$_2$.4 H$_2$O (Sigma, UK) – 0.506g
FeSO$_4$.7H$_2$O (Sigma, UK) – 0.5g
(NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O (Sigma, UK) – 0.11g
CuSO$_4$.5H$_2$O (Sigma, UK) – 0.157g
CoCl$_2$.6H$_2$O (Sigma, UK) – 0.161g
The pH was adjusted to pH 6.0 using 10M KOH, the solution was made up to a final volume of 100mL using distilled H$_2$O. Store at 4$^\circ$C.

2.1.3. Growth of *E. coli* MG1655, *P. putida* KT2440 and *P. putida* DOT-T1E in liquid Medium

An Erlenmeyer flask was filled with an appropriate amount of un-autoclaved medium (25mL of medium in a 100mL flask and 50mL of medium in a 250mL flask). The flask neck was plugged with cotton wool and covered with aluminium foil. Flasks autoclaved at 121$^\circ$C for 20min.

To inoculate from an agar plate, remove a well isolated single colony from the plate using a flame sterilised inoculation loop. Transfer the colony to a pre-autoclaved flask. Replace flask stopper and incubate in an orbital incubator at 200rpm and 37$^\circ$C for *E. coli* MG1655 or 30$^\circ$C for *P. putida* KT2440 or DOT-T1E.

To inoculate from a liquid culture, remove an aliquot of inoculum using a sterile pipette. Transfer the inoculum to a pre-autoclaved flask. Replace flask stopper and incubate in an orbital incubator at 200rpm and 37$^\circ$C for *E. coli* MG1655 or 30$^\circ$C for *P. putida* KT2440 or DOT-T1E.
2.2. Proteomics Methods

2.2.1. Protein Extraction Method

An overnight culture was grown in a 250mL flask containing 50mL of MSX medium (Section 2.1.3). The culture was harvested by centrifugation at 3080g for 10min at 4°C. The supernatant was removed and discarded. The remaining pellet was re-suspended in 1mL of 40mM tris-base (pH 9.5). The tube containing the re-suspended pellet was sonicated at full power in an ice water bath for 10min. After sonication the re-suspended pellet was centrifuged at 11 500g for 5min at 4°C. Following centrifugation the supernatant was removed and retained in a clean micro-centrifuge tube labelled ‘Supernatant 1’.

The remaining pellet was washed twice using 1mL 40mM tris-base (pH 9.5). The washings were removed and discarded. The pellet was then re-suspended in 1mL of Solution A (Table 2.2). The re-suspended pellet was sonicated at full power in an ice water bath for 10min. After sonication the re-suspended pellet was centrifuged at 11 500g for 5min at 4°C. Following centrifugation the supernatant was removed and retained in a clean micro-centrifuge tube labelled ‘Supernatant 2’.

The remaining pellet was washed twice using 1mL 40mM tris-base (pH 9.5). The washings were removed and discarded. The pellet was then re-suspended in 1mL of Solution B (Table 2.2). The re-suspended pellet was sonicated at full power in an ice water bath for 10min. After sonication the re-suspended pellet was centrifuged at 11 500g for 5min at 4°C. Following centrifugation the supernatant was removed and retained in a clean micro-centrifuge tube labelled ‘Supernatant 3’.

The remaining pellet was re-suspended in 100µL of Solution C (Table 2.2). The re-suspended pellet was placed in a pre-heated 95°C water bath for 5min. After heating the sample was centrifuged at 11 500g for 5min at 4°C. The supernatant was removed and combined with ‘Supernatant 3’.
A 100µL aliquot from Supernatant 1 was removed to a clean micro-centrifuge tube and mixed by vortexing, with a 100 µL aliquot of Solution A. From Supernatant 2 a 300µL aliquot was removed to a clean micro-centrifuge tube and mixed by vortexing, with a 300 µL aliquot of Solution B. A 300µL sample of Supernatant 3 was removed to a clean micro-centrifuge tube and mixed by vortexing, with a 300 µL aliquot of Solution B.

All three samples were recombined into a single micro-centrifuge tube and mixed by vortexing. Samples were stored at -20°C until use.

**Table 2.2 - Solutions A, B and C required for protein extraction** (Section 2.2.1)

<table>
<thead>
<tr>
<th></th>
<th>Solution A (10mL)</th>
<th>Solution B (10mL)</th>
<th>Solution C (10mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>4.80g (8M)</td>
<td>3.0g (5M)</td>
<td>NA</td>
</tr>
<tr>
<td>Thiourea</td>
<td>NA</td>
<td>1.52g (2M)</td>
<td>NA</td>
</tr>
<tr>
<td>CHAPS</td>
<td>0.4g (4% w/v)</td>
<td>0.2g (2% w/v)</td>
<td>NA</td>
</tr>
<tr>
<td>DTT</td>
<td>0.1g (1% w/v)</td>
<td>0.1g (1% w/v)</td>
<td>0.05g (50mM)</td>
</tr>
<tr>
<td>SB3-10</td>
<td>NA</td>
<td>0.2g (2% w/v)</td>
<td>NA</td>
</tr>
<tr>
<td>SDS</td>
<td>NA</td>
<td>NA</td>
<td>0.1g (1% w/v)</td>
</tr>
<tr>
<td>Tris Base</td>
<td>8.0mL 50mM stock solution (pH 9.5)</td>
<td>8.0mL 50mM stock solution (pH 9.5)</td>
<td>7.5mL 50mM stock solution (pH 8.8)</td>
</tr>
<tr>
<td>Ampholytes (pH 3-10)</td>
<td>128µL</td>
<td>128µL</td>
<td>NA</td>
</tr>
<tr>
<td>Glycerol</td>
<td>NA</td>
<td>NA</td>
<td>2.5g (25% v/v)</td>
</tr>
<tr>
<td>pH</td>
<td>9.5</td>
<td>9.5</td>
<td>8.8</td>
</tr>
</tbody>
</table>

2.2.2. Protein Quantification Assay

Frozen extracted protein samples were allowed to thaw completely before use, all samples were vortexed and briefly centrifuged before use. Protein quantification was performed using a RC DC protein assay kit (Bio-Rad) in accordance with the manufacturers’ instructions.
2.2.3. Iso-Electric Focusing of Immobilized pH Gradient (IPG) Strips

For a 14cm or 7cm IPG strip an appropriate sample volume to give the required protein concentration was used, according to the manufacturers’ guidelines. For 7cm IPG strips a protein load of between 50-100µg and up to 125µL of sample, for 17cm IPG strips a protein load of 200-400µg and up to 300µL of sample. The appropriate sample volume was pipetted into a lane of a clean iso-electric focusing (IEF) tray. The IPG strip was removed from the -20°C freezer, using clean forceps the plastic backing was carefully removed from the strip. The IPG strip was placed into the IEF tray so that the anode end of the strip was placed on the anode side of the tray. The IPG strip was overlaid with mineral oil and the IEF tray lid replaced.

The IEF tray was inserted into the PROTEAN cell, ensuring that the electrodes were correctly aligned and the pre-programmed method ‘tear2method’ (described in detail below) was run. The first step in the pre-programmed method is the active rehydration of the IPG strip by applying 50mV for 12h. After 12h of active rehydration the PROTEAN cell automatically pauses. The IEF tray was then removed and wicks saturated with distilled water placed between the IPG strip and the electrodes. The IPG strip was checked to ensure it was still sufficiently covered with mineral oil. The IEF tray lid was replaced and the tray returned to the PROTEAN cell. The method was resumed. Protein focusing occurred in 4 ramped stages a) 300V for 1h (linear), b) 1000V for 5h (linear), c) 2500V for 6h (linear) and 5000V 6h (linear). After focusing the strip was held at 500V until the tray was removed from the instrument.

Focused IPG strips which were not required for immediate use were transferred to a clean focusing tray and stored at -20°C until required.

2.2.4. Equilibration of Focused IPG Strips

The focused IPG strip was transferred to a clean focusing tray and covered with 1mL of Equilibration Buffer I (Table 2.3). The tray lid was replaced and the tray placed on a shaking table at room temperature for 10 min. After 10 min the IPG strip was
transferred to a clean lane in the IEF tray. The IPG strip was covered with 1mL of Equilibration Buffer II (Table 2.3). The tray lid was replaced and the tray placed on a shaking table at room temperature for 10 min.

Table 2.3 - Equilibration buffers I and II for focused IPG equilibration

<table>
<thead>
<tr>
<th></th>
<th>Equilibration Buffer I (10mL)</th>
<th>Equilibration Buffer II (10mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>3.6g</td>
<td>3.6g</td>
</tr>
<tr>
<td>SDS</td>
<td>0.2g</td>
<td>0.2g</td>
</tr>
<tr>
<td>Tris – HCl</td>
<td>2.5mL (1.5M pH 8.8)</td>
<td>2.5mL (1.5M pH 8.8)</td>
</tr>
<tr>
<td>20% Glycerol</td>
<td>2mL</td>
<td>2mL</td>
</tr>
<tr>
<td>DTT</td>
<td>0.02g</td>
<td>0.02g</td>
</tr>
<tr>
<td>Iodacetamide</td>
<td>NA</td>
<td>0.418g</td>
</tr>
</tbody>
</table>

2.2.5. Preparation of SDS Gels

The plates, spacers, combs and gaskets were washed with 100% ethanol and allowed to air dry. For mini protein gels (i.e. those for use with a 7cm IPG strip) a separating buffer of 1.5M Tris (Sigma, UK) (pH 8.8) was prepared. For 2D protein gels (i.e. those for use with a 17cm IPG strip) and mini 1D protein gels a separating buffer (see above) and a stacking buffer 0.5M Tris (Sigma, UK) (pH 6.8) were prepared. The gel plates were assembled and checked for leaks using water. A separating gel (12% acrylamide) was prepared as described in Table 2.4.

Table 2.4 - Reagent volumes required for SDS separating gels

<table>
<thead>
<tr>
<th></th>
<th>Mini 2D Gel</th>
<th>2D Gel</th>
<th>Mini 1D Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (37.5:1)</td>
<td>8.0mL</td>
<td>8.0mL</td>
<td>8.0mL</td>
</tr>
<tr>
<td>(BioRad, UK)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>6.8mL</td>
<td>6.8mL</td>
<td>6.8mL</td>
</tr>
<tr>
<td>Separating Buffer</td>
<td>5.0mL</td>
<td>5.0mL</td>
<td>5.0mL</td>
</tr>
<tr>
<td>10% APS (Sigma, UK)</td>
<td>100µL</td>
<td>100µL</td>
<td>100µL</td>
</tr>
<tr>
<td>TEMED (Sigma, UK)</td>
<td>10µL</td>
<td>10µL</td>
<td>10µL</td>
</tr>
</tbody>
</table>
For mini 2D protein gels, the gel mixture was immediately poured to a level ~0.5cm below the top of the short plate. For mini 1D protein gels the gel mixture was poured to a level approx. 1cm below the end of the comb. For 2D protein gels the gel mixture was poured to a level approx. 5cm below the level of the short plate. For all gel types the gel front was covered with water saturated butanol and left to set for 1h.

After 1h the water saturated butanol was removed and the gel front washed five times with distilled water. For mini 1D protein gels and 2D protein gels a stacking gel (Table 2.5) was prepared and poured to approx. 0.5cm below the level of the short plate. For mini 1D protein gels a well forming comb was inserted and the gel allowed to set for 1h. For 2D protein gels the gel front was covered with water saturated butanol as described above.

Table 2.5 - Reagent volumes required for SDS stacking gels

<table>
<thead>
<tr>
<th></th>
<th>Mini 2D Gel</th>
<th>2D Gel</th>
<th>Mini 1D Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (37.5:1) (BioRad, UK)</td>
<td>NA</td>
<td>1.3mL</td>
<td>1.3mL</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>NA</td>
<td>6.1mL</td>
<td>6.1mL</td>
</tr>
<tr>
<td>Separating Buffer</td>
<td>NA</td>
<td>2.5mL</td>
<td>2.5mL</td>
</tr>
<tr>
<td>10% APS (Sigma, UK)</td>
<td>NA</td>
<td>50µL</td>
<td>50µL</td>
</tr>
<tr>
<td>TEMED (Sigma, UK)</td>
<td>NA</td>
<td>5µL</td>
<td>5µL</td>
</tr>
</tbody>
</table>

For 2D gels the equilibrated IPG strip was placed onto the top of the gel so as to be in contact with the gel front. The IPG strip was then covered with an 1% low melt agarose gel containing 10% bromophenol blue dye and left to set for approx. 10min. For the 1D mini protein gels, samples were mixed in a 1:1 ratio with cracking buffer (Table 2.6) and heated in a 95°C water bath for 5min, samples were then stored on ice until 50µL of each sample was loaded into the individual wells.

For all gels a running buffer was prepared (Table 2.7) and used to fill both the inner and outer gel tanks to an appropriate level.
Table 2.6 - 1D SDS PAGE cracking/loading buffer

<table>
<thead>
<tr>
<th></th>
<th>1D SDS PAGE Cracking/Loading Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>0.5M</td>
</tr>
<tr>
<td>SDS</td>
<td>6% w/v</td>
</tr>
<tr>
<td>Glycerol</td>
<td>30% w/v</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>1% v/v</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

Table 2.7 – Running Buffer used for all protein gel types

<table>
<thead>
<tr>
<th></th>
<th>Running Buffer (1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>14.4g</td>
</tr>
<tr>
<td>Tris</td>
<td>3g</td>
</tr>
<tr>
<td>SDS</td>
<td>1g</td>
</tr>
</tbody>
</table>

Mini gels were run at 35mA for approx. 3h or until the dye front reached the bottom of the gel. 2D protein gels were run at 200mA until the dye front reached the bottom of the gel.

2.2.6. Gel Staining Using Colloidal Coomassie Stain

After completion of the run gels were carefully removed from the plates and placed into clean gel boxes. Gels were covered with colloidal Coomassie fixing solution (Table 2.8) and left for 1h at room temperature on a shaking table.

After 1h incubation gels were given two 10min washes with distilled H$_2$O before being transferred to an airtight container and covered with colloidal Coomassie dye working solution (Table 2.8) and left overnight at room temperature on a shaking table.

Following overnight incubation gels were transferred to clean gel boxes and covered with 1% acetic acid and left at room temperature on a shaking table until all of the Coomassie particles had been removed and the gel background was clear.
Table 2.8 – Preparation of colloidal Coomassie reagents

<table>
<thead>
<tr>
<th></th>
<th>Colloidal Coomassie Fixing Solution (800mL)</th>
<th>Colloidal Coomassie Dye Stock (1L)</th>
<th>Colloidal Coomassie Working Solution (1L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>320mL</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>80mL</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Ortho-phosphoric acid</td>
<td>NA</td>
<td>20g (2% w/v)</td>
<td>NA</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>NA</td>
<td>100g (10% w/v)</td>
<td>NA</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue G250</td>
<td>NA</td>
<td>1g (0.1% w/v)</td>
<td>NA</td>
</tr>
<tr>
<td>Colloidal Coomassie Dye Stock</td>
<td>NA</td>
<td>NA</td>
<td>800mL</td>
</tr>
<tr>
<td>Methanol</td>
<td>NA</td>
<td>NA</td>
<td>200mL</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>400mL</td>
<td>Make up to 1000mL</td>
<td>NA</td>
</tr>
</tbody>
</table>

2.3. FT-IR Measurement of Bacterial Stress Response

2.3.1. Preparation of FT-IR Samples to Monitor the Solvent Stress Response of *E. coli* MG1655 and *P. putida* KT2440

Cells were grown in triplicate in liquid medium inoculated from an overnight liquid culture as described in Section 2.1.3.

After an appropriate incubation time an appropriate volume of solvent was added to each flask. After addition of solvent the flasks were sealed with Suba Seals and returned to the orbital incubator. At various time points a 2.0mL aliquot was removed from each flask. From the removed sample OD, readings were made the remaining sample was centrifuged at 11 500g for 5min at 4°C. The supernatant was discarded. The remaining pellet was washed twice with 1mL of autoclaved distilled water. After the final washing the dry pelleted samples were stored at -80°C until use.
After the final sample had been taken at 24h after addition of solvent, the remaining biomass from each of the flasks was harvested into a sterile 50mL centrifuge tube. Samples were centrifuged at 3080g for 10 min at 4°C. The supernatant was discarded and the remaining pellet was washed twice with autoclaved distilled water. After the final wash the dry pelleted samples were stored at -80°C until use.

2.3.2. FT-IR Analysis of Biomass Samples

Samples were removed from the freezer and allowed to thaw. Each sample was re-suspended in distilled water to give an OD at 680nm of ~1. A 20µL aliquot of each sample was spotted onto a silicon FT-IR plate. The plate was dried at 50°C for 10min or until samples were completely dry. The dried plate was loaded onto a motorised microplate module HTS-XT™ attached to an Equinox 55 module (Bruker Optics Ltd., UK). A deuterated sulphate (DTGS) detector was used to record transmission measurements of the samples. An empty well was used for the collection of a background spectrum. Spectra were collected over the wavelength range 4000-600 cm\(^{-1}\) under the control of a computer programmed with OPUS 4, operated under Windows 2000. Spectra were acquired at a resolution of 4 cm\(^{-1}\), a total of 64 spectra were co-added and averaged to improve the signal to noise ratio. The collection time for each spectrum was approx. 1min, spectra displayed in terms of absorbance.

The collected data were converted to ASCII format using an in-house conversion program. The collected FT-IR data were analyzed using MATLAB v6 (The MathsWorks Inc., MA, USA). The analysis performed is described in detail in the relevant results chapter.

2.3.3. Preparation of FT-IR Samples to Monitor the Ionic Liquid Stress Response of *E. coli* MG1655, *P. putida* KT2440 and *P. putida* DOT-T1E

Cells were grown in liquid medium inoculated from an overnight liquid culture as described in Section 2.1.3.
Flasks were prepared in triplicate and after an appropriate incubation time an appropriate volume of solvent was added to each flask. After addition of solvent the flasks were sealed with suba seals and returned to the orbital incubator. At various time points a 2.0mL aliquot was removed from each flask. From the removed sample OD, pH and viable count readings were made. The remaining sample was centrifuged at 11 500g for 5min at 4°C. The supernatant was collected and stored at -80°C until use. The remaining pellet was washed twice with 1mL of autoclaved distilled water. After the final washing the dry pelleted samples were stored at -80°C until use.

After the final sample had been taken at 24h after addition of solvent, the remaining biomass from each of the flasks was harvested into a sterile 50mL centrifuge tube. Samples were centrifuged at 3080g for 10 min at 4°C. The supernatant was discarded and the remaining pellet was washed twice with autoclaved distilled water. After the final wash the dry pelleted samples were stored at -80°C until use.

Biomass samples were prepared for FT-IR analysis as described in Section 2.3.2.

2.3.4. FT-IR Analysis of Supernatant Samples

Samples were removed from the freezer and allowed to thaw. Each sample was vortexed and briefly centrifuged. A 20µL aliquot of each sample was spotted onto a silicon FT-IR plate. The plate was dried at 50°C for 10min or until samples were completely dry. FT-IR analysis was performed as described in Section 2.3.2.
2.4. Rapid Screening Methods for Ionic Liquid Toxicity

2.4.1. Rapid Agar Screening Method for Ionic Liquid Toxicity Using *E. coli* MG1655, *P. putida* KT2440 and *P. putida* DOT-T1E

A series of 6mm x 6mm filter paper discs were produced using a standard office hole punch. The collected discs were autoclaved before a single disc was transferred aseptically into a sterile micro-centrifuge tube. The micro-centrifuge tubes were weighed before a 5µL sample of ionic liquid was added aseptically to the filter paper. The tubes were then re-weighed. Each filter disc was aseptically transferred to the centre of an agar plate which had been pre-inoculated with a lawn of the test organism. Micro-centrifuge tubes were weighed to enable calculation of the weight of the ionic liquid added to the agar plate.

The plates were incubated overnight at a temperature appropriate to enable growth of the test organism, in a static incubator. Following overnight incubation the agar plates were examined and the radius of any growth inhibition zone around the filter disc recorded. A schematic overview of the method is shown in Fig 2.1.

![Fig 2.1 - A Schematic Overview of the Agar Diffusion Toxicity Test used for Ionic Liquids with *E. coli* MG1655, *P. putida* KT2440 and *P. putida* DOT-T1E.](image-url)
2.4.2. Rapid Bioscreen Method for Determination of Ionic Liquid Toxicity Using E. coli MG1655, P. putida KT2440 and P. putida DOT-T1E

A sample of ionic liquid was transferred into a sterile pre-weighed Eppendorf tube. The tube was re-weighed and the amount of ionic liquid in each tube calculated and recorded. For water miscible ionic liquids an appropriate amount of distilled autoclaved water was added to produce ionic liquid at a concentration of 95% (w/v). For water immiscible ionic liquids samples were used without dilution. A 4µL sample of each ionic liquid was added in triplicate to a 100 well Bioscreen plate. Ionic liquid and water samples were vortexed immediately before being added to the Bioscreen plate. An aliquot of 186µL of MSX or LB media was added to each well before the addition of a 10µL aliquot of overnight culture of the test organism (2.1.3), giving a final ionic liquid concentration of 2% (v/v).

The Bioscreen plates were run in the Bioscreen for 24h at an appropriate temperature for the test organism, with intensive shaking. The OD of each well was recorded every 10min throughout the 24h run using a wideband OD filter. Output was recorded in a Microsoft Excel worksheet (Microsoft Office, 2003).

Specific growth rates (μ) were calculated by selecting two points at time t1 and t2 in the exponential growth phase and applying the equation

\[ \mu = \frac{\ln(OD_2/OD_1)}{(t_2 - t_1)} \]

Specific growth rates were reported as a percentage of the growth rate in control cultures grown without ionic liquid.
3. **Results – Solvent Stress Response**

3.1. **Introduction**

An understanding of the effects of organic solvents on the survival of microorganisms and how they respond (both genotypically and phenotypically) is of great importance for applications in both bio-remediation and biocatalysis. By understanding the way in which organic solvents interact with micro-organisms it may be possible to more readily identify those which would be suitable for use in industrial and environmental applications which involve their exposure to high concentrations of organic solvent.

Bioremediation is the process by which harmful chemicals or substances are metabolized (transformed) by micro-organisms to produce non harmful end products (Elliot *et al.*). Most micro-organisms which have been reported as being used in bioremediation applications have been isolated from sites of, or close to, chemical contamination (Masood and Malik, 2011), (Pandey *et al.*, 2010), (Khanna *et al.*, 2010). However the possibility that micro-organisms could be adapted, either through selective enrichment culturing or through transformation of the micro-organism to include a plasmid encoding genes which would allow the micro-organism to metabolise particular solvents enabling them to serve as bioremediation agents for specific chemical contaminants. This would be useful as it may lead to enhanced product yields or increased decontamination rates. In order to explore this it would be necessary to identify micro-organisms which display sufficient tolerance to the chemical contaminant, and as a means to an end an understanding of the mechanisms of chemical toxicity would be greatly beneficial.

Whole cell biocatalysis is another application in which an understanding of the interaction of toxic organic solvents with micro-organisms is of great importance. There are numerous reports of micro-organisms being used for the production of (bio-)chemicals and high value products, which are either difficult or expensive (especially those which require correct stereochemistry) to produce by chemical
means (Winder et al., 2011), (Ojima et al., 2009), (Corre et al., 2008), (Kafshnochi et al., 2010), (Meijnen et al., 2011). In many instances either the starting material or the final product will exhibit some toxicity to the biocatalyst. Therefore an understanding of the mechanisms of toxicity is of great importance in selecting micro-organisms as better biocatalysts.

In this chapter the effect of the exposure to the organic solvent toluene has been investigated using the micro-organisms Escherichia coli MG1655 and Pseudomonas putida KT2440. The effects on the bacteria of exposure to different concentrations of toluene has been investigated by means of optical density (OD) readings for the measurement of specific growth rates and the use of FT-IR spectroscopy to generate biochemical fingerprints from the biomass samples collected during a 24h time course exposure. Data from three separate batches of each of the micro-organisms were collected. Full details of the method used are given in the Materials and Methods Section 2.3.

Additionally, the halogenated solvents chlorobenzene and fluorobenzene were investigated for their effects on the micro-organism P. putida KT2440. Toxicity of these solvents were investigated using the same means as has been described above for the investigation of toluene toxicity, full details of this method are described in the Materials and Methods Section 2.3.

3.2. Materials and Methods

A full description of the materials and methods used is given in Section 2.3, briefly cultures were grown in 50mL of liquid medium inoculated from an overnight culture. After an appropriate incubation time for the culture type an appropriate volume of solvent was added in triplicate to the flasks. After addition of solvent flasks were sealed with Suba seals and returned to the orbital incubator. At various time points over the 24h time course 2.0mL aliquots were removed from each flask.

From the removed sample OD readings were taken. The remainder of the sample was centrifuged at 11 500g for 5min at 4°C. The supernatant was removed and
discarded. The remaining biomass pellet was washed twice with 1mL of autoclaved distilled water. After the final washing step samples were stored at -80°C until use.

FT-IR spectra were collected according to the method in Section 2.3.3.

3.3. Results and Discussion

3.3.1. Physiological measurements of bacteria exposed to toluene

Initially it was necessary to determine the maximum concentration of toluene to which the micro-organisms could be exposed whilst still allowing the collection of growth data and biomass samples. Thus to determine the toluene tolerance of each organism (i.e. the minimum inhibitory concentration (MIC)) a series of 15mL screw cap tubes were prepared. Each tube contained 9mL of sterile MSX medium. A range of toluene concentrations from 0.05% (v/v) to 0.60% (v/v) were tested. Each concentration was tested in triplicate i.e. in three separate tubes. Additionally three tubes were prepared to which no toluene was added, these tubes acted as a positive growth control. To each tube a 10µL aliquot from an overnight culture of the appropriate micro-organism was added. All additions to these tubes were made aseptically. Sealed tubes were incubated overnight in a standing incubator at an appropriate temperature (i.e., 37°C for E. coli and 30°C for P. putida).

Following overnight incubation tubes were inspected for growth of the micro-organism and the results recorded. Results from the toluene MIC experiments are shown in Table 3.1
Table 3.1– Results from the toluene MIC experiments using *E. coli* MG1655 and *P. putida* KT2440. Following overnight incubation cultures were checked for growth, each toluene concentration was tested in triplicate. The presence of growth in a culture is indicated by a √ symbol whilst absence of growth is indicated by an X symbol.

<table>
<thead>
<tr>
<th><em>E. coli</em> MG1655</th>
<th>Growth (√/X)</th>
<th><em>P. putida</em> KT2440</th>
<th>Growth (√/X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene Concentration (v/v)</td>
<td></td>
<td>Toluene Concentration (v/v)</td>
<td></td>
</tr>
<tr>
<td>0% (+VE Control)</td>
<td>√</td>
<td>0% (+VE Control)</td>
<td>√</td>
</tr>
<tr>
<td>0.05%</td>
<td>√</td>
<td>0.05%</td>
<td>√</td>
</tr>
<tr>
<td>0.10%</td>
<td>X</td>
<td>0.10%</td>
<td>X</td>
</tr>
<tr>
<td>0.15%</td>
<td>X</td>
<td>0.15%</td>
<td>X</td>
</tr>
<tr>
<td>0.20%</td>
<td>X</td>
<td>0.20%</td>
<td>X</td>
</tr>
<tr>
<td>0.25%</td>
<td>X</td>
<td>0.25%</td>
<td>X</td>
</tr>
<tr>
<td>0.30%</td>
<td>X</td>
<td>0.30%</td>
<td>X</td>
</tr>
<tr>
<td>0.35%</td>
<td>X</td>
<td>0.35%</td>
<td>X</td>
</tr>
<tr>
<td>0.40%</td>
<td>X</td>
<td>0.40%</td>
<td>X</td>
</tr>
<tr>
<td>0.45%</td>
<td>X</td>
<td>0.45%</td>
<td>X</td>
</tr>
<tr>
<td>0.50%</td>
<td>X</td>
<td>0.50%</td>
<td>X</td>
</tr>
<tr>
<td>0.55%</td>
<td>X</td>
<td>0.55%</td>
<td>X</td>
</tr>
<tr>
<td>0.60%</td>
<td>X</td>
<td>0.60%</td>
<td>X</td>
</tr>
</tbody>
</table>

From the results presented in Table 3.1 it was determined that a range of three toluene concentrations (in addition to positive control cultures) of, 0.025%, 0.05% and 0.1% (v/v) were selected for investigation with flask cultures.

Flask cultures were prepared as described in the Materials and Methods Section 2.1.3 OD measurements were taken over the course of the 24h exposure time course. The results of the OD measurements from batch 1 of the *E. coli* MG1655 cultures can be seen in Fig. 3.1, whilst the results from the first batch of *P. putida* KT2440 cultures are depicted in Fig. 3.2.
Fig. 3.1 – Average growth curves of *E. coli* MG1655 during exposure to toluene

Positive control, ▲ 0.025% (v/v) toluene, △ 0.05% (v/v) toluene, • 0.1% (v/v) toluene, OD readings were measured by preparing a 1/10 dilution of the 100µL samples, OD read at 680nm. The data points plotted are the average of the OD readings from the three replicate flasks, and error bars represent standard deviation between the three flasks.

From the growth curves collected from the first batch of *E. coli* MG1655 cultures in the presence of toluene (Fig. 3.1) it can be seen that there is a clear effect caused by increasing the concentration of the toluene in the flask cultures. Increasing the amount of toluene in the flask cultures causes both a decrease in the final biomass of the samples (as indicated by a decreased final OD reading, compared to the positive control cells). Samples exposed to 0.1% toluene enter exponential growth phase at a later time point than the positive control cells. That growth was recorded in the cultures exposed to 0.1% toluene was not expected based on the initial screening results (Table 3.1), a possible explanation for this discrepancy is that during the flask culture experiment the cultures were given time to become established i.e. they were given incubation time before the solvent was added to the culture. For the screening method in which cultures were grown in sealed
tubes, both the inocula and the solvent were added at the same time, thereby not allowing the culture to become established before the introduction of the solvent. Additionally, it is possible that some solvent was lost from the screw cap tubes due to evaporation; this would result in the concentration of the solvent within the tube being lower than was anticipated.

All three replicate cultures from batch 1 display small standard deviations suggesting that these growth data are reproducible. The specific growth rates of the toluene exposed cultures have been calculated as a percentage of the specific growth rate of the positive control cells. Specific growth rates for the three batches of *E. coli* MG1655 are shown in Table 3.2.

**Fig. 3.2** – Average growth curves of *P. putida* KT2440 during exposure to toluene

- Positive control, ■ 0.025% (v/v) toluene, ▲ 0.05% (v/v) toluene, • 0.1% (v/v) toluene, OD readings were measured by preparing a 1/10 dilution of the 100µL samples, OD read at 680nm. The data points plotted are the average of the OD readings from the 3 replicate flasks, and error bars represent standard deviation between the three flasks.
The growth curves recorded from the first batch of exposures of *P. putida* KT2440 to toluene over a 24h time course do not show consistent sample growth as was seen with the three batches of *E. coli* MG1655 cultures. The growth curves displayed in Fig. 3.2, do not show the pattern with respect to final OD and lag phase differences observed earlier for *E. coli* that could be attributed to the addition of increasing concentrations of toluene to the culture flasks. As can also be seen several of the data points have large standard deviations associated with them. These large standard deviations may indicate that there is inconsistent growth between the three replicate flasks.

The results obtained from the first batch of *P. putida* cultures suggested that there had been a problem with the growth of these cultures. As only a single batch of the *P. putida* cultures had been tested it was not possible to determine whether the unexpected growth curves collected were a result of problems specific to this particular culture batch or whether this was an indication of a problem with the stock culture of the *P. putida*. Therefore it was decided to re-culture the *P. putida* KT2440 from a frozen stock culture. The frozen stock culture was sub-cultured three times on LB agar plates before being used in further experiments. The growth curves obtained from the new culture of *P. putida* KT2440 are shown in Fig. 3.3.
Fig. 3.3– Average growth curves of new culture of *P. putida* KT2440 during exposure to toluene

- Positive control, ■ 0.025% (v/v) toluene, ▲ 0.05% (v/v) toluene, • 0.1% (v/v) toluene, OD readings were measured by preparing a 1/10 dilution of the 100µL samples, OD read at 680nm. The data points plotted are the average of the OD readings from the 3 replicate flasks, and error bars represent standard deviation between the three flasks.

The growth curves in Fig. 3.3, obtained using a new stock culture of *P. putida* KT2440, show growth curves which have similar features to those shown by the *E. coli* MG1655 cultures. In contrast to the *E. coli* cultures there appears to be very little difference in the growth curves of the positive control cultures compared with the cultures exposed to the two lowest concentrations of toluene (0.025% and 0.05% v/v). All three of the cultures appear to achieve a similar final biomass, as indicated by the similar OD readings obtained at the 24h time point. Again, unlike with the *E. coli* cultures there is a large difference between the positive control culture and the culture exposed to the highest concentration (0.1% v/v) of toluene. The *P. putida* culture exposed to the highest concentration of toluene appears to experience significantly lower growth over the duration of the 24h time course of the experiment, with a slightly greater increase in OD readings being observed
between the 8h and 24h time points than had been observed between the other time points.

Although the growth curves presented in Fig. 3.3 were what would be expected from exposure of the organism to increasing concentrations of a toxic solvent it can still be seen that there are large standard deviations associated with the data collected at some of the time points. These large standard deviations may indicate that the triplicate cultures, including the positive control cultures, were not growing at a consistent rate.

Due to the discrepancies seen between the first and second batches of P. putida cultures a third batch of P. putida cultures were prepared, the resulting growth curves from the third batch of P. putida cultures are shown in Fig. 3.4.

![Average growth curves of the third batch of P. putida KT2440 cultures during exposure to toluene.](image)

- Positive control, ▲ 0.025% (v/v) toluene, ▼ 0.05% (v/v) toluene, • 0.1% (v/v) toluene, OD readings were measured by preparing a 1/10 dilution of the 100μL samples, OD read at 680nm. The data points plotted are the average of the OD readings from the 3 replicate flasks, and error bars represent standard deviation between the three flasks.
The growth curves shown in Fig. 3.4 are generally consistent with those seen in Fig. 3.3, in that increasing the concentration of toluene to which the culture is exposed decreased the final biomass of the culture, indicated by a lower final OD value and increases the lag time experienced by the culture before it enters exponential growth phase. The similarity of the growth curves of batches 2 and 3 of the *P. putida* suggest that the results obtained for batch 1 of the *P. putida* cultures are clearly anomalous, although the cause of the differences between the growth curves of batch 1 and batches 2 and 3 can not be determined with certainty from the evidence collected. Therefore it was decided not to use the initial batch in further experiments.

Specific growth rates calculated as a percentage of the specific growth rate of the positive control cells are shown in Table 3.2 for *E. coli* MG1655 and Table 3.3 for *P. putida* KT2440.

**Table 3.2**—Specific growth rates of 3 batches of *E. coli* MG1655 exposed to 3 concentrations of toluene and normalised to the positive control culture

<table>
<thead>
<tr>
<th></th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025%</td>
<td>81 ± 3</td>
<td>86 ± 2</td>
<td>61 ± 7</td>
</tr>
<tr>
<td>0.05%</td>
<td>49 ± 2</td>
<td>82 ± 1</td>
<td>59 ± 1</td>
</tr>
<tr>
<td>0.1%</td>
<td>44 ± 3</td>
<td>60 ± 18</td>
<td>33 ± 9</td>
</tr>
</tbody>
</table>

**Table 3.3**—Specific growth rates of 3 batches of *P. putida* KT2440 exposed to 3 concentrations of toluene and normalised to the positive control culture

<table>
<thead>
<tr>
<th></th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.025%</td>
<td>Not reproducible so not calculated</td>
<td>94 ± 3</td>
<td>98 ± 7</td>
</tr>
<tr>
<td>0.05%</td>
<td></td>
<td>90 ± 9.</td>
<td>88 ± 6</td>
</tr>
<tr>
<td>0.1%</td>
<td></td>
<td>6 ± 7</td>
<td>40 ± 1</td>
</tr>
</tbody>
</table>

From the specific growth rates for the *E. coli* MG1655 cultures shown in Table 3.2 a clear pattern is visible. Increasing the concentration of the toluene to which the cultures are exposed reduces the specific growth rates of the cultures. When considering the specific growth rates for the *P. putida* KT2440 cultures it is more difficult to identify a definite pattern. In general, as with the *E. coli* cultures it can be seen that increasing the concentration of toluene in the culture adversely affects
the specific growth rates of the cultures. The specific growth rates for batch 1 of the *P. putida* cultures have not been calculated, due to the poor growth of the positive control cultures in this batch.

Comparison of these specific growth rates, that have been normalised to the positive, unexposed control, between the *E. coli* MG1655 cultures and the *P. putida* KT2440 cultures suggests that *P. putida* KT2440 cultures have a higher resistance to a sudden addition of toluene at the two lowest concentrations of toluene tested.

That the *P. putida* KT2440 cultures perform better in comparison to the *E. coli* MG1655 cultures in the presence of the lower toluene concentrations is unsurprising based upon a review of the published literature. In work reported by Segrura *et al.* it was noted that following exposure of *P. putida* KT2440 to a toluene shock of 0.1% (v/v) a fraction of the cells ($10^{-5}$) survived the shock (Segura *et al.*, 2003). In comparison *E. coli* K12 (from which the *E. coli* strain MG1655 strain was derived) exhibited no growth when cultures were overlaid with toluene (Aono *et al.*, 1991).

In addition to the collection of optical density readings, biomass samples were also collected from each of the cultures at the recorded time points. The collected biomass samples were analysed using FT-IR. Analysis of microbial cultures to produce metabolic fingerprints through the use of FT-IR spectroscopy has been reported in published literature (Wharfe *et al.*, 2011), (Winder *et al.*, 2006). Recently the use of FT-IR fingerprinting combined with growth data and chemometric cluster analysis has been used to monitor the physiological changes in *E. coli* MG1655 cultures during the biotransformation of toluene to toluene cis-glycol (Winder *et al.*, 2011).

### 3.3.2. FT-IR Spectroscopy of bacteria exposed to toluene

FT-IR spectra were collected from the biomass samples taken from batch 1 of both the *E. coli* MG1655 cultures using the process described in the Materials and Methods Section 2.3.2 collected spectra were CO$_2$ corrected i.e. peaks at 2403-2272 cm$^{-1}$ and 683-656 cm$^{-1}$ were removed and filled with a trend, spectra were then
scaled using EMSC (extended multiplicative signal correction) (Martens et al., 2003).

The EMSC method was originally developed to reduce the effect of light scattering i.e. small particles scatter light more than larger ones, experimentation within the group has shown this method to invaluable in the removal of unavoidable baseline shifts. EMSC normalization takes information registered in the spectra and attempts to separate physical light-scattering effects from the actual light absorbed by the molecules (Martens et al., 2003). The EMSC scaled spectra, showing several of the major band assignments are shown in Fig. 3.5, whilst details of the band assignments are presented in Table 3.4.

From the collected spectra 10 principal components were extracted using PCA and discriminante function analysis (DFA) performed, in the DFA the class structure of the data was based on sample replicates at the collected time points. PCA aims to produce combinations of variables within the spectra which describe the variation in the spectra. The combination of variables which represents the largest degree of variance is named the first principal component, the combination of variables which describes the next largest degree of variance is the second principal component and so on.

Following on from PCA, discriminant function analysis (DFA) was performed on the collected spectra. In this method of analysis variation between samples from the same group or class, in this instance the three biological replicates collected for each culture condition at each time point, is minimised whilst variation between different groups or classes is maximized (Windig et al., 1983).
Fig. 3.5 - EMSC scaled spectra of batch 1 cultures of *E. coli* MG1655 during a 24h exposure to toluene

Table 3.4 – Major band assignments identified from the FT-IR Spectra in Fig 3.5

<table>
<thead>
<tr>
<th></th>
<th>Wavenumber (cm⁻¹) Range</th>
<th>Dominant Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3000-2800</td>
<td>CH₃ stretches from fatty acids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>1700-1500 (1700-1600) (1600-1500)</td>
<td>Proteins C=O from Amide I C=N and C-N-H from Amide II</td>
</tr>
<tr>
<td>C</td>
<td>1450-1200</td>
<td>Carboxylic groups of proteins, free amino acids, polysaccharides</td>
</tr>
<tr>
<td>D</td>
<td>1200-900</td>
<td>C-O or O-H from polysaccharides</td>
</tr>
</tbody>
</table>

Information for the assignment of the FT-IR bands shown in Table 3.4 was adapted from (Wharfe et al., 2010).

From the *E. coli* data 10 PCs were extracted accounting for 99.45% of the total variance within the data set. The resulting PCA plot can be seen in Fig. 3.6.
From this biplot it was difficult to distinguish clear clustering patterns within the data. The spectra collected at time point 0h from all culture conditions do not appear to cluster together. This clustering pattern would be expected as these biomass samples were collected before the addition of toluene to any of the culture vessels, as such conditions within all culture vessels would have been expected to be identical.

Although the expected pattern was not observed from the PCA plot the absence of this clustering pattern has been previously noted in the use of PCA applied to spectroscopic data (Goodacre et al., 1998) and therefore it is necessary to give information regarding replicate numbers to an algorithm such as DFA, this allows for compensation for any small random variability between replicates, which otherwise may mask real variability between different classes. By only providing the DFA algorithm with information regarding replicate values the algorithm is not biased as no information is provided regarding exposure levels or times. DFA was performed on the extracted PCs with the class structure this time based upon biological replicates. The resulting DFA plot can be seen in Fig. 3.9.

From Fig. 3.9 a clearer trend in the data was observed. Firstly a trend based on exposure time can be observed across all culture conditions. Time point 0h samples (labelled X1; where X is A for 0% exposure and B, C and D for 0.025%, 0.05% and 0.1% toluene exposed cultures) are clustered together loosely in the upper right hand corner of the plot area. The data then follows a U shaped trend across the plot area with the final 24h time point samples (labelled X7) for all culture conditions located in the upper left hand corner of the plot area.

In addition it is also noteworthy that whilst samples exposed to 0.025% and 0.05% toluene follow the same trajectory with respect to time within the DFA plot, samples collected from the 0.1% exposed cultures cluster slightly separately from the samples collected from the other three culture conditions at each of the seven time points, with the greatest separations observed at the 8h and 24h time points.
Fig. 3.6 – PCA plot of E. coli MG1655 cultures during a 24h exposure to toluene

Coding used - A – Positive Control, B – 0.025% toluene exposed cultures, C – 0.05% toluene exposed cultures, D – 0.1% toluene exposed cultures. Numbers represent time points, 1 – 0h, 2 – 30min, 3 – 2h, 4 – 4h, 5 – 6h, 6 – 8h and 7 – 24h

In order to investigate the collected FT-IR spectra further the loadings plots for the first and second principal components have been plotted and are shown in Fig. 3.7 and Fig. 3.8. As PC1 accounts for the largest proportion of the variance within the data set it is useful to examine the plot to determine if any relevant biological inference can be drawn from this information.

From Fig. 3.7 and Fig. 3.8 it can be seen that the resulting loadings plots are complex, making the resulting plots difficult to interpret. For PC 1 (Fig. 3.7) the largest degree of variance is recorded between wavenumbers 1500-2000 cm⁻¹. In this area of the spectra it would be expected that the majority of vibrational bands would be associated with the protein component of the sample. This information suggests that the largest proportion of variance over the course of the 24h
experiment and across the tested culture conditions is associated with changes to the protein components of the micro-organism.

For PC 2 (Fig. 3.8) a large degree of variance is again recorded between wavenumbers 1500-2000 cm\(^{-1}\), additional variance was also recorded between wavenumbers 2500- 3500 cm\(^{-1}\), possibly attributable to CH\(_x\) stretching associated with fatty acids.

![PCA loadings plot of PC 1](image)

**Fig. 3.7** – PCA loadings plot of PC 1
Fig. 3.8 - PCA loadings plot of PC 2

The same CO₂ correction, scaling and multivariate clustering analysis of PCA and DFA used for the *E. coli* MG1655 spectra was applied to the spectra collected for the *P. putida* KT2440 spectra (data not shown). From the resulting PCA plot it was difficult to distinguish clear clustering patterns within the data. The spectra collected at time point 0h from all culture conditions cluster together loosely in the centre of the plot area, additionally spectra collected from the later time points of the 0.1% exposed cultures are seen to cluster loosely in the upper left hand corner of the plot area.

As with the FT-IR spectra collected from *E. coli* MG1655 examination of the loadings plot of the first and second principal components (data not shown) revealed that for PC1 the largest degree of variance was detected between 1500-2000 cm⁻¹. As with *E. coli* MG1655 this suggests that the largest proportion of variance over the course of the 24h experiment and across the tested culture conditions is associated with changes to the protein components of the micro-organism. For PC 2 the largest degree of variance was recorded between wavenumbers 1500-2000 cm⁻¹ and wavenumbers 1500-2000 cm⁻¹. Additional variance was also recorded
between wavenumbers 2500-3500 cm\(^{-1}\), possibly attributable to CH\(_x\) stretching associated with fatty acids.

**Fig. 3.9**- DFA plot of EMSC scaled spectra of batch 1 cultures of *E. coli* MG1655 during a 24h exposure to toluene, following extraction of 10 principal components (99.45% total variance) with a class structure based on biological replicates.

Coding used: A – Positive Control, B – 0.025% toluene exposed cultures, C – 0.05% toluene exposed cultures, D – 0.1% toluene exposed cultures.

Numbers represent time points, 1 – 0h, 2 – 30min, 3 – 2h, 4 – 4h, 5 – 6h, 6 – 8h and 7 – 24h.

DFA was performed using the 10 extracted PCs and a class structure based on biological replicates within the data set. The resulting DFA plot can be seen in Fig. 3.10.

As was seen for the DFA plot generated from the *E. coli* MG1655, clustering patterns were more easily identified in the DFA plot than in the previous PCA plot. For the *P. putida* KT2440 cultures the time point 0h samples from all culture conditions are clustered together in the top left hand corner of the plot area. Like
for the *E. coli* MG1655 samples the *P. putida* KT2440 cultures exposed to 0.1% toluene follow the same trend as the samples collected from the positive control culture.

![DFA plot of EMSC scaled spectra of batch 2 cultures of *P. putida* KT2440 during a 24h exposure to toluene, following extraction of 10 principal components (97.80% explained variance) with a class structure based on biological replicates.](image)

**Fig. 3.10** - DFA plot of EMSC scaled spectra of batch 2 cultures of *P. putida* KT2440 during a 24h exposure to toluene, following extraction of 10 principal components (97.80% explained variance) with a class structure based on biological replicates.

Coding used - A – Positive Control, B – 0.025% toluene exposed cultures, C – 0.05% toluene exposed cultures, D – 0.1% toluene exposed cultures. Numbers represent time points, 1 – 0h, 2 – 30min, 3 – 2h, 4 – 4h, 5 – 6h, 6 – 8h and 7 – 24h

The toxic effect of toluene on bacterial cultures has been widely reported. The toxicity of organic solvents is often measured in terms of the logP value of the solvent. LogP is a partition coefficient and gives a measure of the hydrophobicity/lipophilicity of a solvent and is determined by the partitioning of the solvent over an octanol/water 2-phase system. The hydrophobicity of a solvent has been shown to correlate with the solvents ability to partition into and thus
accumulate in the phospholipid membrane of a micro-organism where it kills or harms the microbe (Weber and deBont, 1996), (Sikkema, 1995). Organic solvents with a logP value of between 1.5 – 3 have been shown to be extremely damaging to the membranes of micro-organisms (Segura et al., 2003). Toluene has been calculated to have a logP value of 2.5, making it highly toxic to micro-organisms (Segura et al., 2003).

The logP value of a solvent combined with the intrinsic resistance of the micro-organism to that particular solvent are both factors in determining the effect of the solvent on the culture. Previous studies have shown that toluene, like other toxic organic solvents accumulates in the phospholipid membrane of the organism (Weber et al., 1993), (Weber and deBont, 1996). The accumulation of solvent in the cell membrane affects the membrane in a number of ways including damage to the membrane components, disrupting membrane transport and energy generation as well as disrupting the structure of the phospholipid bilayer by increasing the fluidity of the membrane (Nicolaou et al., 2010), (Sikkema, 1995). To counter the effects of accumulation of organic solvents on the fluidity of the cell membrane, micro-organisms have developed a number of adaption mechanisms. Most of these mechanisms such as the increase in the proportion of unsaturated fatty acid, alteration in the composition of the phospholipid head group and the alteration in the proportion of protein and lipid in the membrane require the synthesis of new lipid molecules. These homoviscous adaptation response mechanisms have been recorded in both E. coli and P. putida. (Ingram, 1976), (Dombek and Ingram, 1984), (Sullivan et al., 1979), (Ramos et al., 2002), (Segura et al., 1999), (Weber and deBont, 1996). However, the synthesis of new molecules requires both time and energy, therefore the response of the organism to the accumulation of toxic solvents is delayed whilst new molecules are synthesised.

However a mechanism which does not require the synthesis of new lipid molecules has been reported in P. putida species. The increase in the proportion of fatty acids in the trans rather than cis configuration, reduces the fluidity of the cell membrane by allowing increased packing of the phospholipid molecules in the membrane (Junker and Ramos, 1999), (Segura et al., 1999), (Heipieper, 2003), (Heipieper et al.,
1992), (von Wallbrunn et al., 2003). This immediate response to the presence of toxic organic solvent may give the *P. putida* KT2440 cultures an advantage over the *E. coli* MG1655 cultures, allowing the *P. putida* cultures to react and adapt more readily to the addition of toluene to the culture medium, explaining the greater specific growth rates seen with the *P. putida* KT2440 cultures as compared to the *E. coli* MG1655 cultures. It should be noted that this *cis to trans* adaption mechanism has not been reported to be observable with FT-IR spectroscopy.

3.3.3. Physiological measurements on *P. putida* KT2440 exposed to chlorobenzene

As the *P. putida* KT2440 cultures had showed superior tolerance compared to *E. coli* MG1655 cultures when exposed to the lower concentrations of toluene, it was decided to use the *P. putida* KT2440 cultures to investigate the effect of a related organic solvent, chlorobenzene, where the methyl group on the toluene was replaced with chlorine. The logP value of chlorobenzene has been reported as being similar to that of toluene (Khadikar et al., 2002). As with toluene an initial MIC screen was performed to determine what concentrations of this solvent should be used in the batch culture experiments. The results of this initial MIC screening are shown in Table 3.5.
Table 3.5- Results from MIC growth experiments using *P. putida* KT2440 with chlorobenzene. Following overnight incubation cultures were checked for growth, each toluene concentration was tested in triplicate. The presence of growth in a culture is indicated by a √ symbol whilst absence of growth is indicated by an X symbol.

<table>
<thead>
<tr>
<th>Chlorobenzene Concentration (v/v)</th>
<th>Growth (V/X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% (+VE Control)</td>
<td>√</td>
</tr>
<tr>
<td>0.05%</td>
<td>√</td>
</tr>
<tr>
<td>0.10%</td>
<td>√</td>
</tr>
<tr>
<td>0.15%</td>
<td>√</td>
</tr>
<tr>
<td>0.20%</td>
<td>√</td>
</tr>
<tr>
<td>0.25%</td>
<td>X</td>
</tr>
<tr>
<td>0.30%</td>
<td>X</td>
</tr>
<tr>
<td>0.35%</td>
<td>X</td>
</tr>
<tr>
<td>0.40%</td>
<td>X</td>
</tr>
</tbody>
</table>

From Table 3.5 it was concluded that the following concentrations of chlorobenzene should be used, 0.1%, 0.15% and 0.2% (v/v). The method used was the same as that used for the toluene exposure experiments and is described in detail in the Materials and Methods Section 2.3 and in Section 3.2 of this chapter.

The growth curves generated from batch 1 of the chlorobenzene exposures (data not shown) were not what had been expected based on the initial MIC screening experiment; they did not follow the previous dosage related trends seen for toluene exposure, the data points for the chlorobenzene exposures were associated with large error bars, suggesting that the levels of chlorobenzene toxicity were too extreme. Therefore the concentration of chlorobenzene added to the cultures was reduced so that concentrations of 0.05%, 0.75% and 0.1% (v/v) were used. The growth curves from two additional batches are shown in Fig. 3.11a and 3.11b.
**Fig. 3.11a** - Average growth curves of *P. putida* KT2440 during exposure to chlorobenzene

- Positive control, ● 0.05% (v/v) chlorobenzene, ▲ 0.075% (v/v) chlorobenzene, • 0.1% (v/v) chlorobenzene, OD readings were measured by preparing a 1/10 dilution of the 100µL samples, OD read at 680nm. The data points are the average of the OD readings from the three replicate flasks, and error bars represent standard deviation between the three flasks.

**Fig. 3.11b** - Average growth curves of *P. putida* KT2440 during exposure to chlorobenzene

- Positive control, ● 0.05% (v/v) chlorobenzene, ▲ 0.075% (v/v) chlorobenzene, • 0.1% (v/v) chlorobenzene, OD readings were measured by preparing a 1/10 dilution of the 100µL samples, OD read at 680nm. The data points are the average of the OD readings from the three replicate flasks, and error bars represent standard deviation between the three flasks.
It is clear from Fig 3.11a and 3.11b that although reducing the concentration of chlorobenzene exposure resulted in a series of reduced growth curves and specific growth rates (Table 3.6) the large standard deviations recorded between the different culture flasks exposed to the same culture conditions indicated that the cultures were not growing in a uniform and reproducible manner. These large standard deviations are especially apparent in the cultures exposed to the highest concentration (0.1% v/v) of chlorobenzene.

**Table 3.6** – Specific growth rates of 2 batches of *P. putida* KT2440 exposed to 3 concentrations of chlorobenzene normalised to the positive control

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05%</td>
<td>19 ± 6</td>
<td>29 ± 7</td>
</tr>
<tr>
<td>0.075%</td>
<td>8 ± 4</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>0.1%</td>
<td>13 ± 14</td>
<td>5 ± 28</td>
</tr>
</tbody>
</table>

3.3.4. FT-IR spectroscopy of *P. putida* KT2440 exposed to chlorobenzene

As with the toluene exposed cultures, biomass samples were collected over the course of the 24h exposure experiment. Samples collected during the chlorobenzene exposure experiments were analysed using FT-IR spectroscopy and the data were CO₂ corrected and processed using EMSC Fig. 3.12, a list of band assignments for the peaks present in these spectra is given in Table 3.4. Due to the poor growth curves obtained from the batch 1 chlorobenzene exposure cultures these samples were not analysed.
From the CO$_2$ corrected scaled data 10 PCs were extracted accounting for 98.05% of the total variance within the data set (data not shown). From the resulting PCA plot it was difficult to distinguish any clustering or separation within the data.
Fig. 3.13 – DFA plot of EMSC scaled spectra of batch 2 cultures of *P. putida* KT2440 during a 24h exposure to chlorobenzene, following extraction of 10 principal components (explaining 98.05% total explained variance) with a class structure based on biological replicates.

Coding used - A – Positive Control, B – 0.05% chlorobenzene exposed cultures, C – 0.075% chlorobenzene exposed cultures, D – 0.1% chlorobenzene exposed cultures. Numbers represent time points, 1 – 0h, 2 – 30min, 3 – 2h, 4 – 4h, 5 – 6h and 6 – 24h.

PCs 1-10 were then used by the DFA algorithm with the class structure based on the biological replicates within the data set. The resulting DFA plot can be seen in Fig. 3.13. From the DFA plot it can be seen that at the final 24h time point there is a separation of the positive control samples from the chlorobenzene exposed samples. Additionally there is an increasing time trend as marked on the biplot.

From the specific growth rates shown in Table 3.6 it can be seen that there is a great reduction in the specific growth rates between the two batches of chlorobenzene exposure. Comparison of the specific growth rates of the *P. putida* KT2440 cultures exposed to chlorobenzene with the specific growth rates of the *P.*
*Pseudomonas putida* KT2440 cultures exposed to toluene (Table 3.3), indicates that chlorobenzene has a more detrimental effect on the growth of this micro-organism than the toluene. However it should be noted that the chlorobenzene has been added at slightly higher concentrations than does toluene, therefore a direct comparison can only be made for two of the solvent concentrations.

From previous work it has been shown that the logP value of chlorobenzene is 2.86 (Lu *et al.*, 2006). As such chlorobenzene falls within the range of logP values which are highly toxic to micro-organisms. However comparison of the specific growth rates of the toluene exposed cultures and the chlorobenzene exposed cultures indicates that chlorobenzene is significantly more toxic to the *P. putida* KT2440 cultures than toluene. Although the logP values of the two solvents are similar, toluene 2.5 and chlorobenzene 2.86, there is a clear difference in the toxic effect of the two solvents. Additionally comparison of the growth curves for the two different solvents appear to show that the chlorobenzene unlike the toluene produces an extended lag time of between 6h to 24h, whilst for the toluene exposed cultures the only extended lag time is observed for the cultures exposed to the highest concentration of toluene tested.

### 3.3.5. Comparison with a third aromatic solvent: fluorobenzene

As has been discussed above micro-organisms are able to counter a sudden addition of toxic organic solvent through a number of adaption mechanisms. One of these is an increase in the proportion of phospholipids in the *trans* rather than *cis* configuration. This homeoviscous adaption has been reported in several strains of *P. putida*, including *P. putida* KT2440. This *cis/trans* change is a short term response to the addition of solvent, which does not require the synthesis of new lipid molecules. As such this response has been reported as occurring within 1 min of solvent addition (*Ramos et al.*, 2002). To examine this adaption mechanism a third related aromatic solvent, fluorobenzene was tested at a single concentration (0.025% v/v) over a solvent exposure time of just 2h.
*P. putida* KT2440 cultures were prepared as has been previously described for other solvent exposure experiments (Section 2.3.1). After incubation for 3h biomass samples were removed from the flasks and fluorobenzene was added to a concentration of 0.025% v/v to half of the culture flasks. Further biomass samples were taken at 15min and 2h after the addition of the solvent.

Biomass samples were prepared for FT-IR analysis using the method which has been previously described. The collected spectra were corrected for CO$_2$, EMSC scaled and then analysed using PCA and DFA. The resulting DFA plot following the extraction of 10 PCs accounting for 97.74% of the total variance within the data set is shown in Fig. 3.14.

![DFA plot of EMSC scaled spectra of *P. putida* KT2440 during a 2h exposure to fluorobenzene, following extraction of 10 principal components (97.74% explained variance) with a class structure based on biological replicates](image)

**Fig. 3.14** - DFA plot of EMSC scaled spectra of *P. putida* KT2440 during a 2h exposure to fluorobenzene, following extraction of 10 principal components (97.74% explained variance) with a class structure based on biological replicates

A – Positive Control, B – 0.025% fluorobenzene exposed cultures. Numbers represent time points, 1 – 0h, 2 – 15min, 3 – 2h.
An additional 2 batches of *P. putida* KT2440 cultures exposed to the same concentration of fluorobenzene and with samples collected at the same time points were tested. All three batches gave similar PC-DFA plots (data not shown). From the above plot it can be seen that there is a clear separation between the cultures exposed to fluorobenzene at the 15min time point and the positive control culture samples collected at the same time point. In contrast there is little separation between the samples collected from the two culture conditions at the 2h time point. This separation pattern may indicate that there is a greater biochemical difference between the positive control cultures and the solvent exposed cultures at the 15min time point than there is between the two culture conditions at the 2h time point.

From the collected data it is not possible to determine why there is a difference between the two culture conditions, and as discussed above FT-IR spectroscopy lacks sufficient resolution to discriminate between *cis* and *trans* isomer lipids, however, it is possible that the difference between the two culture conditions is the result of adaption mechanisms such as solvent pumps which exist to extrude solvent and other damaging substances from the micro-organism, within the culture triggered by the addition of solvent.

### 3.4. Concluding Remarks

In this chapter the response of *E. coli* MG1655 and *P. putida* KT2440 to sub-lethal concentrations of toluene has been investigated. From the data it was observed that *P. putida* KT2440 was able to respond better to the sudden addition of toluene than *E. coli* MG1655.

Examination of the loadings plots of the first principal component from the toluene exposure experiments using both *E. coli* MG1655 and *P. putida* KT2440 showed the largest degree of variance between wavenumbers 1500-2000 cm$^{-1}$. The large degree of variance recorded between these wavenumbers indicates that the largest changes in variance between samples and over the course of the 24h exposure time are associated with changes to the protein content of the test organisms. Reported
literature regarding the adaption of microorganisms to solvent exposure suggest that the majority of adaption to solvent exposure will occur within the membrane of the microorganism, this coupled with the high protein content of the cell membrane suggests that additional analysis of the cell membranes maybe beneficial in gaining a better understanding of the mechanisms of solvent adaption in microorganisms. Additional testing of the P. putida KT2440 culture with the solvent chlorobenzene revealed that although the two solvents had similar logP values, the response of the culture to exposure to the two solvents was very different. The P. putida KT2440 culture was less able to withstand the addition of chlorobenzene than toluene, with poorer specific growth rates recorded for the cultures exposed to chlorobenzene than for the cultures exposed to toluene.
4. Results – Toluene Training

4.1. Introduction

The isolation or development of bacterial strains that are able to grow in the presence of toxic organic solvents are of great interest for applications in both biocatalysis and bioremediation. A number of highly solvent tolerant bacteria have already been isolated from the environment and these include for example *Pseudomonas putida* DOT-T1E, which has been reported to grow in the presence of 90% (v/v) toluene (Ramos et al., 1995), *P. putida* IH-2000 reported to grow in the presence of 50% (v/v) toluene (Inoue et al., 1991) and *P. putida* S12 which can grow in the presence of 1% styrene (Weber et al., 1993).

Although the DOT-T1E, S12 and IH-2000 strains have been isolated due to their high levels of solvent tolerance rather than having been developed to have high levels of tolerance, it is possible to adapt the characteristics of microbial strains to unfavourable culture conditions through a number of reported methods which allow the organism to evolve to tolerate such environments. The micro-organism *E.coli* MG1655 has been studied for its adaption to different nutrient conditions, including nutrient excess and nutrient limitation using a series of batch cultures (Vijayendran et al., 2008). Alternative approaches to adapting bacteria to survive particular culture conditions have included pre-exposure of cultures to sub-lethal doses of toxic solvent (Xin et al., 2009), continuous chemostat culture of the micro-organism in the presence of toxic solvent or unfavourable growing condition (Arense et al., 2010), (Munoz et al., 2009).

This chapter details the use of repeated batch culture in the presence of a sub lethal dose of toluene to attempt to increase the tolerance of the organisms *E. coli* MG1655 and *P. putida* KT2440 to toluene. If any increase in tolerance was developed it would be determined by the measurement of optical density (OD).
readings to record growth and the analysis, by FT-IR spectroscopy, of bacterial biomass collected at the end of a 24h growth/toluene exposure experiment.

4.2. Materials and Methods

Cultures of *P. putida* KT2440 and *E.coli* MG1655 were prepared in 250mL flasks containing 50mL of sterile MSX medium, each culture was prepared from a single colony. Cultures were incubated overnight in a shaking incubator (200rpm) at the appropriate temperature (37°C for *E. coli* and 30°C for *P. putida*). Following incubation 1 mL of each of the cultures was used to inoculate 2x 15mL screw top vials, each vial contained 5mL of sterile MSX medium. To one of the screw top vials from the two cultures toluene was added to give a final toluene concentration of 0.025% (v/v), whilst the other vial received no solvent addition. This vial acted as the negative control. All four screw top vials were sealed using a Suba-Seal and incubated in a standing incubator at the appropriate temperature for 3 days.

Following 3 days of incubation vials were removed from the incubator and 0.5mL of culture was transferred to a new 15mL screw top vial containing 5mL of sterile MSX medium. Toluene (0.025% v/v) was added to the appropriate flasks before the flasks were sealed and incubated. This re-culturing cycle was repeated every 3 days for approximately 2 months.

Following 2 months of continual batch incubation the volume of toluene added to the vials was increased to give a final toluene concentration of 0.05% (v/v). The re-culturing was repeated as described above, every 3 days for a further month.

At the end of this month frozen stocks of each of the cultures were prepared from the mixed populations of adapted micro-organisms, cultures were stored at -80°C until required for testing. Prior to testing frozen stocks were sub-cultured three times from a single colony. After sub-culture a single isolated colony from each agar plate was used to inoculate an overnight culture, in a 50mL flat bottomed flask containing 25mL of sterile MSX medium. A 1mL aliquot of the mixed overnight culture was then used to inoculate 3x 250mL QuickFit flasks. Each flask contained
50mL of sterile MSX medium. Flasks were initially incubated for 3h, after 3h incubation a 100µL sample was removed from each flask, these were the 0h time points (i.e., the point immediately before any toluene exposure). Next toluene (final concentration 0.05% (v/v)) was added to three of the flasks from each of the exposure conditions (i.e., cultures which had been pre-exposed to toluene and cultures which had not been pre-exposed to toluene). All flasks were sealed with Suba-Seals and incubated, at the appropriate temperature, in a shaking incubator at 200rpm. Flasks were incubated for 24h. During the time course of the 24h incubation, 100µL samples were taken from a further three time points (1, 5 and 24h) for OD measurement.

At the 24h time point a 1.5mL sample was taken from each culture flask, 100µL were used for OD readings. The remaining 1.4mL were centrifuged at 11 500g for 2min at 4°C. The supernatant was removed and discarded. The remaining biomass sample was washed twice in 1mL of sterile distilled water before the sample was stored as a dry cell pellet at -80°C until use.

FT-IR samples were prepared and analysed as described in Section 2.3.

4.3. Results and Discussion

4.3.1. Effects on growth of pre-exposure of cultures to toluene

As reported above the two different bacteria underwent ‘training’ with sub-lethal doses of toluene that was aimed at evolving *E. coli* or *P. putida* that were more tolerant to this aromatic solvent. After this adaption phase these cultures as well as non-exposed stock cultures were challenged with 0.05% toluene (v/v), along with negative controls containing no solvent.

The OD readings for the four time points collected over the time course of the 24h flask exposure for *P. putida* KT2440 are shown in Fig. 4.1.
Fig. 4.1– OD measurements from *P. putida* KT2440 cultures taken during 24h exposure experiment.

♦ Negative control – no toluene, ■ Negative control – 0.05% (v/v) toluene, ▲ Pre-exposed cultures – no toluene, • Pre-exposed cultures – 0.05% (v/v) toluene, OD readings were measured by preparing a 1/10 dilution of the 100µL samples. Data points are the average of the OD (680nm) readings from the 3 replicate flasks, error bars represent standard deviation.

From Fig. 4.1 it is clear that the cultures which have been pre-exposed over 3 months to a sub-lethal volume of toluene grow better than the cultures which have not been pre-exposed to toluene. This is particularly clear when the final OD readings are inspected for the toluene challenge: 0.689 for the pre-exposed cultures compared to 0.095 for the cultures which were not pre-exposed to toluene.

As also detailed in the Materials and Methods above (Section 4.2) an identical protocol of toluene pre-exposure over 3 months was performed on *E. coli*. The OD readings collected over the time course of the 24h flask exposure for *E. coli* are shown in Fig. 4.2.
Fig. 4.2 – OD measurements from *E. coli* MG1655 cultures taken during 24h exposure experiment

*♦* Negative control – no toluene, ■ Negative control – 0.05% (v/v) toluene, ▲ Pre-exposed cultures – no toluene, • Pre-exposed cultures – 0.05% (v/v) toluene, OD readings were measured by preparing a 1/10 dilution of the 100μL samples, OD (680nm). Data points are the average of the OD readings from the 3 replicate flasks, error bars represent standard deviation (except for 3h time point for the pre–exposed cultures, in both culture conditions – with and without toluene, for which only 2 measurements are available).

From Fig. 4.2 it is more difficult to distinguish particular growth related patterns in the *E. coli* cultures, than it was for *P. putida* KT2440. In addition, the standard deviation error bars shown in the *E. coli* cultures are generally larger than those calculated from the OD measures from *P. putida*, even for the unexposed cells.

There are a number of possible explanations for the variations seen between the *E. coli* MG1655 cultures and the *P. putida* KT2440. The first possibility is that the *E. coli* cultures had been allowed to incubate and grow for too long before the addition of toluene. Comparison of the starting OD readings between the two cultures shows that the *E. coli* cultures had achieved a significantly higher starting OD (indicating that more biomass was present) than the *P. putida* cultures. Across the 12 flasks of *P. putida* culture the average OD at the 0h time point was recorded as 0.056, whilst for the *E. coli* culture the average OD at the 0h time point was recorded as 0.77. Thus no discussion on lag phase effects is possible.
In addition, it is also possible that by the 24h time point the *E. coli* cultures are limited, due to nutrient limitation and metabolite end product toxicity. This may in part explain the large errors associated with these flask cultures especially at the later time points.

An additional possibility that should be considered is that the *E. coli* cultures have been damaged by repeated culturing under the described conditions, possibly by nutrient limitation within the culture vessels or by the prolonged exposure to toluene. Whilst the prolonged exposure to toluene may be damaging to the cells it would not explain the large error bars associated with the negative control cells, this may suggest that for the *E. coli* cultures it is the repeated culturing under nutrient limited conditions which has damaged the cells.

However, there is also some evidence to suggest that the prolonged exposure to toluene may have had an impact on the cultures. From Fig. 4.2 it can be seen that the pre-exposed cultures had reached a slightly lower starting OD at the 0h time point than the cultures which had not been pre-exposed to sub-lethal doses of toluene. The pre-exposed cultures reached an average OD of 0.57, whilst the cultures which had not been pre-exposed to toluene reached an average starting OD of 0.97. This is in contrast to the *P. putida* cultures in which the pre-exposed cultures were seen to have a higher average starting OD of 0.09 at 0h, compared to the cells which had not been pre-exposed which had an average OD of 0.02 at the 0h time point.

Although the *E. coli* cultures had reached a higher initial OD (at 0h) than was desired, it is still possible to distinguish some features from the 0h and 24h time points. At the 24h time point it can be seen that the OD for both of the flask cultures not exposed to toluene are equivalent. For the flask cultures which have experienced toluene exposure it can be seen that the cultures which have not been previously exposed to toluene experience no growth in the presence of 0.05% (v/v) toluene. By comparison the cultures which have been previously ‘trained’ with sub-lethal concentrations of toluene have undergone some type of adaption and are
now more resistant to the presence of toluene as evidenced by their higher (although variable) final OD readings.

4.3.2. Fourier transform infra red spectroscopy of collected biomass samples

In addition to OD measurements biomass samples were collected at the 24h time point. Collected biomass samples were treated as previously described (Section 2.3).

The collected spectra were corrected for CO$_2$ by removal of peaks at 2403-2272cm$^{-1}$ and 683-656cm$^{-1}$, these removed areas were then filled using a trend and scaled using EMSC scaling to remove any unavoidable artefacts from the baseline (Martens et al., 2003). The collected CO$_2$ corrected and EMSC scaled spectra for the biomass samples collected at the 24h time point can be seen in Fig. 4.3. The large degree of noise associated with the collected spectra may possibly be due to the small amounts of biomass which were collected from some of the culture flasks e.g. *E. coli* MG1655 cells cultured in the presence of toluene.

Following the above pre-processing, 10 principal components (PCs) were extracted from the data set. The extraction of these 10 PCs accounted for 99.56% of the total variance within the data set, with PCs 1 and 2 accounting for 70.80% and 11.97% respectively. Despite the large amount of variance explained no obvious separation was visible in the biplot space when PC1 was plotted against PC2 (data not shown), and no obvious clustering was seen when lower PCs were plotted (data not shown).

Thus following PCA PC-DFA was carried out using the first 10 PCs extracted. In this instance the class structure for the DFA algorithm was based on the biological replicates i.e. the samples collected from the three flasks of the same culture condition were designated as the same class. The resultant PC-DFA plot for the first two discriminant functions (DFs) is shown in Fig. 4.4.
From Fig. 4.4 it can be seen that for the *E. coli* cultures exposed to toluene for three months, there is no difference between the negative control unexposed cultures and the pre-exposed *E. coli* cultures when challenged with toluene. However, for the *P. putida* cultures there is a slight separation of the cultures which have been pre-exposed to sub-lethal concentrations of toluene and those which have not been pre-exposed but which are grown in the presence of toluene. This result is exciting as it shows a clear indication that, for the *P. putida* cultures the FT-IR spectra are reflecting physiological differences in the culture rather than simply a detection of the presence or absence of toluene from the culture medium.

From the data which have been collected it is not possible to state definitively the nature of the changes which have occurred within the cell in order to allow cells to adapt to the presence of solvent following repeated sub culturing. It is possible that the adaption of the cells has been caused by adaption i.e. mutations or alterations to the DNA of the cells, or acclimatisation, i.e. alterations in the expression of the genes. In order to produce cells with the highest tolerance to solvent it would be necessary for the ‘training’ process described in this chapter to produce both adaption and acclimatisation effects.
Fig. 4.4 - PC-DFA plot generated from collected biomass spectra using PCs 1-10 with information about the biological replicates as the a priori class structure for the DFA algorithm.

Coding used - E♦ E. coli MG1655 Negative control – no toluene, E■ E. coli MG1655 Negative control – 0.05% (v/v) toluene, E▲ E. coli MG1655 Pre-exposed cultures – no toluene, E● E. coli MG1655 Pre-exposed cultures – 0.05% (v/v) toluene

P♦ P. putida KT2440 Negative control – no toluene, P■ P. putida KT2440 Negative control – 0.05% (v/v) toluene, P▲ P. putida KT2440 Pre-exposed cultures – no toluene, P● P. putida KT2440 Pre-exposed cultures – 0.05% (v/v) toluene

4.4. Conclusion

Culturing the microorganisms E. coli MG1655 and P. putida KT2440 for three months in the presence of sub-lethal concentrations of toluene did appear to have altered the ability of the cultures to respond to a sudden toluene shock.

On inspection of the growth data for the P. putida cultures (Fig. 4.1), it appears that on toluene challenge the cultures which have been repeatedly cultured in the
presence of sub-lethal concentrations of toluene obtain a higher final OD than the non-exposed cultures and there is evidence to suggest that these pre-exposed cultures experience a shorter lag time.

Whilst in the case of the *E. coli* cultures the growth pattern is not as easy to determine (Fig. 4.2). This may be due to flaws in the experimental design such as collecting OD measurements and FT-IR samples over a limited number of time points as well as using a concentration of solvent above the tolerance limit of the organism, rather than an accurate reflection of the organism’s ability to adapt to a sudden toluene shock. That said comparing the final time point it is clear that the toluene adapted *E. coli* cultures do reach a higher OD than those un-trained bacteria when challenged with toluene. In addition, the two un-challenged controls have equivalent final ODs.

In contrast to the *E. coli* cultures the *P. putida* physiological response, as revealed by FT-IR spectroscopy, does suggest that these cultures have altered their phenotype due to continual culturing in the presence of toluene. This response is clearly seen in the two separate clusters seen in Fig. 4.4 for the 0.05% toluene challenge test.

In conclusion the results discussed above suggest that the successive batch culture of the micro-organism *P. putida* KT2440 in sub lethal concentrations of toluene have produced cultures which have altered their biochemical make up (phenotype) in order to tolerate toluene. Further investigation of these cultures would be required to make a definite determination of the way in which these bacteria have adapted to the presence of toluene. In the future it would be interesting to explore metabolomics in order to investigate changes in the metabolic potential of these bacteria.

As a step towards this it was decided to investigate the alterations in the expression of proteins in these bacteria during solvent exposure.
5. Results – Proteomics Method Development

5.1. Introduction

In the previous chapter it has been reported that FT-IR spectroscopy was used to assess phenotypic changes in the test organisms *E. coli* MG1655 and *P. putida* KT2440 when exposed to aromatic solvents or ionic liquids. As it has been reported that exposure of microorganisms to organic solvents will both detrimentally affect the cell membrane structure and cause the up-regulation of membrane proteins to form efflux pumps it was desirable to examine the impact of solvent exposure on the proteins found in the cell membrane of test organisms (Sikkema, 1995), (Segura et al., 1999).

Proteome analysis can be carried out by the use of any one of a variety of techniques, including both chromatographic and assay based methods. Probably the most widely used technique for the separation of protein samples is gel electrophoresis, which can be either 1D or 2D.

In 2D electrophoresis, which is used in this study, proteins are first separated horizontally across a pH gradient and secondly, separated vertically through an SDS (sodium dodecyl sulphate) polyacrylamide gel. The first dimension of separation uses iso-electric focusing (IEF). The IEF technique exploits the amphoteric nature of the protein. As a protein moves through a pH gradient, under the influence of an electric field the charge on the protein molecule will vary. At a specific point in the pH gradient there will be no net charge on the protein, this point is described as the pI point. The pI point will vary from protein to protein. Once a protein has reached its pI point it will not migrate further (Hamdan, 2005). In general IEF gradients are generated within a gel matrix using non-amphoteric molecules, known as immobilines. These immobiline molecules are either weak acids or weak bases derived from acrylamide (Bjellqvist et al., 1982). Stabilizing the pH gradient within a gel matrix eliminates the problems of cathode drift and plateau effect which had limited the usefulness of older IEF techniques (Bjellqvist et al., 1982).
SDS-PAGE is used as the second dimension of sample separation in 2D gel electrophoresis. In SDS-PAGE the distance travelled by a protein through a polyacrylamide gel depends both on the size of the gel pores and the size of the protein. By running standards containing proteins of known molecular weight on the same gel as the unknown proteins, it is possible to determine the molecular weight of the unknown proteins (Hamdan, 2005).

For 1D gel electrophoresis separation occurs only on the basis of the molecular weight of the protein following separation on a polyacrylamide gel.

Although 2D electrophoresis is probably the most widely used technique for the separation of proteins it is not without its limitations. For the IEF separation in the first dimension a large number of proteins have pI points within the narrow range of pH 4-6, this large clustering of proteins within this span makes complete separation of all proteins within a complex sample difficult (Rabilloud, 2002). It has also been noted that 2D electrophoresis is biased against very large or very small proteins, as well as against very basic or very hydrophobic proteins (Patton et al., 2002). For the analyses of these often liquid chromatography is used coupled with mass spectrometry (Walther and Mann, 2010), but this is labour intensive and expensive.

5.2. Materials and Methods

5.2.1. Protein Extraction

A complete description of the materials and methods used in this section is given in the Materials and Methods chapter Section 2.2. The methods used will be briefly described here.

Protein extraction from P. putida KT2440 cultures used a number of different methods which will be discussed in detail in the text. For E. coli MG1655 cultures, the protein extraction technique used was adapted from the method reported in Molloy et al., (Molloy, 1998). The method of protein extraction is described fully in the Materials and Methods chapter, Section 2.2.1. Briefly, an overnight culture was
centrifuged at 3080g for 10min at 4°C. The supernatant was removed and discarded, the cell pellet was re-suspended in 1mL of 40mM Tris-base (pH 9.5). Samples were disrupted by sonication in an ice-water bath for 10min after which samples were centrifuged at 11 500g for 5min at 4°C. The supernatant was removed, placed into a clean micro-centrifuge tube and labelled ‘supernatant 1’.

The remaining pellet was washed twice using 1mL of 40mM Tris-base (pH 9.5), before re-suspension in 1mL of ‘Solution A’ (recipe Table 2.2). Samples were disrupted by sonication in an ice-water bath for 10min after which samples were centrifuged at 11 500g for 5min at 4°C. The supernatant was removed, placed into a clean microcentrifuge tube and labelled ‘supernatant 2’.

The remaining pellet was washed twice using 1mL of 40mM Tris-base (pH 9.5), before re-suspension in 1mL of ‘Solution B’ (recipe Table 2.2). Samples were disrupted by sonnication in an ice-water bath for 10min after which samples were centrifuged at 11 500g for 5min at 4°C. The supernatant was removed, placed into a clean microcentrifuge tube and labelled ‘supernatant 3’.

The remaining cell pellet was re-suspended in 100µL of ‘Solution C’ (recipe Table 2.2) and placed in a 95°C water bath for 5min after which samples were centrifuged at 11 500g for 5min at 4°C, after which the supernatant was collected and added to supernatant 3. An aliquot of each of the collected supernatants collected was taken and combined with an aliquot of solubilisation buffer. The supernatant were recombined vortexed and briefly centrifuged before storage at -20°C until required for use.

5.2.2. Protein Quantification

If frozen protein samples were analysed these were allowed to thaw completely, all samples were then vortexed and briefly centrifuged before use. Protein quantification was performed using a RC DC protein assay kit (Bio-Rad) and the method was performed in accordance with the manufacturers’ instructions.
5.2.3. Focusing of IPG Strips

For both 7cm and 17cm IPG strips an appropriate sample volume and protein concentration for each IPG strip length was used (7cm IPG strips a protein concentration of between 50-100µg and up to 125µL of sample, for 17cm strips 200-400µg and up to 300µL of sample). The appropriate sample volume was pipetted along the length of a lane on a clean IEF tray. The IPG strip was removed from the -20°C freezer. Using clean forceps the plastic backing was carefully removed from the strip and placed in the IEF lane in the correct orientation; i.e., the anode end of the strip was placed on the anode side of the tray. The strip was overlaid with mineral oil and the lid replaced.

The IEF tray was inserted into the PROTEAN cell, ensuring that the electrodes were correctly aligned and the pre-programmed method ‘tear2method’ was selected. The first step in the pre-programmed method is the active rehydration of the IPG strip by applying 50mV for 12h. After 12h the instrument automatically paused, the IEF tray was removed and wicks saturated with distilled water were placed between the IPG strip and the electrodes. The IPG strip was checked to ensure it was still covered with mineral oil. The IEF tray lid was replaced and the IEF tray was returned to the PROTEAN cell and the method resumed. Protein focusing occurred in 4 ramped stages; a) 300V for 1h (linear), b) 1000V for 5h (linear), c) 2500V for 6h (linear) and d) 5000V 6h (linear). After focusing the strip was held at 500V until the tray was removed from the instrument.

IPG strips which were not required for immediate use were transferred using clean forceps to a clean focusing tray and stored at -20°C until needed.

5.2.4. Equilibration of focused IPG strips

Prior to use focused IPG strips were equilibrated by washing with two separate equilibration buffers (details of buffers in Section 2.2.4). Each wash step was performed at room temperature on a shaking table for 10min.
5.2.5. Preparation of SDS gels

Plates, spacers, combs and gaskets were washed with 100% ethanol and allowed to air dry prior to use. For mini 2D protein gels (i.e. those for use with 7cm IPG strips) a separating buffer (Section 2.2.5) was prepared, for 2D protein gels (17cm IPG strips) and mini 1D protein gels a separating and a stacking buffer were prepared (Section 2.2.5).

Plates were assembled and checked for leaks using water. A separating gel was prepared (Section 2.2.5). For mini 2D protein gels, the gel mixture was poured to fill the plates to approx. 0.5cm below the level of the short plate. For mini 1D protein gels, the gel mixture was poured to a level approx. 1cm below the end of the comb. For 2D protein gels, the gel mixture was poured to fill the plates to approx. 5cm below the level of the short plate. On all gel types the gel front was covered with a layer of water saturated butanol and left to set for 1h.

After 1h the water saturated butanol was removed and the gel front washed five times using distilled water. For mini 1D protein gels and 2D protein gels a stacking gel (Section 2.2.5) was prepared and poured to approx 0.5cm below the level of the short plate. For 1D mini protein gel a well forming comb was inserted and the gel left to set for 1h. For 2D protein gels the gel front was covered with water saturated butanol as described above.

For 2D gels the equilibrated IPG strip was transferred to the top of the gel so as to be in contact with the gel front, the IPG strip was then covered with a 1% agarose gel containing 10% bromophenol blue dye and left to set for 10min. For the 1D mini protein gels, samples were mixed in a 1:1 ratio with loading buffer (Section 2.2.5) and heated in a 95°C water bath for 5min. After heating samples were stored on ice until ready to be loaded onto the gel. A 50µL of sample was loaded into the individual wells. Running buffer was prepared (Section 2.2.5) and used to fill both inner and outer tanks to the appropriate level.
Mini gels were run at 35mA for approx. 3h or until the dye front had reached the bottom of the gel. 2D protein gels were run at 200mA until the dye front reached the bottom of the gel.

5.2.6. Gel staining using colloidal Coomassie stain

After completion of the run gels were carefully removed from the plates and placed into clean gel boxes. Gels were covered with colloidal Coomassie fixing solution (Section 2.2.6) and left for 1h at room temperature on a shaking table.

After 1h incubation gels were removed from the fixing solution and given two 10min washes in distilled water before being covered with colloidal Coomassie working solution (Section 2.2.6), gels were left overnight at room temperature on a shaking table.

Following overnight incubation gels were transferred to clean gel boxes and covered with 1% acetic acid. Gels were left at room temperature on a shaking table until all of the Coomassie particles had been removed and the gel background was clear.

5.3. Results and Discussion

5.3.1. Method development for P. putida KT2440 cultures

In order to understand better the phenotypic changes made to P. putida KT2440 during exposure to solvent it was necessary to find a protein extraction method which could be used effectively with cultures of P. putida KT2440.

Initially, three methods which had been reported in the literature were selected and in some instances adapted due to the availability of equipment or reagents. These were the method of Molloy et al., (Section 5.2.1) (Molloy, 1998) which has been used for extraction of membrane proteins from cultures of E. coli (Method 1), Heim et al., (Heim et al., 2003) (Method 2) and Segura et al., (Segura et al., 2005)
Methods 2 and 3 have been reported as being used successfully for extraction of proteins from *P. putida* cultures.

Each method was used to extract two samples of *P. putida* KT2440, both of the extracted samples were used for gel analysis. All samples were loaded onto a 7cm IPG strip pH 4-7, all samples were at a concentration of 100µg. After focusing and equilibration IPG strips were run on a SDS polyacrylamide gel.

A description of the method used is given the Section 5.2.1. For Method 1 the gels (data not shown) showed a small number of resolved protein spots in the centre of each of the gels; however, both gels showed a large amount of horizontal streaking across the gel. Horizontal streaking across gels can be caused by several different factors including protein overloading, inappropriate iso-electric focusing, poor protein solubilisation or sample preparation problems such as presence of contaminants in the sample (Bio), (Rabilloud, 1996).

In Method 2 a 50mL overnight culture of *P. putida* KT2440 grown in MSX was pelleted by centrifugation at 3080g for 10min at 4°C. The supernatant was removed and discarded. The remaining pellet was re-suspended in 1mL of re-swelling solution (7.4M urea, 2M thiourea, 4% CHAPS, 30mM dithiotheritol (DTT), 20mM Tris-base and 1.28µL ampholytes (per mL)). The re-suspended sample was sonicated at full power for 10min in a bath of ice water. Following sonication samples were centrifuged at 11 500g for 5min at 4°C. The supernatant was transferred to a clean micro-centrifuge tube and stored at -20°C until required.

The resulting gels (data not shown) showed a small number of resolved protein bands, however as with the gels seen in Method 1 there was a significant amount of horizontal streaking visible across both of the gels.

In Method 3 a 50mL overnight culture of *P. putida* KT2440 grown in MSX was pelleted by centrifugation at 3080g for 10min at 4°C. The supernatant was removed and discarded. The remaining sample pellet was snap frozen in -80°C freezer for 30min or until completely frozen. A 1mL aliquot of cold 50mM Tris-HCl (pH 7.6) was added and the pellet re-suspended. The re-suspended sample was sonicated at full
power for 10min in a bath of ice water. Following sonication samples were centrifuged at 11 500g for 5min at 4°C. The process was repeated a twice more all collected supernatants were combined and the sample was diluted in a 1:1 ratio with a solubilisation solution containing 5M urea, 2M thiourea, 2% w/v CHAPS, 2% w/v SB3-10, 1%w/v DTT and 0.5g ampholytes 3-10.

From the resulting gels (data not shown) a handful of resolved protein spots were observed. Unlike Methods 1 and 2, very little horizontal streaking was observed on the gels extracted using this method.

As Method 3 had given a number of resolved protein spots and no horizontal streaking as had been observed with the gels from Methods 1 and 2, it was decided to use this method for further development.

Initially, the freeze / thaw step in which the samples were placed into the -80°C freezer was replaced with a freeze / thaw step in which the samples were snap frozen by immersion in liquid nitrogen, as described in the reported method (Segura et al., 2005). However, use of liquid nitrogen with the tris-HCl buffer in the freeze / thaw step, resulted in gels (data not shown) with more horizontal streaking than had been shown previously when samples where frozen by placing them into the -80°C freezer.

Despite this increase in horizontal streaking it was decided to continue to use liquid nitrogen in the freeze / thaw step as immersion of the samples in liquid nitrogen was a quicker and more efficient way of performing protein extraction than allowing 30min for each of the freezing steps within the method.

When considering the initial gels generated from Method 3 it was noted that there were only a handful of resolved protein spots. In an attempt to visualise more proteins it was decided to replace the two of the three Tris-HCl extractions from the original method with extractions using different solubilising solutions. Two different solubilising solutions were prepared, the first solution (N1) contained 8M urea, 4% w/v CHAPS, 1% w/v DTT, 40mM Tris (pH 9.5) and 128µL (per 10mL) ampholytes (pH 3-10). Each component of the solution (N1) was chosen due to its ability to produce
good solubilisation of extracted proteins. Urea was included in most sample buffers as it is a natural chaotrope (agent which disrupts molecular structure), it is often used in gel electrophoresis as it has a neutral charge and therefore will not migrate during iso-electric focusing (Rabilloud, 1996), (Cordwell, 2002). CHAPS is the most commonly used zwitterionic detergent, and detergents are often included in solubilisation solutions to prevent hydrophobic interactions in proteins which have been denatured due to the presence of urea (Rabilloud, 1996). DTT is used to reduce disulphide bonds between cysteine residues whilst carrier ampholytes are used to minimise charge-charge interactions (Cordwell, 2002).

For the second solubilising solution (N2) the following components were used 5M urea, 2M thiourea, 2% w/v CHAPS, 40mM Tris (pH 9.5), 1% w/v DDT and 128µL (per 10mL) ampholytes (pH 3-10). In this solution thiourea has been included as the presence of thiourea has been shown to increase the solubility of membrane proteins (Rabilloud, 1998).

Method 3 (described above) was therefore modified in the following ways: after the first wash step using 50mM Tris-HCl to generate supernatant 1, either solubilisation solution N1 or N2 was used for the following two freeze/thaw steps to generate supernatants 2 and 3. Additionally, collected supernatants were not pooled at the end of the extraction process and each supernatant was used separately, so that for each extraction with solutions N1 and N2 three gels were prepared.

For the gels run using the samples extracted with solution N1 (data not shown) the results were poor with only supernatant 1 showing any resolved protein spots, supernatant 2 showed large amounts of horizontal streaking across the gel with no resolved protein spots, whilst supernatant 3 showed little either in the way of resolved protein spots or horizontal streaking.

For the gels run with the samples extracted using solution N2 the results were better especially for supernatant 1. The gels from these samples can be seen in Fig. 5.1, Fig. 5.2 and Fig. 5.3.
From Fig. 5.1 it can be seen that there are a number of resolved protein spots however some horizontal streaking is still visible at the edges of the gel.

Fig. 5.1– Supernatant 1 from *P. putida* KT2440 samples extracted using freeze/thaw cycles with solubilisation solution N2.

Although some protein spots are visible on the gels for supernatants 2 and 3, there are fewer than are seen for supernatant 1 and on both gels there is significant horizontal streaking across both gels.
Fig. 5.2 – Supernatant 2 from *P. putida* KT2440 samples extracted using freeze/thaw cycles with solubilisation solution N2.

Fig. 5.3 – Supernatant 3 from *P. putida* KT2440 samples extracted using freeze/thaw cycles with solubilisation solution N2.
Due to the better results obtained from the use of solution N2 it was decided to investigate whether this method would give reproducible results. In order to determine this three identical overnight cultures of *P. putida* KT2440 were harvested and extracted as has been described above. In this instance it was decided to recombine the three supernatants at the end of the extraction process to give a single protein sample from each of the three replicate flasks.

The three identical extractions were used to prepare three mini 2D gels (data not shown), visual comparison of the three gels suggested that the banding pattern observed was not comparable between the three gels, thus suggesting that the method as it had been performed was not reproducible. Additionally, it also appeared that combining the three supernatants resulted in fewer protein spots than can be seen in Fig 6.1a-c, this suggests that it is necessary to run each of the supernatants on a separate gel in order to recover the maximum number of proteins from the extracted samples. By extracting proteins in a number of solubilisation steps rather than in a single extraction the number of overlapping proteins can be reduced thus aiding protein identification and providing a clearer gel (Molloy, 1998).

### 5.3.2. Summary of method development for *P. putida* KT2440 cultures

A number of different methods were tested for the extraction of protein samples from cultures of *P. putida* KT2440. Methods tested included the use of two different cell lysis techniques sonication and freeze/thaw as well as the use of a number of different solubilising solutions in which the components and the concentrations of the components have been varied.

Although some of the tested methods gave better results than others, it was not possible to settle on a method which would provide good, clear and most importantly reproducible results. Although further work on this subject would have been desirable in order to examine the proteomic response of the test culture *P. putida* KT2440 when exposed to solvent it was determined that further work on method development for this end would not make the best use of the time.
available. Therefore the focus of the proteomic work was shifted away from the investigation of *P. putida* KT2440 to look at *E. coli* MG1655 following exposure to ionic liquids.

5.3.3. Proteomic work using *E. coli* MG1655 cultures exposed to ionic liquid

To assess whether it would be possible to examine phenotypic responses in *E. coli* MG1655 samples which had been exposed to ionic liquid it was decided to use a mini 1D protein gel. A series of 250mL flasks were prepared using 50mL of MSX medium, the ionic liquids [C₂mim] [C₈OSO₃], [C₄mim] I, [C₆mim] [NTf₂] and [Ethylidimethyl[(2-quinolyl)-3,6-dioxa-1-hexyl]ammonium] [NTf₂] were added to triplicate flasks to give a final ionic liquid concentration of 1% v/v. To each flask was also added 2mL of an overnight culture of *E. coli* MG1655 was also added, three positive control flasks were also prepared, into which no ionic liquid was added. All flasks were sealed with Suba Seals and incubated at 37°C for 24h with shaking at 200rpm.

After 24h incubation cultures were harvested and samples extracted using the method described in Section 5.2.1. A mini 1D protein gel was prepared as described in Section 5.2.5. Extracted samples were diluted in a 1:1 ratio with loading buffer (Section 2.2.5), 50uL of each sample was loaded per well. The resulting gel (data not shown), showed a great deal of sample streaking and what appeared to be smearing of samples across the lanes.

As has been previously discussed the streaking of samples may be related to the presence of interferants within the sample. In this instance it was considered possible that the presence of the ionic liquids may have been causing the poor gel results as high concentration of salt within an extract can result in poor quality electrophoresis results (Rabilloud, 1996). A protein clean up kit (Ready-Prep 2D Clean-Up Kit, Bio-Rad) was purchased. Samples were re-extracted and treated with the clean-up kit in accordance with the manufacturer’s instructions.
The resulting gel (data not shown) showed a great deal of improvement, no visible streaking, lanes were clearly visible and protein bands could be clearly distinguished. As positive results had been obtained using the clean-up kit on a 1D protein gel it was decided to use include a clean-up step in all future protein extraction and to proceed to using 2D gels.

Final biomass samples were extracted from cultures exposed to the ionic liquids [N1,1,4,8][NTf2], [Ethylidimethyl[(1-naphthyl)-3,6-dioxa-1-hexyl]ammonium] [NTf2] and [Benzyldimethyl[1-{4-(1-dimethyl-2-dimethyl-propyl)phenyl}-3,6-dioxa-1-hexyl]ammonium] [NTf2], plus a positive control sample, full experimental details are given in Chapter 8.

All samples used 7cm IPG strip pH 3-10 and were loaded with 125µL of sample with a protein concentration of 100µg. The resulting gels can be seen in Fig. 5.4, Fig. 5.5, Fig. 5.6 and Fig. 5.7.

![Image](image.jpg)

Fig. 5.4 – *E. coli* MG1655 positive control culture
Fig. 5.5 – *E. coli* MG1655 culture exposed to ionic liquid \([N_{1,1,4,8}]\) [NTf₂]

Fig. 5.6 – *E. coli* MG1655 culture exposed to ionic liquid \([\text{Ethylidimethyl[(1-naphthyl)-3,6-dioxo-1-hexyl]ammonium}]\) [NTf₂]
Fig. 5.7 – E. coli MG1655 culture exposed to ionic liquid 
[Benzylidimethyl[(1-{4-(1-dimethyl-2-dimethyl-propyl)phenyl}-3,6-dioxa-1-hexyl]ammonium] [NTf₂]

The results from the gels shown if Fig. 5.4 -5.7, showed some promise, although there was still some sample streaking visible, even with the use of the cleanup kit, there was also a good number of clearly resolved protein bands visible on each of the gels.

In order to determine whether it would be possible to reproduce these results, a second set of biomass samples (taken from the 1st exposure batch, see Chapter 8), were extracted, cleaned and run using 17cm pH3-10 IPG strips with 300µL of sample per strip at a protein concentration of 300µg.

The result from these gels (data not shown) were not as good as the gels shown in Fig. 5.4-5.7. Although the positive control culture showed a good number of resolved protein spots with only a little sample streaking, the gels for the ionic liquid exposed cultures showed a great deal of horizontal streaking with only a few resolved protein spots.
5.3.4. Summary of proteomic work using \textit{E. coli} MG1655 cultures exposed to ionic liquid

Using the extraction method described it has been possible to produce gels which show a good number of resolved protein spots, whilst also having minimal sample streaking. The introduction of the clean up step into the sample preparation process appears to have greatly improved the quality of the electrophoresis results.

5.4. Conclusion

Although it was not possible to develop a good, reproducible 2D SDS PAGE method for use with the \textit{P. putida} cultures some progress was made towards achieving this end. Any future work may wish to investigate further the possibility of using a sample clean-up step and of using sequential extraction supernatants on separate gels to achieve the best results.

For the \textit{E. coli} cultures, good results were achieved using the method of Molloy et al., (Molloy, 1998), in combination with a sample clean up step. The reproducibility of this method has not been adequately demonstrated yet from the work reported here, any future work in this area may wish to assess the reproducibility of the method before continuing with its use.

Due to the constraints of time it was not possible to pursue these analyses further. However if it had proved possible to develop a robust method for protein extraction the next stage in the process would have been the identification and quantification of the isolated proteins. Protein identification is often performed by the use of protein mass fingerprinting using either MALDI MS or ESI-MS. A protein of interest would be cut from the gel and digested using an enzyme, commonly trypsin to produce peptides. These peptide mixtures are analysed using MS and the results compared against peptide databases in order to determine the identity of the unknown protein (O'Connor, 2008). Quantification is typically performed using a peptide tagging technique such as SILAC or iTRAQ, however label free techniques are becoming increasingly popular although at present they lack the sensitivity of the tagging techniques.
6. Results Ionic Liquid Toxicity Screening with *E. coli* MG1655

6.1. Introduction

A portion of the work contained in this chapter has been accepted for publication by the journal *Green Chemistry*.

The flexible nature of ionic liquids, in which the combination of a different cation and anion will generate an ionic liquid with its own unique set of physical and chemical properties, generates a huge number of potential solvents. This large number of solvents, with widely varying properties allows ionic liquids to be utilized in a diverse range of applications including both whole cell and enzyme biocatalysis (Garcia *et al.*, 2004), (Kragl *et al.*, 2002), (Pfruender *et al.*, 2004). However the identification of an ionic liquid suitable for a particular biocatalysis application, from the millions of potential structures is a difficult and time consuming task. A simple and rapid process for toxicity screening would be of significant advantage not only for the identification of ionic liquids which may be suitable for further analysis and the dismissal of those too toxic to be further considered, but would also allow for the screening of large numbers of structures, so that design rules for the synthesis of non-toxic ionic liquids can begin to be uncovered.

This chapter describes the extensive testing of one such high throughput screening method. In this method agar diffusion plates are used to give an indication of the toxicity of an ionic liquid. The agar screening method is a simple and rapid test which does not require the purchase of expensive laboratory equipment or the training of laboratory personnel in complex testing procedures and allows large numbers of ionic liquid structures to be screened quickly.

The agar diffusion test for determining the toxicity of ionic liquids is based on the well established agar diffusion test for determining antibiotic susceptibility (Vedel *et al.*, 1996). Using the micro organism *E. coli* MG1655 and the agar diffusion screening method described above 150 different ionic liquid structures have been screened.
Comparison of the agar diffusion results with specific growth rates, calculated from a high throughput microplate reader, have been made in order to establish the validity of the agar diffusion method as an initial screen for ionic liquid toxicity.

6.2. Materials and Methods

The agar diffusion testing was performed as described in the Materials and Methods Section 2.4.1. Briefly a filter disc, approximately 6mm in diameter is saturated with ionic liquid is placed onto an agar plate pre-spread with a lawn of micro-organism. The plates are incubated overnight in a static incubator at an appropriate temperature. After overnight incubation the appearance of the plate is observed and the size of any inhibition zone is recorded.

The calculation of specific growth rates from liquid culture was performed as described in the Materials and Methods Section 2.4.2. Briefly, a sample of ionic liquid was transferred into a sterile pre-weighed Eppendorf tube. The tube was re-weighed and the amount of ionic liquid in each tube calculated and recorded. To each sample an appropriate amount of distilled autoclaved water was added to produce ionic liquid at a concentration of 95% (w/v). A 4µL sample of each ionic liquid was added in triplicate to a 100 well Bioscreen plate. Ionic liquid and water samples were vortexed immediately before being added to the Bioscreen plate. An aliquot of 186µL of MSX or LB media was added to each well before the addition of a 10µL aliquot of overnight culture of the test organism. Giving a final ionic liquid concentration of 2% (v/v).

The Bioscreen plates were run in the Bioscreen C instrument for 24h at an appropriate temperature for the test organism, with intensive shaking. The OD of each well was recorded every 10min throughout the 24h run using a wideband OD filter.

With the exception of [nopylPOct$_3$][MeSO$_3$] all ionic liquids tested in this experimental work were synthesised by Queens University Ionic Liquid Laboratory (QUILL, Queens University, Belfast). The ionic liquid [nopylPOct$_3$][MeSO$_3$] was kindly provided by Dr Nazira Karodia, University of Bradford.
6.3. Results and Discussion

6.3.1. Agar Diffusion Screening

Using the micro organism *E. coli* MG1655 and the agar diffusion screening method described above 150 different ionic liquid structures have been screened. A number of these results which are supported by growth rate data will be seen in the tables contained within this chapter. A list of the major ionic liquid cation and anion structures used in the screening can be found in Appendix 1.

As with the agar diffusion test for antibiotics the agar diffusion test for ionic liquids is based on the principle that water miscible ionic liquids will diffuse through the agar to form a stable concentration gradient. However as the interactions between the ionic liquids and the ionic components are not well understood there is the possibility that interactions between the ionic liquid and components of the medium may affect either the formation of an ionic liquid concentration gradient in the agar plate or the stability of such a gradient once formed. Additionally it has been previously reported that some ionic liquids interact with cellulose (Barthel and Heinze, 2005), (Swatloski et al., 2002) suggesting that interaction between the cellulose filter paper and the ionic liquid may be a concern in the production of accurate toxicity information.

From the agar diffusion screening that has been carried out there is some evidence to suggest that such interactions between the ionic liquid and the cellulose filter paper have occurred; i.e., a brown discolouration of the filter paper disc following overnight incubation (Fig. 6.2).

Additional observations of the agar plates following overnight incubation also suggest that there is the possibility of interaction between the ionic liquid and the different growth media tested or its components; i.e., in some instances asymmetric inhibition zones have been observed suggesting a stable, uniform concentration gradient is not being formed or is not stable when formed. Additionally white precipitates have been formed around the filter paper
suggesting interaction between the growth media and the ionic liquid. Examples of these observations can be seen in Fig. 6.1 and Fig. 6.2.

Patterns of ionic liquids (i.e. a particular cation or anion class) which produce unusual results such as the formation of coloured precipitates or discolouration of the cellulose filter paper have been observed, for example, the nopylphosphonium cation nopylP$_{8,8,8}$. Each ionic liquid which contained this particular cation would produce a brown colour change of the filter paper following overnight incubation at 37°C. A number of possible explanations for this consistent colour change have been considered. Firstly the [nopylPOct$_3$] cation may react with a component of the medium to produce the brown colour change. Investigation of this theory is difficult to pursue using LB agar plates, due to the undefined nature of the LB medium. By changing the agar plate medium to MSX it was possible to alter the composition of the medium in a defined way. To investigate possible causes of the brown colour change the carbon source of the medium was altered from glucose to sodium acetate or removed completely from the medium. The agar plates for the ionic liquid [nopylP$_{8,8,8}$] [C$_2$OSO$_3$] can be seen in Fig. 6.3. All plates still show the same brown colour change associated with the initial LB agar test plates. This suggests that either the [nopylP$_{8,8,8}$] cation reacts with a component of the medium other that the carbon source, however this would require the same or a similar component to be present in both MSX and LB media, alternatively the brown colour change may not be linked to a component of the medium, but may be the result of an interaction between the [nopylP$_{8,8,8}$] cation and the cellulose filter paper.
Fig. 6.1 - Asymmetric inhibition zones in the agar diffusion test

An agar diffusion test performed using $[N_{124}][C_4OSO_3]$, the photograph shows that the inhibition zones was asymmetric, rather than forming a uniform circle around the filter paper.

Fig. 6.2 - Formation of precipitates and colour changes in the agar diffusion test

Photographs of the inhibition zones from agar diffusion tests; (a) colour change in the presence of $[C_6mim][NO_3]$, (b) white precipitate around the filter paper disc in the presence of $[C_6mim][AOT]$ (c) colour change in the presence of $[C_4mim][lactate]$. 
Photographs of agar plates and cellulose filters following exposure to nopylPOct$_3$ MeSO$_3$ with (a) LB agar (b) MSX medium (c) MSX agar in which glucose has been replaced by acetate as the sole carbon source (d) MSX medium with no carbon source

A second possibility is that the brown colour change is the result of the Maillard reaction between the cellulose of the filter paper and the amines of the reaction media and may be catalysed by the presence of the ionic liquid. Further investigation would be required to identify this reaction positively as the cause of the observed brown colour change.

In order to determine whether the size of the inhibition zone would be influenced by the choice of media used for the agar plates a small sub-set of six imidazolium
based ionic liquids were selected for testing with MSX medium agar plates. MSX medium is a defined salt solution which contains glucose as its sole carbon source. In this investigation two sets of MSX agar plates were prepared; one set of agar plates containing glucose and the second set without glucose and therefore without a carbon source. The method was performed as has been previously described (Section 6.2) and the results are shown in Table 6.1.

**Table 6.1- Results for agar diffusion tests using LB agar plates, MSX agar plates, both with and without a carbon source.**

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Average Inhibition Zone (cm) on LB Agar plates</th>
<th>Average Inhibition Zone (cm) on MSX Agar plates with Glucose</th>
<th>Average Inhibition Zone (cm) on MSX Agar plates without Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-alkyl-3-ethyl-imidazolium docusate</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>No Growth</td>
</tr>
<tr>
<td>1-alkyl-3-ethyl-imidazolium octylsulfate</td>
<td>0.2 ± 0.2</td>
<td>0 ± 0.1</td>
<td>No Growth</td>
</tr>
<tr>
<td>1-alkyl-3-ethyl-imidazolium bis(trifluoromethylsulfonyl)imide</td>
<td>0.6 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>No Growth</td>
</tr>
<tr>
<td>1-alkyl-3-butyl-imidazolium chloride</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>No Growth</td>
</tr>
<tr>
<td>1-alkyl-3-octyl-imidazolium chloride</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>No Growth</td>
</tr>
<tr>
<td>1-alkyl-3-octyl-imidazolium sacchrinate</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>No Growth</td>
</tr>
</tbody>
</table>

The average inhibition zone is the average of the three agar plates with the standard deviation between the three plates supplied.

From the results presented in Table 6.1 it can be seen that between the two different types of media the agar diffusion test is able to correctly identify highly
toxic ionic liquids 1-alkyl-3-octyl-imidazolium chloride ([C₈mim] Cl) and 1-alkyl-3-octyl-imidazolium sacchrinate ([C₈mim] [Sacch]). As well as correctly identifying the biocompatible ionic liquids 1-alkyl-3-ethyl-imidazolium docusate([C₂mim] [AOT]) and 1-alkyl-3-butyl-imidazolium chloride ([C₄mim] Cl) irrespective of the growth media used for the agar plate. However, for the ionic liquids 1-alkyl-3-ethyl-imidazolium bis(trifluoromethylsulfonyl)imide ([C₂mim] [NTf₂]) and 1-alkyl-3-ethyl-imidazolium octylsulfate ([C₂mim] [C₈OSO₃]), there is a significant difference between the results recorded between the two different types of media. Without further investigation it is not possible to determine if this difference is the result of interaction between the particular ionic liquids tested and the components of the growth media or are indicative of interactions between the specific anions being tested and the growth media. The absence of growth in the MSX plates which do not contain a carbon source suggests that the micro-organism is not able to metabolise any of the ionic liquids tested.

Although the agar diffusion method for determining antibiotic susceptibility is well established the use of this same method to test the susceptibility of microorganisms to ionic liquids has been reported in only a few instances (Rebros et al., 2009), (Saadeh et al., 2009). In order to validate the results collected from the agar toxicity test, the growth of E. coli MG1655 exposed to ionic liquid was measured over a 24h period using a Bioscreen instrument. Bioscreen plates were prepared as described in the Materials and Methods Section 2.4.

6.3.2. Bioscreen growth data

The use of Bioscreen micro-cultures to analyze the toxicity of ionic liquids was found to be problematic for the investigation of certain ionic liquid classed such as ionic liquids which were comprised on the anion docusate (AOT). An example of the type of growth curved produced by an ionic liquid containing this docusate anion is shown in Fig. 6.4.
Fig. 6.4 - Effect of ionic liquid [C₂mim] [AOT] on the optical density of *E. coli* MG1655

The cells were incubated in MSX media with (▲) and without (♦) ionic liquid [C₂mim] [AOT] (2% v/v). A negative control (●) and an uninoculated ionic liquid control (■) are also presented. Means of three replicates are shown and error bars represent standard deviation.

During preparation of the ionic liquid samples for use in the Bioscreen microcultures it was observed that ionic liquids which included the AOT anion formed a paste/gel when mixed with water. It is possible that the addition of these ionic liquids caused a variable light scattering effect within the micro-culture wells leading to the large standard deviations associated with ionic liquids containing this anion, thereby casting doubt on the reliability of the data collected.

The results of the agar diffusion testing and the Bioscreen micro-cultures were correlated in order to check both the accuracy of the agar diffusion test and to give additional information regarding the toxicity of individual ionic liquids than could be determined using either method independently.
Fig. 6.5 – Effect of ionic liquid \( [\text{C}_8\text{mim}] \text{ Br} \) on the optical density of \( \text{E. coli} \) MG1655

The cells were incubated in LB (A) or MSX (B) media with (▲) and without (♦) ionic liquid \( [\text{C}_8\text{mim}] \text{ Br} \) (1% v/v). A negative control (•) and an uninoculated ionic liquid control (■) are also presented. Means of three replicates are shown and error bars represent standard deviation.

The correlation between the two methods was good for the identification of either highly toxic or biocompatible ionic liquids. For example, the ionic liquid 1-alkyl-3-octyl-imidazolium bromide \( [\text{C}_8\text{mim}] \text{ Br} \) which was known to be highly toxic to a range of micro-organisms including \( \text{E. coli} \) (Docherty and Kulpa, 2005), (Cho et al., 2007), (Kulacki and Lamberti, 2008), produced an average inhibition zone of 0.9cm ± 0.15 whilst in the Bioscreen micro-cultures no growth of the micro-organism was observed even with the lowest concentration of ionic liquid (Fig 6.5).
This combination of large agar inhibition zones and inhibition of the growth of the micro-organism in the Bioscreen micro-cultures was observed for a number of other ionic liquids including 1-alkyl-3-hexyl-imidazolium chloride ([C₆mim] Cl), 1-alkyl-3-octyl-imidazolium chloride ([C₈mim] Cl) and 1-alkyl-3-hexyl-imidazolium bromide ([C₆mim] Br) all of these ionic liquids have been previously reported to be toxic (Docherty and Kulpa, 2005), (Luczak et al., 2010).

By contrast the ionic liquid 1-ethyl-3-methylimidazolium 3,6,9-trioxodecylsulfate ([C₂mim] [C₃(OC₂)₃OSO₃]) which has been shown to be biocompatible (Rebros et al., 2009), produced no inhibition zone on the agar diffusion test and produced good specific growth rates of 78% and 88% normalized to the control) in MSX and LB media respectively at the highest concentration of ionic liquid tested (Fig. 6.6).

The results of the agar diffusion test and the Bioscreen micro-cultures show a good correlation for ionic liquids which are either highly toxic or biocompatible with the micro-organism. However, for ionic liquids which display a lower level of toxicity towards the micro-organism it was more difficult to see a correlation between the agar diffusion results and the Bioscreen micro cultures. For ionic liquids that produce small inhibition zones with the agar diffusion test (i.e. diffusion zones between 0.1 and 0.3cm) it was problematic to rank these ionic liquids in order of toxicity. For example the ionic liquid 1-ethyl-3-methylimidazolium thiocyanate([C₂mim] [SCN]) produces an inhibition zone of 0.1cm with the agar diffusion test and specific growth rates of 65% and 79% of the control in MSX and LB media respectively. By contrast the ionic liquids trioctylmethyl phosphonium methylsulfate ([P₁₈₈₈₈] [C₁OSO₃]) and dioctylethyl methyl ammonium hexylsulfate ([N₁₂₈₈₈] [C₆OSO₃]) both give inhibition zones of 0.1cm with the agar diffusion test, but both of these ionic liquids are completely inhibitory of cell growth in the Bioscreen micro-cultures.
**Fig. 6.6** - Effect of ionic liquid 1-Ethyl-3-methylimidazolium 3,6,9-trioxodecylsulfate ([C₂mim] [C₃(OC₂)₃OSO₃]) on the optical density of *E. coli* MG1655

The cells were incubated in LB (A) or MSX (B) media with (▲) and without (♦) ionic liquid 1-Ethyl-3-methylimidazolium 3,6,9-trioxodecylsulfate ([C₂mim] [C₃(OC₂)₃OSO₃]) (2% v/v). A negative control (•) and an uninoculated ionic liquid control (■) are also presented. Means of three replicates are shown and error bars represent standard deviation.

6.3.1.1. Halides

The first set of ionic liquids to be tested using the agar screening method and the micro-culture plates where the imidazolim halide family. As a large amount of literature exists regarding the toxicity and behaviour of this class of ionic liquids
these samples were used to benchmark the behaviour of *E. coli* against previous studies with other living organisms (Table 6.2). Both \([C_2\text{mim}]^+\) and \([C_4\text{mim}]^+\) chlorides and bromides appeared to be biocompatible based on the results obtained from the agar diffusion test, supported by the generation of reasonable specific growth rates for these ionic liquids (excepting \([C_2\text{mim}]\text{Cl}\) for which microculture growth data was not available) in micro-culture. Increasing the length of the alkyl chain to six or eight carbons increased the radii of the associated diffusion circle, indicating an increase in toxicity.

The increased toxicity of longer chained cations appears to be a function of the lipophillic co-efficient (logP value). The logP value of an ionic liquid increases with increasing alkyl chain length. Ionic liquids with larger logP values are able to accumulate within a cell’s membrane to a higher concentration than ionic liquids with lower logP values, thereby increasing the permeability of the cell membrane (Pernak *et al.*, 2004), (Pernak *et al.*, 2003).

The variation between the specific growth rates obtained from the two types of growth media used in the micro-culture plates, most apparent for \([C_4\text{mim}]^+\) cation when paired with all three of the halide anions, suggests that either the \([C_4\text{mim}]^+\) cation is highly reactive with the growth media or that the choice of growth media alters the susceptibility of the micro-organism to the ionic liquid.

Despite the variations between the two types of growth media the observed trend of increasing alkyl chain length producing larger inhibition zones is what would be expected based on previously reported studies which have used a range of test microorganisms (Wells and Coombe, 2006), (Ranke *et al.*, 2007c), (Cho *et al.*, 2007), (Cho *et al.*, 2008), (Couling *et al.*, 2006), (Docherty *et al.*, 2007). This provided an excellent indication not only that the agar diffusion test could identify biocompatible and toxic ionic liquids but also that limited information regarding toxicity series could be interpreted from the collected data.
Table 6.2 - Effect of imidazolium halides on growth of *E. coli*.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition Zone (cm)</th>
<th>μ (%) MSX</th>
<th>μ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C2mim] Cl</td>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C4mim] Cl</td>
<td>0</td>
<td>97 ± 1</td>
<td>45 ± 1</td>
</tr>
<tr>
<td>[C6mim] Cl</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C8mim] Cl</td>
<td>0.8 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C10mim]Cl</td>
<td>1.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C2mim]Br</td>
<td>0</td>
<td>90 ± 2</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>[C4mim]Br</td>
<td>0</td>
<td>56 ± 6</td>
<td>77 ± 5</td>
</tr>
<tr>
<td>[C6mim]Br</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C8mim]Br</td>
<td>0.9 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C4mim] I</td>
<td>0.37 ± 0.1</td>
<td>0</td>
<td>60 ± 16</td>
</tr>
<tr>
<td>[C8mim] I</td>
<td>1.0 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C8mim] I</td>
<td>n.d.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. – data not available. Data are the means of 3 replicates and the standard deviations are shown. All of the ionic liquids were water miscible.

A range of other halide salts was also tested, in which the imidazolium cation was replaced with pyrrolidinium, piperidinium, tetraalkylammonium and tetraalkylphosphonium cations (Table 6.3). For these ionic liquids the trend of increasing alkyl chain length with increasing toxicity is the same as was observed with the imidazolium cations. Similarly (Salminen *et al.*, 2007), there was a reasonable correlation between the agar diffusion results for the imidazolium and the corresponding pyrrolidinium and piperidinium ionic liquid with the same alkyl chain length.

In earlier studies (Couling *et al.*, 2006), (Wells and Coombe, 2006), (Cooper, 1988), increasing the alkyl chain length of tetra-alkyl ammonium cations produced increasingly toxic ionic liquids. The data collected from this screening process also follows this pattern, for example [N1,1,2,4]Br produced less than 12% inhibition of the
growth rate whereas the halides of \([N_{1,1,4,8}]^+\) and \([N_{1,8,8,8}]^+\) were completely toxic with specific growth rates of 0 in both types of growth media.

The substitution of one or more of the tetra alkyl ammonium alkyl chains for an alcohol group considerably decreased the ionic liquids toxicity. However, the ethanolamine and propanolamine salts contained shorter alkyl substituents and it would be desirable to screen both hexylamine and octylamine salts in order to get a clearer impression of the effect on toxicity of the substitution of an alcohol group. Introduction of the hydroxyl group into quaternary ammonium cations also decreases inhibition of the enzyme, acetylcholinesterase, by an order of magnitude (Arning et al., 2008).

In general, the initial data provide a good correlation with already published literature, thus providing a good indication that the agar diffusion method can be used to screen a range of structurally diverse ionic liquids. However, discrepancies were observed between the agar diffusion tests and the growth rate studies as well as between the two types of growth media used in the micro-culture plates.
Table 6.3 - Effect of pyrrolidinium, piperidinium and quaternary ammonium halides on growth of E. coli.

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₄mpyr] Cl S,M</td>
<td>0.08 ± 0.03</td>
<td>91 ± 7</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>[C₄mpyr] Br S,M</td>
<td>0</td>
<td>0</td>
<td>53 ± 1</td>
</tr>
<tr>
<td>[C₆mpyr] Br S,M</td>
<td>0.16 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₆mpip] Br S,M</td>
<td>0.05</td>
<td>83 ± 5.69</td>
<td>3.35</td>
</tr>
<tr>
<td>[C₆mpip] Br S,M</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₆mpip] Br S,M</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,1,2,4] Br S,M</td>
<td>0.13 ± 0.06</td>
<td>88 ± 4</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>[N₁,1,4,8] Br S,M</td>
<td>0.9 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,1,4,8] I S,N</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,8,8,8] Cl L,N</td>
<td>0.3 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,8,8,8] Br L,N</td>
<td>0.3 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,1,4,3OH] Cl S,M</td>
<td>0</td>
<td>110 ± 1</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>[N₂,4,2OH,2OH] Br L,M</td>
<td>0.1 ± 0.1</td>
<td>108 ± 8</td>
<td>122 ± 3</td>
</tr>
<tr>
<td>[N₁,1,2,3OH] Br S,M</td>
<td>0</td>
<td>104 ± 2</td>
<td>96 ± 1</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. – data not available. Data are the means of 3 replicates and the standard deviations are shown. * Solid, † Liquid, M – Water miscible, N – water immiscible

3 – Brown discolouration of filter disc following overnight incubation,* - only 2 replicates available

The acquired data suggest a number of apparently biocompatible structures suitable for further in depth toxicological investigation such as EC₅₀, LC₅₀ or viable count studies. In general shorter chained cations were preferred with hydroxyl containing tetra-alkyl ammonium structures appearing to be biocompatible. With regard to the anion, both bromide and chloride anions produced reasonably biocompatible ionic liquids when combined with short chain cations, whereas the iodides were invariably toxic.
6.3.1.2. Nitrates and Dicyanamides

A range of imidazolium and quaternary ammonium nitrates were tested for toxicity, but none allowed growth of *E. coli* (Table 6.4). Even in combination with the \([\text{C}_2\text{mim}]^+\) cation, the nitrate anion completely inhibited the growth of *E. coli*. It should be noted that some of the nitrates caused black discoloration of the filters. The black discolouration of the filter paper possibly suggested silver contamination in the ionic liquid.

In contrast to the nitrates, dicyanamide and thiocyanate salts were relatively well tolerated by *E. coli* MG1655, although as only short chained cations e.g. \([\text{C}_4\text{mim}]^+\) and \([\text{C}_4\text{mpyrr}]^+\) were available for screening, a larger collection of dicyanamide and thiocyanate salts, including long chained cations such as \([\text{C}_6\text{mim}]^+\) and \([\text{C}_8\text{mim}]^+\), should be screened to give a better understanding of the toxicity profile of this set of ionic liquids.

Although the dicyanamide salts produced small diffusion zones using the agar diffusion test, the associated errors with these tests were relatively large. A possible explanation is the observation during the preparation of the filter paper discs that dicyanamide salts were not readily absorbed by the filter discs, resulting in variations between the amount of ionic liquid on each of the replicate agar plates. This may also explain why there were some discrepancies between the results of the agar diffusion tests and the growth rates studies; for example, \([\text{N}_{2,4,20H,20H}[\text{N(CN)}_2]\) produced a large inhibition zone but had very little effect on the growth rate (Table 6.4).
### Table 6.4 – Effect of nitrate, thiocyanate and dicyanamide salts on growth of *E. coli*.

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Inhibition Zone (cm)</th>
<th>μ (%) MSX</th>
<th>μ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{C}_2\text{mim}] \text{[NO}_3\text{]}^{\text{S,M}})</td>
<td>n.d.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([\text{C}_4\text{mim}] \text{[NO}_3\text{]}^{\text{L,M}})</td>
<td>0.9 ± 0.2(^1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([\text{C}_6\text{mim}] \text{[NO}_3\text{]}^{\text{L,M}})</td>
<td>0.8 ± 0.1(^1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([\text{C}_4\text{mpip}] \text{[NO}_3\text{]}^{\text{S,M}})</td>
<td>0.6 ± 0.1(^2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([\text{N}_{1,1,4,8}] \text{[NO}_3\text{]}^{\text{L,M}})</td>
<td>0.8 ± 0.1(^1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([\text{C}_2\text{mim}] \text{[SCN]}^{\text{L,M}})</td>
<td>0.1</td>
<td>65 ± 0.41</td>
<td>79 ± 2</td>
</tr>
<tr>
<td>([\text{C}_4\text{mim}] \text{[N(CN)}_2\text{]}^{\text{L,M}})</td>
<td>0.03 ± 0.06</td>
<td>0</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>([\text{C}_4\text{mpyr}] \text{[N(CN)}_2\text{]}^{\text{L,M}})</td>
<td>0</td>
<td>45 ± 4</td>
<td>82 ± 1</td>
</tr>
<tr>
<td>([\text{N}_{1,1,4,2OH}] \text{[N(CN)}_2\text{]}^{\text{L,M}})</td>
<td>0.07 ± 0.06</td>
<td>92 ± 1</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>([\text{N}_{2,4,2OH,2OH}] \text{[N(CN)}_2\text{]}^{\text{L,M}})</td>
<td>0.2 ± 0.1</td>
<td>90 ± 3</td>
<td>80 ± 1</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. \(^5\) – Solid, \(^1\) – Liquid, \(^M\) – Water miscible, \(^N\) – water immiscible. \(^1\) – Black discoloration of filter disc after incubation overnight. \(^2\) – Halo effect

#### 6.3.1.3. Saccharinates

Saccharin-based ionic liquids have been suggested as a good starting point to produce non-toxic ionic liquids, as saccharin has already been approved for human consumption and is often used as a non-nutritive sweetener (Carter *et al.*, 2004). Therefore, we tested a range of imidazolium and quaternary ammonium saccharinates for toxicity. Unfortunately, all of the saccharin based ionic liquids completely inhibited the growth of *E. coli* except for \([\text{C}_4\text{-3mpy}][\text{Sacch}]\) (Table 6.5). However all of the saccharin based ionic liquids tested comprised of cations with long alkyl chains of six or eight carbons, these cations had previously been shown to be toxic when combined with either halide or nitrate anions. Shorter chained cations were not available at the time of screening however for published literature
it has been reported that the toxicity of choline saccharinate to *Daphnia magna* is reported to be two orders of magnitude lower than the corresponding imidazolium or pyridinium salts (Nockemann *et al.*, 2007) making further investigation of this anion class worth pursuing.

An additional point of note is the apparent alteration of pH produced by the saccharinate containing ionic liquids. This is the only class of ionic liquid screened which appeared to significantly alter the pH of the growth media i.e caused an alteration of more than 0.5 pH units.

6.3.1.4. Alkanoates and lactates

The possibility of producing ionic liquids from naturally occurring anions (*e.g.* alkanoates, lactate) (Cybulski *et al.*, 2008) and cations (*e.g.* choline, [N_1,1,12OH] (Nockemann *et al.*, 2007)), is of great interest since the component ions are biodegradable and relatively non-toxic. Initial tests showed that the imidazolium and pyrrolidinium lactates tested were toxic (Table 6.5). However the halo effect produced during the agar diffusion testing suggested the possibility of an interaction between the ionic liquid and either the growth media or the filter paper. Later in the screening process a [C_4 mim][Lactate] which had been synthesised using a different method become available. This ionic liquid was found to be less toxic than the [C_4 mim][Lactate] originally tested. As the original [C_4 mim][Lactate] had been synthesised from a silver salt it is possible that the toxicity observed was the result of silver contamination rather than the inherent toxicity of the ionic liquid.

A series of choline based alkanoates were also screened and gave good specific growth rates for the shorter chained cations. Again the trend of increasing alkyl chain length with decreasing growth rates (increased toxicity) was observed.
Table 6.5 – Effect of Saccharinate and alkanoic acid salts on growth of *E. coli*

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₆mim][Sacch] S,M</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₆mim][Sacch] L,M</td>
<td>0.7 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₁,₄,₈][Sacch] L,N</td>
<td>0.8 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₈,₈,₈][Sacch] L,N</td>
<td>0.07 ± 0.06</td>
<td>0</td>
<td>n.d</td>
</tr>
<tr>
<td>[C₄mim][C₁COO] L,M</td>
<td>0.06 ± 0.05</td>
<td>27 ± 6</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>[C₈mim][C₁COO] S,M</td>
<td>0.9 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₁,₁,₂OH][C₁COO] L,M</td>
<td>0</td>
<td>93 ± 1</td>
<td>92 ± 1</td>
</tr>
<tr>
<td>[N₁,₁,₁,₂OH][C₂COO] S,M</td>
<td>0</td>
<td>77 ± 7</td>
<td>64 ± 3</td>
</tr>
<tr>
<td>[N₁,₁,₁,₂OH][C₃COO] S,M</td>
<td>0</td>
<td>50 ± 3*</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>[C₄mim][Lactate] L,M</td>
<td>0.33 ± 0.06¹</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mim][Lactate] L,M</td>
<td>0.33 ± 0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mim][Lactate] L,M,₂</td>
<td>0</td>
<td>67 ± 14</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>[C₈mim][Lactate] L,M</td>
<td>0.1 ± 0.1</td>
<td>n.d</td>
<td>n.d</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. ⁵ – Solid, ¹ – Liquid, ² – Water miscible, ³ – water immiscible. ¹ – Halo Effect, ² - silver-free synthesis * - only 2 reps used

6.3.1.5. Sulfates and dimethylphosphates

In a previous study of ionic liquid toxicity towards the anaerobe, *Clostridium butyricum*, alkyl sulfate salts were identified as non-toxic salts (Rebros et al., 2009). In this screening process the toxicity of a much wider range of aryl and alkyl sulfates towards *E. coli* (Table 6.6). For this class of ionic liquids there were discrepancies between the results of the agar diffusion tests and the growth rate measurements.

The inhibition zones observed in the agar diffusion tests had large standard deviations. There is no clear explanation for these large errors, only a single ionic liquid in this class produced a precipitate which may be indicative of ionic liquid interaction with either media or filter paper, no observation was recorded regarding the absorption of the ionic liquid onto the filter paper, as was noted for the dicyanamide ionic liquids. Furthermore, there was a poor correlation between the growth rates observed in MSX and LB medium for ionic liquids containing cation
with either hydroxyl groups or ether linkages. As the large standard deviations in the agar diffusion test can not be accounted for discussion of ionic liquid toxicity for this set of ionic liquids will be based primarily on the growth rate data, rather than the agar diffusion data.

As might be expected \([\text{C}_2\text{mim}][\text{C}_1\text{OSO}_3]\), \([\text{C}_2\text{mim}][\text{C}_2\text{OSO}_3]\) and \([\text{C}_2\text{mim}][\text{tosylate}]\) were relatively non-toxic for \(E. \text{coli}\). However, increasing the alkyl sulfate chain length to eight carbons in length did produce inhibition as reflected the specific growth rates in which inhibition of 31% and 23% was observed in MSX and LB, respectively, as compared to the ethyl sulfate anion, suggesting that the length of the alkyl chain associated with the anion may also influence the toxicity of the ionic liquid. As has been previously discussed introducing ether links into the alkyl chain produced little effect on the specific growth rate of \(E. \text{coli}\).

Extension of the methyl group in the cation to ethyl (\([\text{C}_4\text{eim}][\text{C}_2\text{OSO}_3]\)) made the ionic liquid toxic. Unfortunately this was the only example of this particular cation structure available for screening. The radical increase in toxicity which appeared to be associated with this alteration in cation structure would be of interest in further study. The introduction of branching in the anion appeared to have little effect on the overall toxicity of the ionic liquid.

From all of the classes of ionic liquids screened those containing the sulfate anion appeared to offer the most scope for the identification of biocompatible ionic liquids. Although further detailed toxicological study would be required, the range of diverse structures of both cation and anion possible within this class suggest the possibility that biocompatible ionic liquids with a range of both physical and chemical properties could be identified.

Additionally two dimethylphosphate ionic liquids were screened, \([\text{C}_4\text{mpyrr}][\text{DMPO}_4]\) and \([\text{N}_{1,1,1,4}][\text{DMPO}_4]\). On average,\([\text{C}_4\text{mpyrr}][\text{DMPO}_4]\) produced a small inhibition zone in the agar diffusion test, but the variation between replicates was very large. However, the growth rate measurements in LB and MSX medium showed that the ionic liquid was relatively non-inhibitory for \(E. \text{coli}\) and \([\text{N}_{1,1,1,4}]\)
[DMPO$_4$] was non-inhibitory. Therefore, dimethylphosphates also appear to be potentially promising as non-toxic ionic liquids.

**Table 6.6** – Effect of sulphate anions on growth of *E. coli*

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C$_2$im] [C$_1$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C$_2$im] [C$_2$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>93 ± 3</td>
<td>147 ± 8</td>
</tr>
<tr>
<td>[C$_2$im] [C$_8$OSO$<em>3$]$</em>{LM}$</td>
<td>0.17 ± 0.15$^1$</td>
<td>68 ± 6</td>
<td>77 ± 16</td>
</tr>
<tr>
<td>[C$_2$im][C$_1$(OC$_2$)$_3$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>78 ± 1</td>
<td>88 ± 5</td>
</tr>
<tr>
<td>[C$_2$im] [Tosylate]$^{S,M}$</td>
<td>0</td>
<td>101 ± 8</td>
<td>111 ± 7</td>
</tr>
<tr>
<td>[C$_4$im] [C$_1$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>82 ± 2</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>[C$_4$im] [C$_2$OSO$<em>3$]$</em>{LM}$</td>
<td>0.06 ± 0.05</td>
<td>74 ± 2</td>
<td>69 ± 2</td>
</tr>
<tr>
<td>[C$_4$im] [(C$_1$C$_2$OSO)$<em>3$]$</em>{LN}$</td>
<td>0.1</td>
<td>73 ± 1</td>
<td>76 ± 1</td>
</tr>
<tr>
<td>[C$_4$im] [C$_3$OSO$<em>3$]$</em>{LM}$</td>
<td>0.07 ± 0.06</td>
<td>78 ± 3</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>[C$_4$im] [C$_2$OC$_2$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>65 ± 1</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>[C$_4$im] [(C$_1$C$_3$OSO)$<em>3$]$</em>{SN}$</td>
<td>0.1 ± 0.1</td>
<td>71 ± 6</td>
<td>47 ± 1</td>
</tr>
<tr>
<td>[C$_4$im] [C$_2$OC$_2$OSO$<em>3$]$</em>{LM}$</td>
<td>0.06 ± 0.06</td>
<td>82 ± 2</td>
<td>71 ± 1</td>
</tr>
<tr>
<td>[C$_4$eim] [C$_2$OSO$<em>3$]$</em>{LN}$</td>
<td>0.03 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C$_4$mypyr] [C$_1$OSO$<em>3$]$</em>{SM}$</td>
<td>0.02 ± 0.02</td>
<td>85 ± 9</td>
<td>91 ± 7</td>
</tr>
<tr>
<td>[C$_6$mip] [C$_1$OSO$<em>3$]$</em>{SM}$</td>
<td>0.5 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N$_{1,1,2,4}$] [C$_2$OSO$<em>3$]$</em>{SM}$</td>
<td>0</td>
<td>80 ± 9</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>[N$_{1,1,2,4}$] [C$_4$OSO$<em>3$]$</em>{SM}$</td>
<td>0.2 ± 0.1</td>
<td>89 ± 2</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>[N$_{1,1,8,8}$] [C$_1$OSO$<em>3$]$</em>{SM}$</td>
<td>0.8 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N$_{1,1,2,8}$] [C$_2$OSO$<em>3$]$</em>{SM}$</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N$_{1,2,8,8}$] [C$_2$OSO$<em>3$]$</em>{SM}$</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N$_{1,2,8,8}$] [C$_3$OSO$<em>3$]$</em>{SM}$</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N$<em>{1,2,8,8}$] [Tosylate]$</em>{SN}$</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N$_{1,1,2,2OH}$] [C$_2$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>94 ± 6</td>
<td>139 ± 11</td>
</tr>
<tr>
<td>[N$_{2,4,(2OH)2}$] [C$_2$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>85 ± 4</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>[N$_{1,(2OH)3}$] [C$_1$OSO$<em>3$]$</em>{LM}$</td>
<td>0</td>
<td>106 ± 1</td>
<td>84 ± 1</td>
</tr>
<tr>
<td>[P$_{1,8,8,8}$] [C$_1$OSO$<em>3$]$</em>{LM}$</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C$_4$mypyr] [(C$_1$O)$_2$PO$<em>2$]$</em>{LM}$</td>
<td>0.03 ± 0.03</td>
<td>97 ± 5</td>
<td>89 ± 1</td>
</tr>
<tr>
<td>[N$_{1,1,1,4}$] [(C$_1$O)$_2$PO$<em>2$]$</em>{LM}$</td>
<td>0</td>
<td>102 ± 1</td>
<td>111 ± 1</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. – data not available. Data are the means of 3 replicates and the standard deviations are shown. $^S$ – Solid, $^L$ – Liquid, $^M$ – Water miscible, $^N$ – water immiscible, $^1$ – white precipitate formed around filter paper following overnight incubation.
6.3.2.6. Water Immiscible Ionic Liquids

The agar diffusion test was originally developed to test the toxicity of water miscible ionic liquids (Rebros *et al.*, 2009). However, it was desirable to examine the potential to use the test for assaying toxicity of water-immiscible salts. In this study, we avoided the use of BF$_4^-$ and PF$_6^-$ salts due to the formation of hydrofluoric acid when these ionic liquids are exposed to water. Instead, we focussed on ionic liquids containing the bis(trifluoromethylsulfonyl)imide (NTf$_2$) anion, since they are extremely insoluble in water and have low viscosities. Also screened were a range of docusate (AOT) salts and bis(2,4,4-trimethylpenty1)phosphinate (DIOPN) salts (Table 6.7).

For hydrophobic ionic liquids, it was not anticipated that the agar diffusion test would work in the same way as for the water miscible ionic liquids; i.e. the water immiscible ionic liquids would not dissolve into the aqueous component of the media to form a concentration gradient through the plate.

Also of concern was the possibility of interaction between the ionic liquid and either the cellulose filter paper or the components of the growth media. Indeed the ionic liquids containing the (AOT) anion do seem to react either with the filter paper or with a component of the growth media as evidenced by the formation of a white precipitate around the filter paper ring, following overnight incubation. Comparison of the agar diffusion data with specific growth rates obtained from micro-culture appears to show that the presence of water-immiscible solvents does not usually affect the accuracy of growth rate measurements to a significant extent (Cornmell *et al.*, 2008a), (Pfruender *et al.*, 2006), (Pfruender *et al.*, 2004). However, care should be taken when examining the data collected for the AOT salts due to the possibility of interaction between the salt and a component of the agar test kit and the difficulties associated with obtaining reliable specific growth rates due to the light scattering effect of the salt when combined with water, as has been previously discussed previously (Fig. 6.4).
Table 6.7 - Effect of bis-triflamide salts on growth of *E. coli*

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Inhibition Zone (cm)</th>
<th>μ (%) MSX</th>
<th>μ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C&lt;sub&gt;2&lt;/sub&gt;mim] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.63 ± 0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.77 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C&lt;sub&gt;6&lt;/sub&gt;mim] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C&lt;sub&gt;8&lt;/sub&gt;mim] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.4 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mpyrr] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C&lt;sub&gt;3&lt;/sub&gt;mpip] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1,1,4,8&lt;/sub&gt;] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.4 ± 0.1</td>
<td>28 ± 3</td>
<td></td>
</tr>
<tr>
<td>[N&lt;sub&gt;1,1,4,10&lt;/sub&gt;] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.07 ± 0.06</td>
<td>68 ± 7</td>
<td>89 ± 20</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1,8,8,8&lt;/sub&gt;] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0</td>
<td>98 ± 2</td>
<td>65 ± 1</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1,12,2OH&lt;/sub&gt;] [NTf&lt;sub&gt;2&lt;/sub&gt;]</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. Data are the means of 3 replicates and the standard deviations are shown. All of the ionic liquids were liquid at room temperature and were water immiscible.

For the [NTf<sub>2</sub>] salts screened good correlation was observed between the agar diffusion inhibition zones and the specific growth rates with large inhibition zones combined with the complete inhibition of growth in liquid. These findings agree with earlier studies in which [C<sub>2</sub>mim] – [C<sub>6</sub>mim][NTf<sub>2</sub>] salts inhibit growth by affecting membrane integrity (Cornmell *et al.*, 2008a), (Weuster-Botz, 2007). This suggests that these water-immiscible imidazolium salts are more toxic than the water-miscible equivalents, since [C<sub>2</sub>mim] and [C<sub>4</sub>mim] halides and sulfates were relatively non-toxic.

Also tested was a number of DIOPN salts: [C<sub>4</sub>-mpy][DIOPN], [N<sub>1,1,4,8</sub>][DIOPN] and [N<sub>1,8,8,8</sub>][DIOPN]. All produced large inhibition zones and all inhibited growth in both MSX and LB (Table 6.8). Therefore, it may be concluded that they are toxic.
Table 6.8 - Effect of docusate, DIOPN and linoleate salts on growth of E. coli

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%MSX)</th>
<th>µ (%LB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₂mim] [AOT]⁻ LN</td>
<td>0</td>
<td>57 ± 14</td>
<td>84 ± 35</td>
</tr>
<tr>
<td>[C₄mim] [AOT]⁻ LN</td>
<td>0¹</td>
<td>88 ± 8</td>
<td>53 ± 38</td>
</tr>
<tr>
<td>[C₆mim] [AOT]⁻ LN</td>
<td>0¹</td>
<td>57 ± 6*</td>
<td>198 ± 76</td>
</tr>
<tr>
<td>[C₄mpyr] [AOT]⁻ S,N</td>
<td>0</td>
<td>62 ± 6</td>
<td>66 ± 8</td>
</tr>
<tr>
<td>[P₆,6,6,14] [AOT]⁻ LN</td>
<td>0</td>
<td>97 ± 2</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>[P₈,8,8,14] [AOT]⁻ LN</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,1,4,8][DIOPN]⁻ LN</td>
<td>0.4 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,8,8,8][DIOPN]⁻ LN</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,1,4,8][Linoleate]⁻ S,N</td>
<td>0.4 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. * n.d. – data not available. Data are the means of 3 replicates and the standard deviations are shown. All of the ionic liquids were liquid at room temperature and were water immiscible.

¹ – white precipitate formed around filter paper following overnight incubation, * - only two replicates available

Although the agar diffusion test appeared to be a reliable method to screen hydrophobic ionic liquids for toxicity, it was difficult to obtain quantitative measurements of toxicity for water-immiscible ionic liquids with unusual phase behaviour i.e. the docusate (AOT) or linoleate anions which formed a gel/paste when added to water. The formation of a paste/gel possibly causing interference, due to light scattering in the micro-culture plates and thereby decreasing the accuracy of the growth rate measurements.

6.4. Conclusion

The agar diffusion test that has been developed provides a quick simple and cost effective method of screening a large number of ionic liquid structures. The test needs only standard laboratory equipment and can be carried out using only a few micro litres of sample. As such it is an effective high throughput screening system.
The agar diffusion test is in no way intended to replace detailed toxicological assessment of any of the ionic liquids tested and care should be taken when assessing information from ionic liquid plates on which precipitate formation, filter paper discolouration or large standard deviations between replicate plates are observed as this may indicate chemical interactions which could potentially affect the reliability of the generated data. However, if the limitations of the method are appreciated, the results obtained show it is possible to identify biocompatible or highly toxic salts quickly, thus allowing suitable ionic liquids to be easily identified as suitable for further study or to be discarded as inappropriate for a particular application.
7. Results - Ionic Liquid Toxicity Screening with *P. putida*

7.1. Introduction

In this set of experiments the effects of ionic liquid exposure to two different strains of *Pseudomonas putida* were investigated. The two *P. putida* strains were *P. putida* KT2440 and *P. putida* DOT-T1E. *P. putida* DOT-T1E was isolated from wastewater treatment plant and has been shown to tolerate high levels of organic solvent, being able to grow in the presence of 90% (vol/vol) toluene (Ramos *et al.*, 1995). By contrast *P. putida* KT2440 has been shown to be sensitive to the presence of organic solvents (Segura *et al.*, 2003).

By screening a number of different ionic liquids against both strains of *P. putida* it was hoped to determine whether having high intrinsic resistance to organic solvent would improve the survival and specific growth rates of the microorganisms following exposure to ionic liquid. A review of the available literature has suggested that there are no other reports of these particular highly solvent resistant microorganisms being screened against ionic liquids.

To investigate the response of the test organism *P. putida* DOT-T1E to exposure to a range of ionic liquids further two efflux pump knockout mutants of *P. putida* DOT-T1E were screened. The first of these strains *P. putida* DOT-T1E 18 is a toluene sensitive mutant of the wild type strain in which a *pho A* gene has been inserted into a gene coding for the efflux pump protein *ttgB*. The efflux pump *ttg* ABC has been reported to be involved in not only the extrusion of the aromatic hydrocarbon toluene, but also in the extrusion of the antibiotics chloramphenicol, tetracycline and ampicillin (Ramos, 1998).

The second strain to be investigated was *P. putida* DOT-T1E PS28, a toluene sensitive mutant of the wild type DOT-T1E strain in which a Streptomycin resistance cassette was inserted into the gene coding for the efflux pump protein (*ttgH*). The efflux pump *ttg* GHI has been reported to have importance in the extrusion of toluene from the cell; however, it does not appear to have involvement in the
extrusion of other hydrocarbons such as ethylbenzene, propylbenzene and heptane (Rojas et al., 2001).

Toxicity of ionic liquids towards the test organisms was assessed by the use of two independent, high throughput screening assays. The screening assays used were the agar diffusion test and specific growth rates calculated as a percentage of a positive control culture grown in the absence of ionic liquid. Growth rates were calculated from optical density (OD) readings collected from micro-cultures grown in a Bioscreen C instrument over a 24h time period.

7.2. Materials and Methods

Full details of the materials and methods used are given in Section 2.4. Briefly, for the agar screening process a series of 6mm x 6mm filter paper discs were produced using a standard office hole punch. The collected discs were autoclaved before a single disc was transferred aseptically into a sterile microcentrifuge tube. The microcentrifuge tubes were weighed before a 5µL sample of ionic liquid was added aseptically to the filter paper. The tubes were then re-weighed. Each filter disc was aseptically transferred to the centre of an agar plate which had been pre-inoculated with a lawn of the test organism. Micro-centrifuge tubes were weighed to enable calculation of the weight of the ionic liquid added to the agar plate.

The plates were incubated overnight at a temperature of 30°C which is appropriate for the test organisms, in a static incubator. Following overnight incubation the agar plates were examined and the radius of any growth inhibition zone around the filter disc recorded.

For the Bioscreen C growth assay a sample of ionic liquid was transferred into three sterile pre-weighed Eppendorf tubes. The tubes were re-weighed and the amount of ionic liquid in each tube calculated. To each sample of water miscible ionic liquid an appropriate amount of distilled autoclaved water was added to produce ionic liquid at concentrations of 95% (w/v). A 4µL sample of each concentration was added in triplicate to a 100 well Bioscreen plate. Ionic liquid and water samples were vortexed immediately before being added to the Bioscreen plate. An aliquot
of 186µL of MSX or LB media was added to each well before the addition of a 10µL aliquot of overnight culture of the test organism, giving a final ionic liquid concentration of 2% (v/v).

The Bioscreen plates were run in the Bioscreen for 24h at an appropriate temperature for the test organism, with intensive shaking. The OD of each well was recorded every 10min throughout the 24h run. Output was recorded in a Microsoft Excel worksheet.

Growth curves are reported as a percentage of the positive control culture and are the average of 3 replicates unless otherwise stated.

All ionic liquids tested in this experimental work were synthesised by Queens University Ionic Liquid Laboratory (QUILL, Queens University, Belfast).

7.3. Results and Discussion

7.3.1. Halide containing ionic liquids

The results from the screening of *P. putida* KT2440 and *P. putida* DOT-T1E, screened against ionic liquids containing a halide anion (bromide, chloride or iodide) with a number of different cation structures can be seen in Table 7.1 and Table 7.2.

As was observed with the previous screening results using the test organism *E. coli* MG1655 (Chapter 6), increasing the length of the alkyl chain associated with the imidazolium cation causes a decrease in the specific growth rate of the test organism, whilst also causing an increase in the inhibition zone associated with the agar diffusion test of that ionic liquid.

A similar pattern is observed for the ionic liquids containing pyrrolidinium and piperidinium cations with increasing alkyl chain length in the cation associated with an increase in the inhibitory nature of the ionic liquid.

For the tetra-alkyl ammonium ionic liquids the inclusion of an alkyl chain of eight carbons appears to increase the inhibitory nature of the ionic liquid greatly as
reflected by the absence of growth in liquid culture. However unlike with the other
cation classes tested a reduction in the specific growth rates recorded is not
reflected by an increase in the inhibition zone observed with the agar diffusion test.

The addition of one or more hydroxyl groups to the cation structure appears to
improve the specific growth rates of the cultures exposed to those ionic liquids.
However, only tetra-alkyl ammonium structures with short alkyl chains, up to four
carbons in length and containing hydroxyl groups were available for testing. It was
not therefore possible to determine whether the good specific growth rates
associated with these ionic liquids are a result of the introduction of one or more
hydroxyl groups or a result of the short alkyl chain structure of the cations, or are a
combination of these two factors.

From the results shown in Table 7.1 and 7.2, there is no apparent correlation
between the cation or anion class of the ionic liquid being screened and the strain
of *P. putida*. The *P. putida* DOT-T1E strain performs better than the *P. putida*
KT2440 strain when screened against the ionic liquids [C₄mim] Br, [C₄mim] I and
[N₂,4,2OH,2OH] Br. However, the *P. putida* KT2440 strain performs better when
screened against the other ionic liquids reported.

Also there is no apparent pattern within the results between the type of growth
medium used and the ionic liquid tested or between the *P. putida* strain tested and
the type of growth medium.
### Table 7.1 – Effect of imidazolium, pyrrolidinium, piperidinium and quaternary ammonium halides on growth of *P. putida* KT2440.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition Zone (cm)</th>
<th>μ (%) MSX</th>
<th>μ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₄mim] Cl⁸,M</td>
<td>0.07 ± 0.06</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C₆mim] Cl⁻,L,M</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mim] Cl⁻,L,M</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₁₀mim]Cl⁻,L,M</td>
<td>0.7 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mim]Br⁻,S,M</td>
<td>0</td>
<td>79 ± 5</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>[C₆mim]Br⁻,L,M</td>
<td>0.1</td>
<td>93 ± 4</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>[C₆mim]Br⁻,L,M</td>
<td>0.4 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mim]Br⁻,L,M</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mim] I⁻,L,M*</td>
<td>0.17 ± 0.1</td>
<td>59 ± 2</td>
<td>62 ± 7</td>
</tr>
<tr>
<td>[C₆mim] I⁻,LM</td>
<td>0.7 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄pyrr] Cl⁻,LM</td>
<td>0</td>
<td>86 ± 6</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>[C₄pyrr] Br⁻,S,M</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>[C₄mpip] Br⁻,S,M</td>
<td>0</td>
<td>87 ± 2</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>[C₄mpip] Br⁻,S,M</td>
<td>0.07 ± 0.1</td>
<td>53 ± 5</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>[C₄mpip] Br⁻,S,M</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₁,₂,₄] Br⁻,LM</td>
<td>0</td>
<td>96 ± 2</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>[N₁,₁,₄,₈] Br⁻,LM</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₁,₄,₈] I⁻,LM</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₈,₈,₈] Br⁻,LM</td>
<td>0.07 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₈,₈,₈] Cl⁻,LM</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₂,₄₂OH,₂OH]Br⁻,LM</td>
<td>0.1</td>
<td>124 ± 8</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>[N₁,₁,₂,₃₀OH]Br⁻,LM</td>
<td>0</td>
<td>116 ± 7</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>[N₁,₁,₄,₃₀OH] Cl⁻,LM</td>
<td>0</td>
<td>104 ± 7</td>
<td>87 ± 1</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. ⁸ – Solid, ⁻ – Liquid, ᵀ – Water miscible, ᵃ – water immiscible.
Table 7.2 – Effect of imidazolium, pyrrolidinium, piperidinium and quaternary ammonium halides on growth of *P. putida* DOT-T1E.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₄mim] Cl ⁵M</td>
<td>0.02 ± 0.03</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C₆mim] Cl ⁵M</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₈mim] Cl ⁵M</td>
<td>0.4 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₁₀mim] Cl ⁵M</td>
<td>0.8 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₂mim] Br ⁵M</td>
<td>0</td>
<td>53 ± 1</td>
<td>88 ± 2</td>
</tr>
<tr>
<td>[C₄mim] Br ⁵M</td>
<td>0.08 ± 0.03</td>
<td>100 ± 12</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>[C₆mim] Br ⁵M</td>
<td>0.4 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₈mim] Br ⁵M</td>
<td>0.7 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mim] I ⁵M</td>
<td>0.05 ± 0.05</td>
<td>45 ± 7</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>[C₆mim] I ⁵M</td>
<td>0.4 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄pyrr] Cl ⁵M</td>
<td>0</td>
<td>51 ± 1</td>
<td>94 ± 9</td>
</tr>
<tr>
<td>[C₄pyrr] Br ⁵M</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mpip] Br ⁵M</td>
<td>0.02 ± 0.02</td>
<td>34 ± 1</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>[C₄mpip] Br ⁵M</td>
<td>0.02 ± 0.06</td>
<td>0</td>
<td>63 ± 4</td>
</tr>
<tr>
<td>[C₄mpip] Br ⁵M</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁₁₂₄] Br ⁵M</td>
<td>0</td>
<td>37 ± 1</td>
<td>57 ± 1</td>
</tr>
<tr>
<td>[N₁₁₄₈] Br ⁵M</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁₁₄₈] I ⁵M</td>
<td>0.2 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁₈₈₈] Br ⁵M</td>
<td>0.3 ± 0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁₈₈₈] Cl ⁵M</td>
<td>0.02 ± 0.03</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₂₄₂₀₂₀] Br ⁵M</td>
<td>0.02 ± 0.03</td>
<td>98 ± 4</td>
<td>103 ± 4</td>
</tr>
<tr>
<td>[N₁₁₂₃₀] Br ⁵M</td>
<td>0</td>
<td>104 ± 5</td>
<td>74 ± 5</td>
</tr>
<tr>
<td>[N₁₁₄₃₀] Cl ⁵M</td>
<td>0.03 ± 0.03</td>
<td>99 ± 16</td>
<td>84 ± 5</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *N.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. ⁵ – Solid, ⁶ – Liquid, ⁷ – Water miscible, ⁸ – water immiscible.
7.3.2. Sulfate containing ionic liquids

The next group of ionic liquids screened were those which contained a sulfate cation. From the screening testing performed with the test organism *E. coli* MG1655 (Chapter 6), the sulfate containing ionic liquids emerged as relatively non-inhibitory, giving good specific growth rates in liquid culture and small inhibition zones in the agar diffusion test.

The results for the sulphate containing ionic liquids screened against the test organisms *P. putida* KT2440 and *P. putida* DOT-T1E are shown in Table 7.3 and Table 7.4.

As with previous screening results from *E. coli* MG1655 (Chapter 6), it can be seen that the ionic liquids which contained short chained cations combined with a sulfate anion gave good specific growth rates with the test organism *P. putida* KT2440. Increasing the length of the alkyl chain associated with the cation from 2 carbons to 4 carbons in length does not appear to have a detrimental impact on the specific growth rate of the *P. putida* KT2440 cultures. However, the substitution of a long alkyl chain of eight carbon atoms in place of either a one or two carbon chain on either the cation or the anion greatly reduces the specific growth rate of the culture in both types of growth media reducing the specific growth rate by almost a half in MSX media.

Additionally, the introduction of ester linkages or alkyl chain branching into the structure of the anion does not appear to affect the specific growth rates obtained from exposure to these ionic liquids. These results tie in well with those recorded in Chapter 6, when the same ionic liquid structures were screened against *E. coli* MG1655, the introduction of branching into the alkyl chain was also shown to have little effect on the toxicity of the ionic liquid as was the introduction of ester linkages. The observation regarding the potential of adding ester linkages into a structure to potentially produce less toxic ionic liquids has also been reported in the literature (Gathergood *et al.*, 2006), in which the introduction of ester linkages into an ionic liquid structure was seen to reduce the overall toxicity of the ionic liquid.
**Table 7.3-** Effect of imidazolium, pyrrolidinium, piperidinium and quaternary ammonium sulfates on growth of *P. putida* KT2440.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C&lt;sub&gt;2&lt;/sub&gt;mim] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0</td>
<td>72 ± 2</td>
<td>68 ± 1</td>
</tr>
<tr>
<td>[C&lt;sub&gt;2&lt;/sub&gt;mim] [C&lt;sub&gt;8&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.06 ± 0.06</td>
<td>38 ± 1</td>
<td>47 ± 5</td>
</tr>
<tr>
<td>[C&lt;sub&gt;2&lt;/sub&gt;mim] [C&lt;sub&gt;1&lt;/sub&gt;(OC&lt;sub&gt;2&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.1</td>
<td>89 ± 4</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>[C&lt;sub&gt;2&lt;/sub&gt;mim] [Tosylate]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0</td>
<td>119 ± 2</td>
<td>94 ± 5</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [C&lt;sub&gt;3&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.1</td>
<td>76 ± 1</td>
<td>79 ± 4</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [C&lt;sub&gt;1&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.06 ± 0.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.1</td>
<td>95 ± 3</td>
<td>86 ± 6</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [(C&lt;sub&gt;1&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;)&lt;sup&gt;LN&lt;/sup&gt;</td>
<td>0.17 ± 0.15</td>
<td>101 ± 2</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [(C&lt;sub&gt;1&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;)&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0.03 ± 0.06</td>
<td>81 ± 2</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [C&lt;sub&gt;1&lt;/sub&gt;C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.07 ± 0.06</td>
<td>63 ± 1</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mim] [(C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;)&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.03 ± 0.06</td>
<td>106 ± 13</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mpyr] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LN&lt;/sup&gt;</td>
<td>0.15</td>
<td>54*&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>[C&lt;sub&gt;4&lt;/sub&gt;mpyr] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0</td>
<td>96 ± 2</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>[C&lt;sub&gt;6&lt;/sub&gt;mpyr] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0.1</td>
<td>0</td>
<td>73 ± 7</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1&lt;/sub&gt;,1,2,4] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0</td>
<td>99 ± 18</td>
<td>59 ± 14</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1&lt;/sub&gt;,1,2,4] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0.2 ± 0.1</td>
<td>83 ± 2</td>
<td>81 ± 3</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1&lt;/sub&gt;,1,2,8] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1&lt;/sub&gt;,1,2,8] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1&lt;/sub&gt;,2,8,8] [C&lt;sub&gt;6&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1&lt;/sub&gt;,2,2,8] [Tosylate]&lt;sup&gt;SM&lt;/sup&gt;</td>
<td>0.3 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N&lt;sub&gt;2&lt;/sub&gt;,4,(2OH)&lt;sub&gt;2&lt;/sub&gt;] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.2 ± 0.1</td>
<td>96 ± 7</td>
<td>0</td>
</tr>
<tr>
<td>[N&lt;sub&gt;2&lt;/sub&gt;,4,(2OH)&lt;sub&gt;3&lt;/sub&gt;] [C&lt;sub&gt;2&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.2 ± 0.1</td>
<td>97 ± 9</td>
<td>0</td>
</tr>
<tr>
<td>[N&lt;sub&gt;1&lt;/sub&gt;,(2O&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;1&lt;/sub&gt;)&lt;sub&gt;3&lt;/sub&gt;] [C&lt;sub&gt;1&lt;/sub&gt;OSO&lt;sub&gt;3&lt;/sub&gt;]&lt;sup&gt;LM&lt;/sup&gt;</td>
<td>0.2 ± 0.1</td>
<td>99 ± 1</td>
<td>96 ± 1</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. – data not available. Data are the means of 3 replicates and the standard deviations are shown. S – Solid, L – Liquid, M – Water miscible, N – water immiscible, *<sup>1</sup> only 1 replicate.
### Table 7.4 - Effect of imidazolium, pyrrolidinium, piperidinium and quaternary ammonium sulfates on growth of *P. putida* DOT-T1E.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition Zone</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₂mim] [C₂OSO₃]</td>
<td>0</td>
<td>74 ± 1</td>
<td>87 ± 1</td>
</tr>
<tr>
<td>[C₂mim] [C₈OSO₃]</td>
<td>0</td>
<td>38 ± 1</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>[C₂mim] [Tosylate]</td>
<td>0</td>
<td>60 ± 6</td>
<td>86 ± 1</td>
</tr>
<tr>
<td>[C₄mim] [C₂OSO₃]</td>
<td>0.05</td>
<td>97 ± 1</td>
<td>69 ± 1</td>
</tr>
<tr>
<td>[C₄mim] [C₈OSO₃]</td>
<td>0.03 ± 0.05</td>
<td>43 ± 4</td>
<td>74 ± 2</td>
</tr>
<tr>
<td>[C₄mim] [C₁OSO₃]</td>
<td>0.07 ± 0.06</td>
<td>47 ± 6</td>
<td>75 ± 4</td>
</tr>
<tr>
<td>[C₄mim] [(C₄)C₂OSO₃]</td>
<td>0.17 ± 0.06</td>
<td>37 ± 2</td>
<td>52 ± 3</td>
</tr>
<tr>
<td>[C₄mim] [(C₄)C₁OSO₃]</td>
<td>0.05 ±0.05</td>
<td>0</td>
<td>67 ± 1</td>
</tr>
<tr>
<td>[C₄mim] [C₆OC₂OSO₃]</td>
<td>0.06 ± 0.03</td>
<td>43 ± 8</td>
<td>56 ± 6</td>
</tr>
<tr>
<td>[C₄mim] [C₂OC₃OSO₃]</td>
<td>0.03 ± 0.06</td>
<td>46 ± 3</td>
<td>69 ± 3</td>
</tr>
<tr>
<td>[C₄eim] [C₄OSO₃]</td>
<td>0.05 ± 0.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄mpyr] [C₆OSO₃]</td>
<td>0.1 ± 0.1</td>
<td>66 ± 10</td>
<td>77 ± 12</td>
</tr>
<tr>
<td>[C₄mpip] [C₄OSO₃]</td>
<td>0.1</td>
<td>0</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>[N₁,₁,₂,₄] [C₁OSO₃]</td>
<td>0</td>
<td>54 ± 5</td>
<td>112 ± 2</td>
</tr>
<tr>
<td>[N₁,₁,₂,₄] [C₂OSO₃]</td>
<td>0.08 ± 0.03</td>
<td>53 ± 7</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>[N₁,₁,₂,₈,₈] [C₁OSO₃]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₁,₂,₈] [C₂OSO₃]</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₁,₂,₈] [C₂OSO₃]</td>
<td>0.7 ± 0.3</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[N₁,₁,₂,₈] [C₄OSO₃]</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[N₁,₁,₂,₈] [C₆OSO₃]</td>
<td>0.3 ± 0.1</td>
<td>37 ± 3</td>
<td>23 ± 1*</td>
</tr>
<tr>
<td>[N₁,₂,(2O₂O₁)₃] [C₂OSO₃]</td>
<td>0.1 ± 0.1</td>
<td>98.7 ± 6</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. – data not available. Data are the means of 3 replicates and the standard deviations are shown. S – Solid, L – Liquid, M – Water miscible, N – water immiscible, * Only 2 replicates available.

However, the introduction of one or more hydroxyl groups into the structure of the tetra-alkyl ammonium cation structure appeared to greatly increase the inhibitory
nature of the ionic liquid to the test organism when grown in LB medium with no
growth recorded in cultures exposed to two of the four hydroxyl containing ionic
liquids. In comparison it was shown during the screening of the same ionic liquids
that introduction of an hydroxyl group into the structure of the ionic liquid reduced
the toxicity of that ionic liquid towards \textit{E. coli} MG1655.

For the test organism \textit{P. putida} DOT–T1E the affect of exposure to the sulphate
containing ionic liquids appeared to be more pronounced. Only cultures exposed to
the ionic liquids \([\text{C}_2\text{mim}]\ [\text{C}_2\text{OSO}_3]\) and \([\text{N}_{2(2\text{O}_2\text{O}_1)}] \ [\text{C}_2\text{OSO}_3]\) produced specific
growth rates of greater than 50\% when grown in MSX medium. In contrast for \textit{P. putida} KT2440 only the ionic liquids \([\text{C}_2\text{mim}]\ [\text{C}_8\text{OSO}_3]\) and \([\text{C}_4\text{eim}]\ [\text{C}_2\text{OSO}_3]\)
producing specific growth rates of less than 50\% of the positive control culture.

In general the test organism \textit{P. putida} DOT-T1E performed better in LB medium
than in MSX medium with only the ionic liquids \([\text{C}_6\text{mpip}]\ [\text{C}_2\text{OSO}_3]\) and \([\text{N}_{1,2,8,8}\]
[Tosylate] producing specific growth rates of less than 50\% of the positive control
culture (Table 7.4).

\textit{P. putida} DOT-T1E has adapted to grow in hostile environments, containing high
concentrations of organic solvents. However from the initial screening results
generated here it does not appear that these adaptive mechanisms have conferred
any significant advantage to the \textit{P. putida} DOT-T1E cultures when exposed to a
range of halide containing ionic liquids.

7.3.3. Effect of halide containing ionic liquids on the \textit{P.putida} DOT-T1E
efflux pump knockout mutants \textit{P. putida} DOT-T1E 18 and \textit{P. putida} DOT-T1E PS28

The \textit{P. putida} DOT-T1E efflux pump knockout mutants were screened against a
range of halide containing ionic liquids. The cation structures for the tested ionic
liquids spanned the immidazolium, pyridinium, piperidinium and ammonium
families. The screening result for *P. putida* DOT T1E 18 can be seen in Table 7.5 whilst the screening results for *P. putida* DOT-T1E PS28 can be seen in Table 7.6.

**Table 7.5-** Effect of imidazolium, pyrrolidinium, piperidinium and quaternary ammonium halides on growth of *P. putida* DOT-T1E 18.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₆mim] Cl₅LM</td>
<td>0.3 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₈mim] Cl₅LM</td>
<td>0.6 ± 0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₁₀mim]Cl₅LM</td>
<td>1.0 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₂mim]Br₅SM</td>
<td>n.d.</td>
<td>26 ± 1</td>
<td>94 ± 2</td>
</tr>
<tr>
<td>[C₄mim]Br₅LM</td>
<td>0.02 ± 0.02</td>
<td>60 ± 12</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>[C₆mim]Br₅LM</td>
<td>0.5 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₈mim]Br₅LM</td>
<td>0.9</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C₄mim] I₅LM</td>
<td>0.13 ± 0.06</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C₆mim] I₅LM</td>
<td>0.6 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₄pyrr] Br₅SM</td>
<td>n.d.</td>
<td>17± 5</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>[C₄pyrr] Cl₅SM</td>
<td>0</td>
<td>183 ± 28</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>[C₄mpip] Br₅SM</td>
<td>0.05</td>
<td>118 ± 17</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>[N₁,1,2,4] Br₅LM</td>
<td>0.02 ± 0.03</td>
<td>128 ± 17</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>[N₁,1,4,6] Br₅LM</td>
<td>0.3 ± 0.06</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>[N₁,1,4,6] I₅LM</td>
<td>n.d.</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>[N₁,8,8,8] Br₅LM</td>
<td>0.1 ± 0.1</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>[N₂,4,20H,20H] Br₅LM</td>
<td>0</td>
<td>60 ± 1</td>
<td>122 ± 1</td>
</tr>
<tr>
<td>[N₁,1,2,30H]Br₅LM</td>
<td>0</td>
<td>97 ± 6</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>[N₁,1,4,30H] Cl₅LM</td>
<td>0.05</td>
<td>70 ± 10</td>
<td>103 ± 1</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. S – Solid, L – Liquid, M – Water miscible, N – water immiscible.
Comparison of the screening results of *P. putida* DOT-T1E 18 and *P. putida* DOT-T1E PS28 with the wild type *P. putida* DOT-T1E did not show a consistent pattern between the two cultures i.e. the wild type *P. putida* DOT-T1E test organism did not consistently produce higher specific growth rates than the efflux pump knockout mutant *P. putida* DOT-T1E 18.

For both the *P. putida* DOT-T1E wild type cultures and the *P. putida* DOT-T1E 18 cultures the highest specific growth rates are generally achieved when the culture is grown in MSX rather than LB media. This is in contrast for the *P. putida* DOT-T1E PS28 cultures the highest specific growth rates were generally recorded with LB medium rather than MSX.

As with previously reported screening results for *E. coli* MG1655 as well as previously published literature (Cho *et al.*, 2007), (Ranke *et al.*, 2004), (Kulacki and Lamberti, 2008), (Latala *et al.*, 2009) it was apparent that increasing the length of the alkyl chain associated with either the cation increasing the inhibitory nature of the ionic liquid towards all of the organisms tested, with the exception on [C\text{2}mim] Br ionic liquid, which appears to give lower specific growth rates than the [C\text{4}mim] Br ionic liquid. This trend was observed across all 4 of the test organisms and across all cation classes of ionic liquid tested.

Comparison of the screening data of the efflux pump knockout mutant screening data with the screening data obtained from the wild type *P. putida* DOT-T1E samples revealed several unexpected results. For the ionic liquids [C\text{2}mim] Br, [C\text{4}mim] I, [C\text{6}mim] I, [C\text{4}pyrr] Cl, [C\text{4}mpip] Br, [N\text{1,1,2,4}] Br, [N\text{1,1,2,3OH}] Br and [N\text{1,1,4,3OH}] Cl the wild type *P. putida* DOT-T1E cultures gave specific growth rates lower than for at least one of the efflux pump knockout mutants.
### Table 7.6 - Effect of imidazolium, pyrrolidinium, piperidinium and quaternary ammonium halides on growth of *P. putida* DOT-T1E PS 28.

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition Zone (cm)</th>
<th>µ (%) MSX</th>
<th>µ (%) LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>([C_6\text{mim}]\text{Cl}^{LM})</td>
<td>0.3 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([C_8\text{mim}]\text{Cl}^{LM})</td>
<td>0.5 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([C_{10}\text{mim}]\text{Cl}^{LM})</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([C_2\text{mim}]\text{Br}^{SM})</td>
<td>0</td>
<td>34 ± 13</td>
<td>76 ± 4</td>
</tr>
<tr>
<td>([C_4\text{mim}]\text{Br}^{LM})</td>
<td>0.6</td>
<td>75 ± 8</td>
<td>97 ± 13</td>
</tr>
<tr>
<td>([C_6\text{mim}]\text{Br}^{LM})</td>
<td>0.4 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([C_8\text{mim}]\text{Br}^{LM})</td>
<td>0.7 ± 0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([C_4\text{mim}]\text{I}^{LM})</td>
<td>0.1</td>
<td>0</td>
<td>80 ± 7</td>
</tr>
<tr>
<td>([C_6\text{mim}]\text{I}^{LM})</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([C_4\text{pyrr}]\text{Br}^{SM})</td>
<td>n.d.</td>
<td>54 ± 3</td>
<td>63 ± 1</td>
</tr>
<tr>
<td>([C_4\text{pyrr}]\text{Cl}^{SM})</td>
<td>0.02 ± 0.03</td>
<td>68 ± 2</td>
<td>119 ± 12</td>
</tr>
<tr>
<td>([C_4\text{mpip}]\text{Br}^{SM})</td>
<td>0.07 ± 0.03</td>
<td>118 ± 3</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>([N_{1,1,2,3}\text{OH}]\text{Br}^{LM})</td>
<td>0.3 ± 0.1</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>([N_{1,1,2,3}\text{OH}]\text{Br}^{LM})</td>
<td>0.3 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>([N_{2,4,2OH,2OH}]\text{Br}^{LM})</td>
<td>0.02 ± 0.03</td>
<td>131 ± 9</td>
<td>76 ± 11</td>
</tr>
<tr>
<td>([N_{1,1,2,3OH}]\text{Br}^{LM})</td>
<td>0</td>
<td>123 ± 2</td>
<td>129 ± 4</td>
</tr>
<tr>
<td>([N_{2,1,4,3OH}]\text{Cl}^{LM})</td>
<td>0.05</td>
<td>145 ± 4</td>
<td>120 ± 6</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (µ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. *n.d.* – data not available. Data are the means of 3 replicates and the standard deviations are shown. ^s^ – Solid, ^l^ – Liquid, ^m^ – Water miscible, ^n^ – water immiscible.

In general the test organism *P. putida* DOT – T1E PS28 performed better i.e. this strain gave better specific growth rates as a percentage of the control, than the test organism *P. putida* DOT-T1E 18 across both types of liquid media screened.
The *P. putida* DOT-T1E 18 strain gave the highest percentage of failed Bioscreen plates, i.e. plates in which the positive control cultures failed to grow, of any of the screened test organisms (*E. coli* MG1655 and *P. putida* strains). Additionally, it was also noted after a visual examination of the growth curves of the *P. putida* DOT-T1E 18 positive control cultures that the lag time for these cultures was longer than that of any of the other microorganisms screened. As such the *P. putida* DOT-T1E cultures still appear to be in exponential growth phase at the end of the 24h Bioscreen run. This difference in the growth profile of the *P. putida* DOT-T1E cultures suggests that care should be taken in making a direct comparison between the specific growth rates for this microorganism and those obtained from the other microorganisms screened.

### 7.4. Conclusion

In conclusion, from the four strains of *P. putida* which have been screened in this work it is difficult to draw any definitive conclusions. This difficulty in establishing firm conclusions is exacerbated by two limiting factors.

Firstly a limited number of ionic liquid structures were available for screening with all four *P. putida* strains. This limitation in the number and diversity of available ionic liquid structures means that it is more difficult to identify specific structure related patterns.

Second, the difficulties presented in producing repeatable and reliable Bioscreen cultures for the test organism *P. putida* DOT- T1E reduces still further the amount of data available for comparison. The increasing lag time shown by the *P. putida* DOT-T1E 18 cultures as compared to the other test organisms screened means that care must be taken in interpreting the data collected from these cultures.

However, from the collected screening data it appears that the intrinsic tolerance of the test organism *P. putida* DOT-T1E wild type culture does not confer a significant survival advantage to the cultures when exposed to ionic liquids of various
structures. The good specific growth rates demonstrated by the efflux pump knockout mutants *P. putida* DOT-T1E 18 and *P. putida* DOT-T1E PS28 also suggests that the efflux pumps *ttg* ABC and *ttg* GHI are not significant factors in the survival of the test organisms following exposure to ionic liquid. That certain ionic liquids do impact the growth of microorganisms is also clearly apparent, as such it would be advantageous to establish whether the effect of the presence of ionic liquid in the culture medium is reflected by a change in the phenotypic profile of the culture.
8. Results – Exposure of \textit{E. coli} MG1655 to Water Immiscible Ionic Liquids

8.1. Introduction

One potential application of ionic liquids which is of considerable interest is the use of ionic liquids as replacements for conventional solvents in whole cell biotransformation reactions (Seddon, 1997). The use of whole cell biocatalysts in place of chemical catalysts can have considerable advantages in that they can offer enhanced chemoselectivity (catalysis of a single functional group), regioselectivity (catalysis of a specific functional group on different regions of the substrate) and enantioselectivity (ability to produce product of a single isomer) (Faber, 2004). Additionally, whole cell biocatalysts typically operate at mild temperatures and pressures, compared to chemical catalysts which often require both high temperatures and pressures. The use of a biological catalyst thereby reduces the energy demand and environmental impact of a process.

The drawback to using a biological system is that often either the substrate the end product or both will be toxic to the biocatalyst. To overcome this problem biocatalysts are often used in 2 phase reaction systems. Whilst the use of a 2 phase system is potentially beneficial it requires careful experimental design in order to find a second phase solvent which will not only be a good solvent for both the substrate and end products but which is also non toxic to the biocatalyst.

In a 2 phase reaction system there are several advantages of using an ionic liquid in place of a conventional solvent. These advantages include the large number of ionic liquids which are available and the possibility to design an ionic liquid through the combination of different cations and anions to produce an end product exactly tailored to the user needs. In comparison, there are only a limited number of solvents (approximately 600) (Rogers and Seddon, 2003) which can be considered and it is not possible to redesign these solvents in order to tailor their properties to a particular application.
Additionally the non-volatile nature of ionic liquids coupled with their negligible vapour pressure makes them safer to work with than conventional solvents by reducing the risk to the operator and reducing the possibility of negative environmental impact through loss of the solvent to the atmosphere due to evaporation, making ionic liquids a safer and more environmentally benign option.

Several instances of the use of ionic liquids in biotransformation processes have already been reported (Pfruender et al., 2004), (Pfruender et al., 2006), (Brautigam et al., 2007), (Brautigam et al., 2009), (Lou et al., 2009), (Sendovski et al., 2010). In this chapter the phenotypic response of *E. coli* MG1655 to a series of three water immiscible ionic liquids was investigated through the use of FT-IR fingerprinting, OD measurement, pH measurements and viable counts.

8.2. Materials and Methods

A full description of the materials and methods used for this work is reported in Section 2.3. Briefly, a series of 12x 250mL QuickFit flasks were prepared with 50mL of MSX medium. To each flask 2mL of innocula from an overnight culture of *E. coli* MG1655 was added. Flasks were incubated for 3h at 37°C with shaking at 200rpm.

After 3h, 500µL of each ionic liquid was added in triplicate to the flasks (3x flasks contained no ionic liquid and served as positive control cultures), all flasks were sealed using Suba-seals and incubated as described above.

At time points 0, 2, 6, 8, 20 and 24h 2mL samples were removed from each of the flasks and used for OD measurement (100µL) and pH measurement (100µL), the remaining sample was centrifuged at 11500g for 5min at 4°C and the supernatant retained. The biomass pellet was washed twice using sterile distilled water. Both the pellet and supernatant were stored at -80°C until required.
Additionally, at time points 0, 6 and 24h viable count readings were made. At the 0h time point viable count readings were taken only from the positive control flasks.

After the 24h time point the remaining biomass was collected by centrifugation at 3080g for 10 min at 4°C, the supernatant was removed and discarded. The biomass pellet was washed twice using sterile distilled water and stored at -80°C.

The collected biomass and supernatant samples were analysed using FT-IR spectroscopy. Samples were removed from the freezer and allowed to thaw completely, the supernatant samples were briefly vortxed and centrifuged before a 20µL aliquot was pipetted onto a 96 well silicon FT-IR plate. For the biomass samples, each sample was re-suspended in sterile distilled water, so as to give an OD reading at 680nm of approximately 1. A 20 µL aliquot of the re-suspended samples was then pipetted onto a 96 well silica FT-IR plate. Plates were dried at 50°C for 10min, or until samples were completely dry.

All ionic liquids used in these experiments were synthesized by QUILL (Queens University Ionic Liquid Laboratory) Queens University, Belfast.
8.3. Results and Discussion

8.3.1. Physiological effects of ionic liquid exposure

The structures of the three ionic liquids investigated are shown in Fig 8.1.

![Ionic liquid structures](image)

**Fig 8.1**– Ionic liquid structures used at 1% (v/v) (a) \([\text{N}_{1,1,4,8}][\text{NTf}_2]\) (IL 1), (b) [Ethyldimethyl\([(1\text{-napthyl})-3,6\text{-dioxo-1-hexyl}]\text{ammonium}]\)[\text{NTf}_2] (IL 2), (c) [Benzyldimethyl\([(1\text{-[4-(1\text{-dimethyl-2-dimethyl-propyl})phenyl]-3,6-dioxo-1-hexyl}]\text{ammonium}] \)[\text{NTf}_2] (IL 3).
With the exception of $[\text{N}_{1,1,4,8}] [\text{NTf}_2]$, very little is known about the toxicity profiles and potential impact on the phenotype of the test organism. These particular ionic liquids were selected in part due to their unknown and untested nature; however, also of consideration was the large volumes of each of the ionic liquid which would be required to complete testing of three separate culture batches. At each of the time points OD readings were made in order to assess the growth of the cultures. The plotted growth curves for each of the three batches are shown in Fig 8.2.

At each of the time points pH readings were also taken and the results of the pH measurements tested with Universal indicator from the three culture batches can be seen in Fig 8.3.

From the growth curves shown in Fig 8.2 it can be seen that there is a great deal of variation between the growth measurements recorded between the three replicate flasks, as indicated by the large error bars associated with some of the time points. A probable explanation for these large errors bars associated with some of the recorded time points is that the E. coli cultures were allowed to incubate for too long prior to the addition of ionic liquid to the flask cultures. Due to the large standard deviations associated with these measurements specific growth rates were not calculated.

Although there are large standard deviations associated with some of the time points, especially for the positive control cultures, it was decided not to repeat the growth curve cultures. This was because all three of the culture batches show the same growth pattern, i.e. growth is seen in the positive control cultures and the cultures exposed to IL 3. In contrast no growth is observed in the cultures exposed to IL 1 and IL 2, this trend is consistent across all three culture batches.

The information presented in Fig 8.2 shows that the addition of ionic liquid to the culture medium does appear to affect the overall pH of the flask culture. The degree to which the pH of the cultures is influenced by the addition of ionic liquid seems to be very much dependent on the particular ionic liquid. For the positive control cultures, across all three batches, the pH remains fairly constant at values between 7.0 and 7.5. The largest variation in pH is for cultures exposed to IL 1 and
IL2. For these cultures the recorded pH values, across the three culture batches fluctuates across the range of 7.5 to 6.5. By comparing these results to the growth data (Fig 8.2) it can be seen that the cultures exposed to IL1 and IL 2 showed no growth across any of the three tested batches. From the data collected it is not possible to determine whether it is the fluctuations in the pH of the culture medium which repress the growth of the *E. coli* cultures, or whether the fluctuations in pH are indicative of other biochemical reactions occurring in the culture medium as the result of the addition of these two ionic liquids. However, the observed fluctuations in pH are unlikely to have repressed the growth of the *E. coli* cultures.

Also collected at time points 0h (positive controls only because this was before the addition of any ionic liquid), 6h and 24h were samples for viable count readings. The results of these readings for batch 1 of the experiment can be seen in Table 8.1.

From Table 8.1 it can be seen that there is a large degree of variation between the viable count values from the replicate flasks, this degree of variation is not surprising as it is also seen in the growth curves generated from the optical density readings.
Fig 8.2 – Average growth curves of 3 batches of *E. coli* MG1655 during ionic liquid exposure (a) Batch 1, (b) Batch 2, (c) Batch 3

Positive control, ■ IL 1, ▲ IL 2, • IL 3, OD readings were measured by preparing a 1/100 dilution of the 100µL samples, OD read at 680nm. The data points plotted are the average of the OD readings from the 3 replicate flasks, error bars represent standard deviation between the three flasks. All ionic liquids were tested at a concentration of 1% (v/v).
**Fig 8.3** - Average pH values of 3 batches of *E. coli* MG1655 during ionic liquid exposure (a) Batch 1, (b) Batch 2, (c) Batch 3. Positive control – blue, IL 1 – red, IL 2 – yellow and IL 3 – green. pH measurements taken by adding 20µL of universal indicator to a 100µL aliquot of culture.
It is interesting to note from the viable count readings from the cultures exposed to IL1 and IL2 showed some colony growth at both the 6h and 24h time points. This suggests that IL1 and IL2 had a biostatic effect on the test organism rather than a biocidal effect. Once removed from the presence of the ionic liquid cultures were able to grow. The number of colony forming units recorded was in general far lower than the numbers which were recorded for both the positive control culture and the cultures exposed to IL3. This pattern suggests that although IL1 and IL2 may not be completely toxic to the test organism they do negatively affect the number of viable cells within the culture.

The viable count information collected for batches 2 and 3 of the cultures are not shown here, however the viable count numbers followed the same trends as had been seen for the batch 1 cultures. Viable count information was included within the results if the viable count readings fell within the range of 30-330 colony forming units (CFU). Plates with more than 300 CFU were too difficult to count accurately whilst plates with fewer than 30 CFU were considered to have too few colonies to be statistically significant.
Table 8.1– Viable count readings from *E. coli* cultures taken over the course of the 24h ionic liquid exposure experiment.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sample Type</th>
<th>Dilution</th>
<th>Colony Forming Units Counted</th>
<th>Colony Forming Units from (per mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>10^-6</td>
<td>41</td>
<td>4.1x10^8</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>10^-6</td>
<td>49</td>
<td>4.9x10^8</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>10^-6</td>
<td>253</td>
<td>2.53x10^9</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>10^-6</td>
<td>274</td>
<td>2.74x10^9</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>IL 1</td>
<td>10^-6</td>
<td>21</td>
<td>2.1x10^8</td>
</tr>
<tr>
<td>6h</td>
<td>IL 1</td>
<td>10^-6</td>
<td>85</td>
<td>8.5x10^8</td>
</tr>
<tr>
<td>6h</td>
<td>IL 2</td>
<td>10^-6</td>
<td>29</td>
<td>2.9x10^8</td>
</tr>
<tr>
<td>6h</td>
<td>IL 2</td>
<td>10^-6</td>
<td>136</td>
<td>1.3610^9</td>
</tr>
<tr>
<td>6h</td>
<td>IL 2</td>
<td>10^-6</td>
<td>52</td>
<td>5.2x10^8</td>
</tr>
<tr>
<td>6h</td>
<td>IL 3</td>
<td>10^-6</td>
<td>66</td>
<td>6.6x10^8</td>
</tr>
<tr>
<td>6h</td>
<td>IL 3</td>
<td>10^-6</td>
<td>78</td>
<td>7.810^9</td>
</tr>
<tr>
<td>6h</td>
<td>IL 3</td>
<td>10^-6</td>
<td>119</td>
<td>1.19x10^9</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>10^-8</td>
<td>193</td>
<td>1.93x10^11</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>10^-8</td>
<td>162</td>
<td>1.62x10^11</td>
</tr>
<tr>
<td>24h</td>
<td>IL 1</td>
<td>10^-8</td>
<td>77</td>
<td>7.710^10</td>
</tr>
<tr>
<td>24h</td>
<td>IL 1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>IL 1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>IL 2</td>
<td>10^-8</td>
<td>39</td>
<td>3.9x10^10</td>
</tr>
<tr>
<td>24h</td>
<td>IL 2</td>
<td>10^-8</td>
<td>68</td>
<td>6.8x10^9</td>
</tr>
<tr>
<td>24h</td>
<td>IL 2</td>
<td>10^-8</td>
<td>46</td>
<td>4.6x10^10</td>
</tr>
<tr>
<td>24h</td>
<td>IL 3</td>
<td>10^-8</td>
<td>196</td>
<td>1.96x10^11</td>
</tr>
<tr>
<td>24h</td>
<td>IL 3</td>
<td>10^-8</td>
<td>101</td>
<td>1.01x10^11</td>
</tr>
<tr>
<td>24h</td>
<td>IL 3</td>
<td>10^-8</td>
<td>136</td>
<td>1.31x10^11</td>
</tr>
</tbody>
</table>

NA – CFU count did not fall within the accepted 30-300 CFU range

8.3.2. FT-IR spectroscopy of samples exposed to water immiscible ionic liquids

FT-IR spectra were collected from both the supernatant and biomass samples taken over the course of the 24h exposure course. The method used for the collection of the FT-IR spectra is described fully in the Materials and Methods Section 2.3.3 and briefly recapped in Section 8.2. After collection all spectra were corrected for CO₂ by removal of peaks at 2403-2272cm⁻¹ and 683-656cm⁻¹, these removed areas were
then filled with a trend. The CO$_2$ corrected spectra were then scaled using EMSC (Martens et al., 2003). EMSC scaling was used to remove any unavoidable artefacts from the baseline. The CO$_2$ and EMSC scaled spectra for the biomass samples collected from culture batch 3 can be seen in Fig 8.4 whilst the spectra for the supernatant samples can be seen in Fig 8.5.

![Absorbance vs Wavenumber](image)

**Fig 8.4** – CO$_2$ corrected EMSC scaled FT-IR spectra showing of biomass samples from batch 3 cultures of *E. coli* MG1655 during a 24h exposure to 3 water immiscible ionic liquids

Following CO$_2$ correction and EMSC scaling 10 principal components (PCs) were extracted using PCA. The PCA plot for the biomass samples from the batch 3 cultures can be seen in Fig 8.8 and the equivalent from the corresponding supernatant samples in Fig 8.9. In both cases PC 1 and PC 2 have been plotted against each other. For the biomass samples the extraction of 10 PCs accounted for 97.06% of the total variance, whilst for the supernatant samples the extraction of 10 PCs accounted for 99.45% of the total variance.
From examination of the PCA biplots shown in Fig 8.8 and Fig 8.9 it is difficult to determine any clear clustering patterns. On both of the plots the 0h time points cluster loosely together (upper left corner in Fig 8.8 and the upper right corner in Fig 8.9). Additionally for the FT-IR spectra collected from biomass samples (Fig 8.8) the largest separation appears to be based on culture condition with the positive control cultures clustering in a vertical line on the left hand side of the plot space. However for the supernatant samples the largest separation appears to be loosely based on time, with samples moving across the plot space from left to right corresponding with increasing exposure time.

To investigate further the information gathered from the PCA plot the loadings plots of the first two principal components from the biomass samples were plotted and are shown in Fig 8.6 and Fig 8.7. From the loadings plot of PC1 it can be seen that the resulting plot is fairly complex. As has been seen previously multiple changes occur within the loadings plots however the largest degree of variance
appears to be recorded between wavenumbers 2000-1000 cm\(^{-1}\). This region of the spectra contains a large number of vibrations including vibrations associated with proteins as well as vibrations associated with polysaccharides.

From the PC2 loadings plot it is difficult to determine which area of the spectra is showing the largest amount of variance, it is therefore not possible to extract further information from this plot.

Fig 8.6 – PCA loading plot for PC1
Fig 8.7 - PCA loading plot for PC2

As the clustering was not so clear in PCA DFA was performed using the 10 extracted PCs and a class structure based on biological replicates within the data set. The resulting DFA plot for the biomass samples can be seen in Fig 8.10, whilst the plot for the supernatant samples can be seen in Fig 8.11.
Fig 8.8 - PCA plot of *E. coli* MG1655 biomass samples collected during a 24h exposure to 3 water immiscible ionic liquids.

Coding used - A – Positive Control, B – IL 1 exposed cultures, C – IL 2 exposed cultures, D – IL 3 exposed cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 3 – 6h, 4 – 8h, 5 – 20h and 6 – 24h.

In DFA the variation between samples from the same group or class, in this instance the three biological replicates collected for each culture condition at each time point, is minimised whilst variation between different groups or classes is maximized (Manly, B, 2005), (Windig et al., 1983). By only providing the algorithm with information relating to the 3 biological replicates (rather than anything about time or the different ionic liquids) collected at each time point any bias introduced into the analysis is minimised.
Fig 8.9 - PCA plot of *E. coli* MG1655 supernatant samples collected during a 24h exposure to 3 water immiscible ionic liquids.

Coding used - A – Positive Control, B – IL 1 exposed cultures, C – IL 2 exposed cultures, D – IL 3 exposed cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 3 – 6h, 4 – 8h, 5 – 20h and 6 – 24h.

From inspection of the PC-DFA plot of the biomass samples (Fig 8.10) it is possible to distinguish two distinct trends in the data. Firstly there is a clear trajectory based on time, visible in the plot space (and annotated with arrows), with samples from later exposure time points following a trend from left to right across the plot space. Secondly there is a clear separation between the different culture conditions. The positive control cultures and the cultures exposed to IL 3 cluster together in the top of the plot space whilst the cultures exposed to IL 1 and IL 2 occupy the bottom half of the plot space. However, there is also a significant separation between the cultures exposed to IL 1 and IL 2 suggesting that these have affected the bacteria differently and this is reflected in the FT-IR phenotype.
Again for the supernatant samples (Fig 8.11) the same two trends are clearly distinguishable as was seen for the biomass samples. The fact that the supernatant samples for the positive control cluster very tightly with the supernatant samples collected from the culture exposed to IL 3 is somewhat surprising. However, it must be noted that IL 3 was an extremely viscous solvent and it is therefore possible that it was not collected effectively during the sampling processes due to the difficulty of pulling such a viscous material through the narrow bore of the collection syringe.

**Fig 8.10** – PC-DFA plot constructed from the of EMSC scaled spectra of batch 3 culture biomass samples of *E. coli* MG1655 collected during a 24h exposure to 3 water immiscible ionic liquids, following extraction of 10 principal components (accounting for 97.06% total explained variance) with a class structure based on sample replicates.

Coding used - A – Positive Control, B – IL 1 exposed cultures, C – IL 2 exposed cultures, D – IL 3 exposed cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 3 – 6h, 4 – 8h, 5 – 20h and 6 – 24h.
Fig 8.11 - PC-DFA plot constructed from the of EMSC scaled spectra of batch 3 culture supernatant samples of *E. coli* MG1655 collected during a 24h exposure to 3 water immiscible ionic liquids, following extraction of 10 principal components (accounting for 99.45% total explained variance) with a class structure based on sample replicates.

Coding used - A – Positive Control, B – IL 1 exposed cultures, C – IL 2 exposed cultures, D – IL 3 exposed cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 3 – 6h, 4 – 8h, 5 – 20h and 6 – 24h.

To examine this possibility further an individual supernatant spectra from each culture condition collected at the 24h time point was plotted. The individual spectra can be seen in Fig 8.12.
Fig 8.12 – Individual supernatant spectra collected at the 24h time point, (a) – positive control, (b) – IL 1 exposed cultures, (c) – IL 2 exposed cultures and (d) – IL 3 exposed cultures.

Visual comparison of the spectra of the supernatant from the positive control culture with the spectra with the spectra obtained from the culture exposed to IL 3, do not reveal any obvious differences. However, comparison of the spectra from the positive control culture and the culture exposed to IL3 with the spectra from
the cultures exposed to IL 1 and IL 2 reveal a number of differences. There are a number of differences between approximate wavenumbers 1400-800cm\(^{-1}\). A list of the major band assignments is given in Table 3.4.

The absence of the spectral variation between wavenumbers 1400-700cm\(^{-1}\) in the spectra for the positive control cultures and the cultures exposed to IL 3 may explain why the samples for these two culture conditions cluster together so closely. The lack of spectral vibrations in the spectra from the culture exposed to IL 3 would also suggest that the ionic liquid has not been collected in the supernatant samples collected. Unfortunately, there are many vibrations in this region and this part of the FT-IR spectrum from 1400-700cm\(^{-1}\) is often referred to as the ‘fingerprint region’ as many functional groups vibrate here so almost all organic chemical species produce a pattern in this area (Stuart, 1997).

8.4. Conclusion

The reported literature regarding the toxicity of water immiscible ionic liquids is partly contradictory, with different studies reporting different levels of toxicity for the same ionic liquid. The ionic liquid \([\text{C}_4\text{mim}]\text{NTf}_2\) has been reported both as being toxic to \(E.\ coli\) (Cornmell et al., 2008a) and as being compatible with \(E.\ coli\) and having no negative impact on the integrity of the cell membranes (Pfruender et al., 2006).

From the results presented in this chapter it is apparent that the water immiscible ionic liquids tested do affect the biochemical fingerprint of the cultures, as evidenced by the separation observed between the culture conditions in the PC-DFA plot of the biomass samples. A different trajectory is seen in the DFA plot, indicating that the phenotype of the cell is affected differently depending on the structure of the ionic liquid.

Additionally, the results of the viable count readings suggest that there is a negative effect on the test organism as a result of adding ionic liquid to the culture flasks. However the growth of some colonies on the agar plates after removal from the
presence of the ionic liquids suggests that the impact of the ionic liquid addition may be biostatic rather than biocidal.
9. Results Exposure of \textit{P. putida} and \textit{E. coli} to water miscible ionic liquids

9.1. Introduction

In this series of experiments three test organisms \textit{Escherichia coli} MG1655, \textit{Pseudomonas putida} strains KT2440 and DOT-T1E, exposed to a series of imidazolium chloride ionic liquids in flask culture and sampled over a 24h time course. The purpose of these experiments was not only to determine the toxicity of the ionic liquids to the test organisms but also to collect both biomass and supernatant samples for FT-IR spectroscopic analysis. It was hoped that through the collection of these samples it may be possible to identify mechanisms of toxicity of the ionic liquids to the test organisms and to determine the response mechanisms of the organism to exposure to ionic liquids.

\textit{Escherichia coli} MG1655 was selected for use in this experiment as it is widely used laboratory strain, and as such comparison of the results generated from its use are more readily comparable to work which has been previously published. In particular the accumulation of several different ionic liquid structures within the membrane of this particular strain of \textit{E. coli} has already been reported (Cornnell \textit{et al.}, 2008b).

\textit{Pseudomonas putida} strains were selected for testing in this experiment based on the similarity of the mechanisms of toxicity for both ionic liquids and organic solvents. The accumulation of ionic liquids in cell membranes has been reported in several publications (Cornnell \textit{et al.}, 2008b), (Pernak \textit{et al.}, 2004), (Pernak \textit{et al.}, 2003). This interaction and potential damage to the cell membrane is also the mode of toxicity of organic solvents such as toluene, phenol and benzene (Keweloh \textit{et al.}, 1991), (Ingram, 1977), (Ingram, 1976). A number of strains of solvent tolerant \textit{Pseudomonas putida}, including the solvent tolerant \textit{P. putida} DOT-T1E used in this study have been isolated (Ramos \textit{et al.}, 1995). As such it was desirable to compare the response of two strains of \textit{P. putida} (KT2440 and DOT-T1E) both to each other and to \textit{E. coli} MG1655 to determine whether the known solvent resistance of the \textit{P. putida} organisms would also affect their response to ionic liquid exposure.
9.2. Materials and Methods

The method used for the collection of this set of results is detailed in the Materials and Methods Section 2.3.3. Briefly, 15x 250mL Quick Fit flasks were prepared with 50mL of MSX medium. Each flask was inoculated with 2mL of inoculum from an overnight culture of either *E.coli* MG1655, *P. putida* KT2440 or *P. putida* DOT-T1E. For *E. coli* MG1655 cultures flasks were incubated for 1½h at 37°C with shaking at 200rpm, for *P. putida* cultures flasks were incubated for 3h at 30°C with shaking at 200rpm. After incubation for the stated time, 2mL aliquots were removed from each flask to appropriately labelled tubes. The ionic liquids [C$_2$mim] Cl, [C$_4$mim] Cl, [C$_6$mim] Cl and [C$_8$mim] Cl (*E. coli* MG1655 cultures only) were added to the appropriate number of flasks (each ionic liquid was tested in triplicate, with three flasks containing no ionic liquid as positive controls). Each flask was sealed with a rubber Suba-Seal before further incubation at either 37°C or 30°C with shaking at 200rpm.

From the 2mL sample removed from each flask, 100µL was retained for optical density measurement, 100µL used for pH measurement and 100µL used for viable count measurement. The remaining 1.7mL of sample was centrifuged at 11 500g for 2min at 4°C. The supernatant was removed and stored at -80°C for FT-IR spectroscopy. The cell pellet was washed twice using sterile, distilled water, before storage as a dry cell pellet at -80°C for FT-IR spectroscopy. These samples form the T0 (0h) data points.

Additional samples were removed at 2h, 4h, 6h, 8h, 10h and 24h following addition of ionic liquid to the flasks. The matrix for sample testing is shown in Table 9.1.
Table 9.1 – Matrix to show sample tests performed at each time point

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>OD</th>
<th>pH</th>
<th>Viable Count</th>
<th>FT-IR Supernatant</th>
<th>FT-IR Cell Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2h</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>4h</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>6h</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>8h</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>10h</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>24h</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

After the 24h time point the remaining culture was collected by centrifugation at 3080g, for 10min at 4°C. The resulting pellet was washed twice in sterile distilled water before storage as a cell pellet at -80°C.

At time points 0h, 6h and 24h samples were collected for viable count measurements. At each of the above time points 100µL of culture was used to inoculate a 15mL screw top filled with 9mL of sterile distilled water, from this serial dilutions of 10^{-4}, 10^{-6} and 10^{-8} were prepared. From the dilutions 10^{-6} and 10^{-8} a 100µL sample was removed and used to spread an LB agar plate. Following inoculation, LB plates were incubated for 24h in a standing incubator at 37°C. Following 24h of incubation plates were removed from the incubator and the number of distinct colony forming units (CFUs) were counted. Plates which produced more than 300 CFUs were not counted.

At the 7 time points both biomass and supernatant samples were collected for FT-IR analysis. Biomass samples were re-suspended in sterile distilled water to give an OD reading of approximately 1.0. After re-suspension 20µL of the re-suspended sample was applied to a silica FT-IR plate. For supernatant, samples were allowed to thaw completely before being vortexed and briefly centrifuged, before application of a 20µL sample to a clean silicon FT-IR plate. For both types of sample plates were placed in a 50°C drying oven and dried completely before being loaded into the FT-IR instrument.
After collection of the spectra the ASCII file was imported into MATLAB v.7.3.0 (2000b) (Maths Works). The collected spectra were corrected for CO$_2$ by removal of peaks at 2403-2272cm$^{-1}$ and 683-656cm$^{-1}$, removed peaks are then filled with a trend and scaled using extended multiplicative signal correlation (EMSC). This spectral processing method allows for the separation of physical light scattering effects from chemical (vibrational) light absorbance effects in spectra which are collected from turbid solutions (Martens et al., 2003).

All ionic liquids used in these experiments, with the exception of [C$_8$mim] Cl were purchased from Sigma – Aldrich (Sigma – Aldrich, UK). The ionic liquid [C$_8$mim] Cl was synthesized by QUILL (Queens University Ionic Liquid Laboratory) Queens University, Belfast.

**9.3. Results and Discussion**

**9.3.1. Physiological measurements of E. coli MG1655 exposed to imidazolium ionic liquids**

The OD data collected at 680nm over the course of the 24h growth time have been averaged over the three flask replicates and plotted to give the growth curves shown in Fig. 9.1.

The design of this experiment could be considered to be a ‘scaled up’ version of the micro-culture toxicity screening described previously in Chapter 6. Increasing the culture size allows additional data to be collected from the cultures viz viable counts and FT-IR analysis.
**Fig. 9.1** - Average growth curves of *E. coli* MG1655 during exposure to a series of imidazolium chloride ionic liquids

- Positive control, ♦ [C₂mim] Cl, ▲ [C₄mim] Cl, ♦ [C₆mim] Cl, • [C₈mim] Cl, OD readings were measured by preparing a 1/10 dilution of the 100µL samples, OD read at 680nm and data points are the average of the OD readings from the 3 replicate flasks, error bars represent standard deviation between the three flasks.

The selection of this series of imidazolium chloride ionic liquids investigated here was based in part on the specific growth rates and ionic liquid volumes used in the screening process. The imidazolium chlorides were selected for a number of reasons -: first, at the time of testing this was the most complete ionic liquid series (i.e., a range of cations from C₂ up to C₁₀) available in sufficient quantities to allow for testing of not only *E. coli* but also *P. putida* KT2440 and *P. putida* DOT-T1E at a scale that allowed the appropriate number of replicates within a batch and sufficient batches to check the reproducibility of the data--; second, the toxicity behaviour of the imidazolium halides has been reported in a large number of
publications (Bailey et al.), (Latala et al.) (Latala et al., 2009), (Pernak et al., 2003), (Ranke et al., 2007a), (Romero et al., 2008), (Wells and Coombe, 2006).

Experimental data collected from screening of *E.coli* suggested that the selected range of ionic liquids would cover both; (1) the biocompatible – C₂mim ionic liquids (although [C₂mim] Cl was not available at the time of screening, [C₂mim] Br appeared to be highly biocompatible giving a specific growth rate of 92% of the unexposed control cells in MSX medium at 2%v/v concentration of the ionic liquid); (2) and highly toxic [C₈mim]⁺ ionic liquids; - in the previous screening experiments [C₈mim] Cl gave a large inhibition zone of 0.8cm ± 0.12, whilst giving specific growth rates of 0 in MSX medium at 1%v/v.  

From the OD based growth curves (Fig. 9.1), two main trends are readily distinguishable. Firstly the ionic liquid [C₂mim] Cl is more biocompatible than the other ionic liquids investigated. As the other three ionic liquids are composed of cations with increasing alkyl chain length, this trend is as would be expected based both on published literature and on the toxicity trends established during the ionic liquid screening process reported previously.

The second trend is the apparent absence of growth in the flasks containing [C₄mim] Cl exposed cultures until between the 10-24h time points. From the growth curves previously collected this trend had not been anticipated. Previous screening of [C₄mim] Cl had shown good specific growth rates of 97% of the positive control cells in micro-culture plates with cell growth (indicated by increasing optical density readings) occurring at the start of the incubation period without an extended lag time.

The difference in the growth trend observed for cultures exposed to the ionic liquid [C₄mim] Cl in micro-culture as opposed to those exposed during a larger scale flask culture can not be definitively explained. One possibility is a potential difference in the [C₄mim] Cl ionic liquid itself. The [C₄mim] Cl used in the flask culture exposures was obtained from a different source than the [C₄mim] Cl used in the micro-culture screening plates. Although both ionic liquids were believed to be ‘pure’ the possibility of variation between the synthesis methods and processes for the two
Ionic liquids must be considered as a possible explanation for the variance observed between the two sets of growth curves. However, without chemical comparison of the two different ionic liquids, it is not possible to determine if a contaminant is present in either ionic liquid or what any possible contaminant may be.

To test this possibility, it was hoped to use the original [C₄mim] Cl (i.e., the [C₄mim] Cl used during the screening experiments) in a flask culture experiment in which the two different batches of [C₄mim] Cl ionic liquids would be used in parallel flask cultures to determine whether the source of the observed variation in growth rates was the effect of using different [C₄mim] Cl ionic liquids or was related to the increase in the size of the culture volume. Unfortunately, insufficient [C₄mim] Cl from the original screening experiment was available to allow this comparison to be performed.

That the observed lag time is a genuine trend rather than an anomaly associated with this particular batch of culture is confirmed by this trend being observed in all three batches of E. coli MG1655 culture which were exposed to [C₄mim] Cl.

Comparison of the pH measurements collected over the same 24h time course supports the above observations made from the growth data (Fig. 9.2). That E. coli metabolises glucose to carboxylic acid and acetate, especially during active growth, has been widely reported (Papoutsakis and Meyer, 1985a), (Papoutsakis and Meyer, 1985b). This metabolism of glucose to form acetate can result in the alteration of the pH of the growth media. This effect was the rationale between the choice of MSX growth medium which has a high buffering capacity, rather than the LB medium which had been used in previous agar plate screening tests. As can be seen from Fig. 9.2, the pH of the flasks containing [C₄mim] Cl exposed cultures decreases from pH 7.5 to pH 7.0 between the time points 10h and 24h, indicating that the culture was actively fermenting glucose after 8h.
Fig. 9.2 - pH measurements recorded from *E. coli* MG1655 cultures over a 24h time course following exposure to imidazolium chloride ionic liquids

Sample type is indicated by the colour of the bar in the above graph, *Positive control, [C₂mim] Cl culture, [C₄mim] Cl culture, [C₆mim] Cl culture* and *[C₈mim] Cl culture*.

At time points 0h, 6h and 24h samples were collected for viable count measurements, the collected information collected from the viable counts is shown in Table 9.2. Viable count plates which did not fall within the range of 30 to 300 CFU are not included in the results table.
Table 9.2 - Viable count information for *E. coli* MG1655 recoded at 0h, 6h and 24h.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sample Type</th>
<th>Dilution</th>
<th>Colony Forming Units Counted</th>
<th>Colony Forming Units (per mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>+VE</td>
<td>$10^{-6}$</td>
<td>64</td>
<td>$6.4 \times 10^8$</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>$10^{-6}$</td>
<td>60</td>
<td>$6.0 \times 10^8$</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>$10^{-6}$</td>
<td>72</td>
<td>$7.2 \times 10^6$</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>$10^{-8}$</td>
<td>107</td>
<td>$1.07 \times 10^8$</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_{2}$ mim] Cl</td>
<td>$10^{-6}$</td>
<td>220</td>
<td>$2.20 \times 10^9$</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_{2}$ mim] Cl</td>
<td>$10^{-6}$</td>
<td>258</td>
<td>$2.58 \times 10^9$</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_{2}$ mim] Cl</td>
<td>$10^{-6}$</td>
<td>217</td>
<td>$2.17 \times 10^9$</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_{4}$ mim] Cl</td>
<td>$10^{-6}$</td>
<td>45</td>
<td>$4.5 \times 10^8$</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_{4}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>6h</td>
<td>[C$_{6}$ mim] Cl</td>
<td>NA</td>
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<td>[C$_{6}$ mim] Cl</td>
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</tr>
<tr>
<td>6h</td>
<td>[C$_{8}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_{8}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>$10^{-8}$</td>
<td>$9.9 \times 10^{10}$</td>
<td>$9.9 \times 10^{10}$</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>$10^{-8}$</td>
<td>$1.61 \times 10^{11}$</td>
<td>$1.61 \times 10^{11}$</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>$10^{-8}$</td>
<td>$5.3 \times 10^{10}$</td>
<td>$5.3 \times 10^{10}$</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{2}$ mim] Cl</td>
<td>$10^{-8}$</td>
<td>$1.07 \times 10^{11}$</td>
<td>$1.07 \times 10^{11}$</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{2}$ mim] Cl</td>
<td>$10^{-8}$</td>
<td>$7.4 \times 10^{10}$</td>
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</tr>
<tr>
<td>24h</td>
<td>[C$_{4}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{4}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{4}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{6}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{6}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{8}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_{8}$ mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA – CFU count did not fall within the accepted 30-300 CFU range

From the number of CFUs recorded it can be seen that the ionic liquids [C$_{6}$ mim] Cl and [C$_{8}$ mim] Cl appeared to be immediately toxic to the test organism i.e. no or
very few CFUs were recorded at either the 6h or 24h time points. By contrast, the cultures exposed to the ionic liquid [C₄mim] Cl produced a small number of CFUs at the 6h time point before appearing to enter exponential growth phase by the 24h time point, at which time more than 300 CFUs were recorded.

Also of interest from the viable count information is the presence of 5 CFUs recorded from the 10⁻⁶ dilution for the cultures exposed to the ionic liquid [C₆mim] Cl at the 24h time point. This small number of colonies would in general be discarded as being too small to count, the typical accepted range being between 30-300 CFUs. However the long lag time associated with the [C₄mim] Cl ionic liquid exposed cultures suggest that additional incubation time may allow for some culture growth even in following exposure to ionic liquids of alkyl chain length C₆ or longer.

9.3.2. FT-IR measurement of E. coli MG1655 exposed to imidazolium chloride ionic liquids

In addition to the tests already described, at each of the 7 time points both biomass and supernatant samples were collected for FT-IR analysis. The collected spectra are shown in Fig. 9.3. This spectral processing method allows for the separation of physical light scattering effects from chemical (vibrational) light absorbance effects in spectra which are collected from turbid solutions (Martens et al., 2003).
All collected biomass spectra from the E. coli – these spectra have been corrected for CO₂ and EMSC scaled.

Multivariate analysis of the collected spectra was performed, initially principal component analysis (PCA). From the collected spectra 10 (PCs) were extracted and PCs 1 and 2 were plotted against each other, shown in Fig. 9.4. The extraction of these 10 PCs accounts for 98.16% of the total variance within the collected spectral data set, with 42.14% and 20.47% of that variance accounted for by PCs 1 and 2 respectively.
Fig. 9.4 – PCA showing PC1 versus PC2 from the biomass spectra

Numbers represent time points, 0 – 0h, 1 – 2h, 2 – 4h, 3 – 6h, 4 – 8h, 5 – 10h and 6 – 24h

From the above PCA plot it can be seen that there is no obvious separation between the different ionic liquids or time points, although there is some suggestion that cultures exposed to the [C₆mim] Cl and [C₈mim] Cl ionic liquids are generally located on the right side of the plot area.

To investigate further the information gathered from the PCA plot the loadings plots of the first two principal components from the biomass samples were plotted and the loadings plot for principal component 1 is shown in Fig. 9.5. From the loadings plot of PC1 it can be seen that the resulting plot is fairly complex. However as has been previously seen the largest degree of variance appears to be recorded between wavenumbers 2000-1000 cm⁻¹. This region of the spectra contains a large number of vibrations including vibrations associated with proteins as well as vibrations associated with polysaccharides.
From the PC2 loadings plot (data not shown) it is difficult to determine which area of the spectra is showing the largest amount of variance, it is therefore not possible to extract further information from this plot.

Following on from PCA, discriminate function analysis (DFA) was performed on the first 10 PCs. In this method of analysis the algorithm is given some information on the number of classes, in this instance the three biological replicates collected for each culture condition at each time point. DFA then minimises the variation between the samples from the same group or class, whilst the variation between different groups or classes is maximized (Windig et al., 1983). The PC-DFA plot generated from the collected spectra is shown in Fig. 9.6.
In this plot separation across the two axes is clearly visible. In the first instance it can be seen that all spectra collected at the 0h time point are clustered together on the extreme right side of the plot area. As the samples used to collect these spectra were collected from samples experiencing identical growth conditions i.e. before exposure of the culture to ionic liquid this clustering pattern would be expected. Clustering is further observed with separation visible based on both time and ionic liquid type. Samples A1-A6 and samples B1-B6 cluster together in the bottom right of the plot area. As these points represent the positive control and the biocompatible [C$_2$mim] Cl exposed cultures it would be expected that these points would cluster together and that this clustering pattern is an accurate representation of the growth curves observed for these two sample sets. In addition there can also be observed some separation based on time within these clustered samples, however this separation is not well defined.
To the left side of the plot area are the spectra generated from the cultures exposed to the ionic liquids $[C_6\text{mim}]\text{Cl}$ and $[C_8\text{mim}]\text{Cl}$. These are the two ionic liquids which were shown to be completely toxic to the *E. coli* MG1655 cultures according to the growth curves generated (Fig. 9.1) and the viable count information presented in Table 9.2. Again some separation based on time is observed and is more clearly defined than that which is observed for the biocompatible and positive control samples.

The separation of samples based on time course is an important indicator that the observed separations are not merely the result of the detection of the different ionic liquids.

The feature of most interest in the plot space is the separation of the cultures exposed to the ionic liquid $[C_4\text{mim}]\text{Cl}$ (codes C1-C6). These samples cluster away from both the biocompatible and positive control samples as well as away from the cultures exposed to the toxic ionic liquids. The exception to this clustering pattern are the $[C_4\text{mim}]\text{Cl}$ samples collected from the final 24h time point. The spectra collected from these samples cluster with the $[C_2\text{mim}]\text{Cl}$ and positive control samples. Based on the observations from the OD measurements, pH measurements and viable count information as well as the clustering pattern observed suggests that for *E. coli* MG1655 the ionic liquid $[C_4\text{mim}]\text{Cl}$ may have a biostatic effect on the test organism rather than being completely toxic.

Comparison of the individual spectra for biomass samples exposed to the four different ionic liquids and positive control is shown in Fig. 9.7. Visual comparison of the spectra of the positive control cells and the ionic liquid exposed biomass cultures does not reveal any obvious differences between the different spectra. This lack of obvious difference between the exposed and the unexposed cultures suggests that the observed clustering patterns (Fig. 9.6) must be the result of more subtle differences in the spectra, possibly the result of physiological differences within the cells, rather than simply reflecting the addition of ionic liquid to some of the flask cultures.
Fig. 9.7 - Individual biomass spectra recorded from different culture conditions at the 2h and 24h time points

(a) – Positive Control 2h
(b) – [C₂mim] Cl 2h
(c) – [C₄mim] Cl 2h
(d) – [C₆mim] Cl 2h
(e) – [C₈mim] Cl 2h
(f) – Positive Control 24h
(g) – [C₂mim] Cl 24h
(h) – [C₄mim] Cl 24h
(i) – [C₆mim] Cl 24h
(j) – [C₈mim] Cl 24h
In addition to the biomass samples, supernatant samples were also collected and analysed by FT-IR spectroscopy. The collected spectra were also corrected for CO$_2$ and scaled using the same EMSC scaling as was applied to the biomass samples. The CO$_2$ corrected and EMSC scaled spectra can be seen in Fig. 9.8.

![Absorbance vs. Wavenumber](image)

**Fig. 9.8**– EMSC scaled and CO$_2$ corrected spectra collected from supernatant samples from *E. coli* MG1655 cultures exposed to imidazolium chloride ionic liquids

The same multivariate analysis protocol of PCA and DFA were applied to the supernatant spectra. The result of the PCA can be seen in Fig. 9.9. The total variance accounted for by the extraction of 10 PCs is 99.77% of the total variance within the data set whilst PC1 accounted for 87.61% of the variance and PC 2 accounted for 2.47% of the variance. The large percentage of variance accounted for by the first PC indicates that there is a large degree of similarity in the collected data i.e., a large amount of the measured data is the same.
Fig. 9.9 - PCA plot showing PC1 versus PC2 from the supernatant spectra

Coding used - A – Positive Control, B – [C$_2$mim] Cl cultures, C – [C$_4$mim] Cl cultures, D – [C$_6$mim] Cl cultures, E – [C$_8$mim] Cl cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 2 – 4h, 3 – 6h, 4 – 8h, 5 – 10h and 6 – 24h

Inspection of the PCA plot shows clustering of the 0h samples in the centre of the plot area. As with the biomass samples this is the clustering pattern which would be anticipated as the 0h samples were collected before the addition of ionic liquid to any of the culture flasks. However, unlike the biomass samples the largest separation is seen between the positive control supernatants and the supernatants collected from the cultures exposed to different ionic liquids. The ionic liquid culture supernatants collected from [C$_2$mim] Cl and [C$_4$mim] Cl cluster separately whilst there is no apparent separation between the supernatants collected from the flasks containing [C$_6$mim] Cl and [C$_8$mim] Cl exposed cultures.

Comparison of the individual spectra plotted from each of the exposure conditions at 2h and 24h are shown in Fig. 9.10. Unlike the spectra collected from the biomass samples (Fig. 9.7) there are a number of differences clearly visible between the
spectra collected from the positive control cells and the spectra collected from the flasks in which cultures were exposed to ionic liquids. The main areas of variation appear to be between wavenumbers 3500-2500 cm\(^{-1}\) and 1750-500 cm\(^{-1}\), in these areas a number of spectral features not observed in the positive control cultures can be seen. The bands observed between wavenumbers 3500-2500 cm\(^{-1}\) are likely due to CH\(_x\) stretching vibrations, possibly associated with the increasing alkyl chain of the ionic liquid.

The collection of spectra from the pure ionic liquids used in this experiment proved to be difficult. From the spectra collected using the high throughput FT-IR system (HTS), it was not possible to distinguish any spectral features. However from the published literature spectra from a number of imidazolium ionic liquids have been collected (Noack et al., 2010). The spectra of [C\(_2\)mim] [NTf\(_2\)] and [C\(_4\)mim] [NTf\(_2\)] have been collected by the use of attenuated total reflection (ATR) and a number of spectral features have been identified. A series of spectral bands have been observed between the 1500-700 cm\(^{-1}\) wavenumbers, similar to those which can be observed from the spectra collected from the supernatant as seen in Fig 9.10. The spectral bands observed in this region are likely due to C-O or O-H stretching vibrations.

From the results obtained from the E. coli MG1665 batch culture experiments using a series of imidazolium chloride ionic liquids a number of trends were observed. As was expected based on previous high throughput screening experiments and published literature, there was a correlation between increasing the length of the alkyl chain length of the cation and an increase in the toxicity of the ionic liquid. However what had not been anticipated was the apparent biostatic effect of the [C\(_4\)mim] Cl ionic liquid, associated with the extended lag time of between 10-24h, this effect had not been observed in the initial high throughput screening experiments, nor to date has this been described in published literature.
Fig. 9.10 - Individual supernatant spectra recorded from different culture conditions at the 2h and 24h time points

(a) – Positive Control 2h
(b) – [C<sub>2</sub>mim] Cl 2h
(c) – [C<sub>4</sub>mim] Cl 2h
(d) – [C<sub>6</sub>mim] Cl 2h
(e) – [C<sub>8</sub>mim] Cl 2h

(f) – Positive Control 24h
(g) – [C<sub>2</sub>mim] Cl 24h
(h) – [C<sub>4</sub>mim] Cl 24h
(i) – [C<sub>6</sub>mim] Cl 24h
(j) – [C<sub>8</sub>mim] Cl 24h
9.3.3. Physiological measurements of *P. putida* KT2440 exposed to imidazolium ionic liquids

From the results collected from the *E. coli* flask exposure experiments, some modifications were made to the experimental design before repetition with two strains of *P. putida*. The largest change to the experimental design was the exclusion of the ionic liquid [C₈mim] Cl from further testing. The toxicity of this ionic liquid was felt to be such that further testing would not produce significant data for analysis. For testing with both *P. putida* KT2440 and *P. putida* DOT-T1E, the incubation temperature was reduced from 37°C to 30°C and the interval between inoculation of the Suba Seal flask from the overnight culture to the addition of the ionic liquid was increased from the 1½h to 3h. This alteration in incubation time was based on previous experimental work, which had indicated that increased incubation time was required to allow *P. putida* cultures to reach approximately the same optical density as the *E. coli* cultures.

Comparison of the growth curves obtained from the *E. coli* exposure experiment (Fig. 9.1) with the growth curves obtained from the exposure of *P. putida* KT2440 (Fig. 9.11), shows an immediate difference with regard to the [C₄mim] Cl exposed cultures.
Fig. 9.11 - Average growth curves of *P. putida* KT2440 during exposure to a series of imidazolium chloride ionic liquids

- Positive control, ♦ [C₂mim] Cl, ▲ [C₄mim] Cl, • [C₆mim] Cl, OD readings were measured by preparing a 1/10 dilution of the 100μL samples, OD read at 680nm and data points are the average of the OD readings from the 3 replicate flasks, error bars represent standard deviation between the three flasks.

From the *E. coli* batches it was noted that cell growth in the cultures exposed to [C₄mim] Cl did not occur until between the 10h and 24h time point. However for the *P. putida* KT2440 batches exposed to the same ionic liquid, at the same concentration there is no extended lag time, with exponential growth occurring in the *P. putida* KT2440 cultures within 4h following exposure to the ionic liquid. A consideration when comparing the two data sets (*E. coli* MG1655 and *P. putida* KT2440) must be that the *P. putida* cultures were given double the amount of incubation time prior to the addition of the ionic liquid, thereby allowing the *P. putida* cultures additional time to grow and perhaps lessening the impact of a sudden exposure to a potentially toxic substance. However comparison of the OD data collected from all three batches of *E. coli* MG1655 with all three batches of *P. 
*P. putida* KT2440 at timepoint 0h, shows that the two microorganisms had comparable OD readings. This observation suggests that the improved response of *P. putida* KT2440 to *E. coli* MG1655 is not the result of the extended incubation time given to the *P. putida* KT2440 cultures.

As with *E. coli* MG1655 comparison of the growth curves with the pH measurements recorded at the same time points, supports the observations made from the growth curves (Fig. 9.12). As with *E. coli* MG1655 a drop in pH is observed at the time point at which the cultures are actively growing, for *P. putida* KT2440 this pH shift is observed at 4h. Compared to the pH measurements taken from the *E. coli* MG1655 cultures (Fig. 9.2), the alteration in pH for the positive control and the [C₂mim] Cl exposed cultures occurs at a later suggesting that the *P. putida* cultures have a slightly extended lag time as compared with the *E. coli* cultures. No drop in pH is observed for the cultures exposed to [C₆mim] Cl exposed cultures, concurring with the recorded OD measurements, suggesting that this ionic liquid is completely toxic to the test organism.

![Fig. 9.12](image-url)

**Fig. 9.12** - pH measurements recorded from *P. putida* KT2440 cultures over a 24h time course following exposure to imidazolium chloride ionic liquids

Culture type is indicated by the colour of the bar in the above graph, Positive control, [C₂mim] Cl culture, [C₄mim] Cl culture, and [C₆mim] Cl culture.
As with the *E. coli* MG1655 cultures viable count measurements were made at 0 and 24h. The results from the viable count plates are seen in Table 9.3. Viable count information was included within the results if the viable count readings fell within the range of 30-330 CFU. Plates with more than 300 CFU were too difficult to count accurately whilst plates with fewer than 30 CFU were considered to have too few colonies to be statistically significant.

**Table 9.3 – Viable count information from timepoints 0h, 6h and 24h**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sample Type</th>
<th>Dilution</th>
<th>Colony Forming Units Counted</th>
<th>Colony Forming Units (per mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_2$mim] Cl</td>
<td>10$^{-8}$</td>
<td>256</td>
<td>2.56x10$^8$</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_2$mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_2$mim] Cl</td>
<td>10$^{-8}$</td>
<td>228</td>
<td>2.28 x10$^8$</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_4$mim] Cl</td>
<td>10$^{-8}$</td>
<td>130</td>
<td>1.30 x10$^8$</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_4$mim] Cl</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_4$mim] Cl</td>
<td>10$^{-8}$</td>
<td>255</td>
<td>2.55x10$^8$</td>
</tr>
<tr>
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<td>[C$_6$mim] Cl</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_6$mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>[C$_6$mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>10$^{-8}$</td>
<td>173</td>
<td>1.73 x10$^8$</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>+VE</td>
<td>10$^{-8}$</td>
<td>164</td>
<td>1.64 x10$^8$</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_2$mim] Cl</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_2$mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_4$mim] Cl</td>
<td>10$^{-8}$</td>
<td>203</td>
<td>2.03 x10$^8$</td>
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<tr>
<td>24h</td>
<td>[C$_4$mim] Cl</td>
<td>10$^{-8}$</td>
<td>198</td>
<td>1.98 x10$^8$</td>
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<tr>
<td>24h</td>
<td>[C$_4$mim] Cl</td>
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<td>146</td>
<td>1.46 x10$^8$</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_6$mim] Cl</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_6$mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_6$mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA – CFU count did not fall within the accepted 30-300 CFU range
9.3.4. FT-IR measurement of *P. putida* KT2440 exposed to imidazolium chloride ionic liquids

As with previous batches, FT-IR spectra were collected from both biomass and supernatant samples. Collected FT-IR spectra were analysed using PCA and PC-DFA. The plot obtained from the PCA using 10 PCs accounting for 98.16% of the total variance can be seen the Fig. 9.13.

From the PCA plot in Fig. 9.13 it is seen that the extraction of 10 PCs from the *P. putida* KT2440 data, accounts for roughly the same amount of variance as the extraction of 10 PCs from the *E. coli* data unlike in the *E. coli* PCA plot a clear separation of the time point 0h samples is visible following PCA. Additionally, there also appears to be some separation of the samples collected following exposure to the ionic liquid [C₆mim] Cl from the rest of the collected samples.

![Figure 9.13](image-url)

**Fig. 9.13** - PCA plot showing PC1 versus PC2 from the biomass spectra

A – Positive Control, B – [C₂mim] Cl cultures, C – [C₄mim] Cl cultures, D – [C₆mim] Cl cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 2 – 4h, 3 – 6h, 4 – 8h, 5 – 10h and 6 – 24h.
To further investigate the information gathered from the PCA plot the loadings plots of the first two principal components from the biomass samples were plotted and the loadings plot for principal component 1 is shown in Fig. 9.14. From the loadings plot of PC1 it can be seen that the resulting plot is fairly complex, however the largest degree of variance appears to be recorded between wavenumbers 2000-1000 cm\(^{-1}\). This region of the spectra contains a large number of vibrations including vibrations associated with proteins as well as vibrations associated with polysaccharides.

From the PC2 loadings plot (data not shown) it is difficult to determine which area of the spectra is showing the largest amount of variance, it is therefore not possible to extract further information from this plot.

![PCA loading plot for PC1](image)

**Fig. 9.14** – PCA loading plot for PC1

Further analysis using PC-DFA (Fig. 9.15) in which the class structure is based on the biological replicates, shows a distinct separation based upon both time and toxicity of the ionic liquid. As might be expected the largest observed separation is determined by the toxicity of the ionic liquid to which the culture is exposed.
Cultures which have been exposed to the most toxic ionic liquid ([C₆mim] Cl), in which no culture growth was observed, cluster away from the cultures exposed to the less toxic ionic liquids [C₂mim] Cl and [C₄mim] Cl as well as the positive control cells, in all of which good culture growth was observed.

In addition to the clustering patterns based around the toxicity of the ionic liquids, there can also be observed a separation along a time axis, with samples clustering along an axis of increasing exposure time. Even within the cultures exposed to the toxic [C₆mim] Cl ionic liquid a time dependent separation can be observed within the plot area. This separation based on time indicates that the FT-IR spectra are reflecting physiological changes within the cell cultures rather than simply detecting the presence of absence of the different ionic liquids.

**Fig. 9.15** — PC-DFA plot generated from collected *P. putida* KT2440 biomass spectra using PCs 1-10 which account for 98.26% of the total variance

A – Positive Control, B – [C₂mim] Cl cultures, C – [C₄mim] Cl cultures, D – [C₆mim] Cl cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 2 – 4h, 3 – 6h, 4 – 8h, 5 – 10h and 6 – 24h
9.3.5. Physiological measurements of *P. putida* DOT-T1E exposed to imidazolium ionic liquids

The final round of testing with this series of imidazolium chloride ionic liquids saw the substitution of *P. putida* KT2440 with *P. putida* DOT-T1E. The experimental procedure was performed as has previously been described for *P. putida* KT2440.

The growth curves generated from the collection of OD data can be seen in Fig. 9.16.

![Average growth curves of *P. putida* DOT-T1E during exposure to a series of imidazolium chloride ionic liquids](image)

**Fig. 9.16 - Average growth curves of *P. putida* DOT-T1E during exposure to a series of imidazolium chloride ionic liquids**

- Positive control, ♦ [C$_2$mim] Cl, ▲ [C$_4$mim] Cl, • [C$_6$mim] Cl, OD readings were measured by preparing a 1/10 dilution of the 10µL samples, OD read at 680nm and data points are the average of the OD readings from the 3 replicate flasks, error bars represent standard deviation between the three flasks.

As with the growth curves recorded from *P. putida* KT2440 and unlike those recorded for *E. coli* MG1655, it can be seen that the cultures exposed to [C$_4$mim] Cl
did not experience a lag time significantly longer than that experienced by the cultures exposed to the ionic liquid \([C_2\text{mim}]\text{Cl}\) or the positive control cells.

Comparison of the OD data with the pH measurements collected at the same time points shows, as with the other batches that the OD measurements are supported by the pH measurements (Fig. 9.17). No alteration in the pH measurement is observed for the cultures exposed to the ionic liquid \([C_6\text{mim}]\text{Cl}\), whilst a drop in pH is observed for the positive control cultures as well as the cultures exposed to the ionic liquids \([C_2\text{mim}]\text{Cl}\) and \([C_4\text{mim}]\text{Cl}\).

![Fig. 9.17 - pH measurements recorded from *P. putida* DOT-T1E cultures over a 24h time course following exposure to imidazolium chloride ionic liquids](image)

**Fig. 9.17 -** pH measurements recorded from *P. putida* DOT-T1E cultures over a 24h time course following exposure to imidazolium chloride ionic liquids

Culture type is indicated by the colour of the bar in the above graph, **Positive control**, \([C_2\text{mim}]\text{Cl}\) culture, \([C_4\text{mim}]\text{Cl}\) culture, and \([C_6\text{mim}]\text{Cl}\) culture.

These results are further supported by the viable count information collected at time points 0h, 6h and 24h. The results from these viable count readings can be seen in Table 9.4. Again from these results it is clear that no growth was recorded in the cultures exposed to the ionic liquid \([C_6\text{mim}]\text{Cl}\), whilst good culture growth was
recorded in both the positive control cultures as well as the cultures exposed to the other two ionic liquids.

Table 9.4 – Viable count information from timepoints 0h, 6h and 24h

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Sample Type</th>
<th>Dilution</th>
<th>Colony Forming Units Counted</th>
<th>Colony Forming Units (per mL)</th>
</tr>
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<tbody>
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<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>$10^{-6}$</td>
<td>62</td>
<td>$6.2 \times 10^5$</td>
</tr>
<tr>
<td>0h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>6h</td>
<td>+VE</td>
<td>NA</td>
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<td>NA</td>
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</tr>
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<td>$2.81 \times 10^8$</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_2$mim] Cl</td>
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<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_2$mim] Cl</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>24h</td>
<td>[C$_2$mim] Cl</td>
<td>$10^{-8}$</td>
<td>170</td>
<td>$1.70 \times 10^8$</td>
</tr>
</tbody>
</table>

NA – CFU count did not fall within the accepted 30-300 CFU range

9.3.6. FT-IR measurement of *P. putida* KT2440 exposed to imidazolium chloride ionic liquids

In addition to OD, pH and viable count readings, FT-IR spectra were also collected from both biomass and supernatant samples. These spectra were analysed using the methods of PCA and PC-DFA as previously described.
The plot from the analysis of the biomass samples using PCA and using PCs 1 vs. 2 is shown in Fig. 9.18.

Analysis was performed on both biomass samples and supernatant samples as described for the previous test organisms. PCA was carried out on the collected FT-IR spectra from the biomass samples and is shown in Fig. 9.18.

**Fig. 9.18** - PCA plot showing PC1 versus PC2 from the biomass spectra

- A – Positive Control, B – [C₂mim] Cl cultures, C – [C₄mim] Cl cultures, D – [C₆mim] Cl cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 2 – 4h, 3 – 6h, 4 – 8h, 5 – 10h and 6 – 24h

From the above PCA plot it can be seen that not only does the extraction of 10 PCs account for less of the total variance within the data set, 96.77%, than for *P. putida* KT2440, 98.16% and *E. coli* MG1655 99.77%. Also the combination of PCs 1 and 2 accounts for less of the total variance than for the other two test organisms.

Visual examination of the PCA plot shows no distinct clustering patterns, unlike for *P. putida* KT2440 in which the time point samples from 0h were clearly separated from the other samples.
To further investigate the information gathered from the PCA plot the loadings plots of the first two principal components from the biomass samples were plotted and the loadings plot for principal component 1 is shown in Fig. 9. From the loadings plot of PC1 it can be seen that the resulting plot is fairly complex, however the largest degree of variance appears to be recorded between wavenumbers 2000-1000 cm\(^{-1}\). This region of the spectra contains a large number of vibrations including vibrations associated with proteins as well as vibrations associated with polysaccharides.

From the PC2 loadings plot (data not shown) it is difficult to determine which area of the spectra is showing the largest amount of variance, it is therefore not possible to extract further information from this plot.

Further analysis of the collected biomass samples was performed using PC-DFA, the resulting plot can be seen in Fig. 9 20. The class structure was again based on the biological sample replicates. In this instance it can be seen as for the other test organisms that there is a clustering separation based upon both ionic liquid toxicity and exposure time.
As for the *P. putida* KT2440 samples the largest separation is based upon exposure to the different ionic liquids with the biggest separation being between the cultures exposed to the [C₆mim] Cl ionic liquid and the samples exposed to the less toxic ionic liquids, [C₂mim] Cl and [C₄mim] Cl as well as the positive control samples.

Additionally within each of the culture conditions, i.e. cultures exposed to different ionic liquids and the positive control samples, there is a clear clustering pattern based along increasing exposure time to the ionic liquid. Again this separation is indicative of detection of physiological changes within the culture, rather than a simple detection of the different ionic liquids.

![Discriminant Function Plot](image)

**Fig. 9** 20 - PC-DFA plot generated from collected *P. putida* DOT-T1E biomass spectra using PCs 1-10 which account for 96.77% of the total variance

A – Positive Control, B – [C₃mim] Cl cultures, C – [C₄mim] Cl cultures, D – [C₆mim] Cl cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 2 – 4h, 3 – 6h, 4 – 8h, 5 – 10h and 6 – 24h

As with the previous two bacteria samples were collected from the supernatant, samples were analysed using the same chemometric methods as for the biomass
samples. The PC-DFA plot, from 10 extracted PCs with class structure based on the biological replicates within the experiment is shown in Fig. 9.21. From the plot it is apparent that the positive control samples cluster away from cultures exposed to any of the ionic liquids. However there is also a clear separation between the cultures exposed to the ionic liquid \([C_2\text{mim}]\text{Cl}\) and \([C_4\text{mim}]\text{Cl}\) and the toxic ionic liquid \([C_6\text{mim}]\text{Cl}\). This suggests that the FT-IR spectra are detecting not only the differences between the ionic liquids i.e. the difference in the alkyl chain length of the cation, but is also detecting differences in the growth media, caused by actively growing cultures as opposed to non-growing or dead cultures.

![PC-DFA plot](image)

**Fig. 9.21** - PC-DFA plot generated from collected *P. putida* DOT-T1E supernatant spectra using PCs 1-10 which account for 99.90% of the total variance

A – Positive Control, B – \([C_2\text{mim}]\text{Cl}\) cultures, C – \([C_4\text{mim}]\text{Cl}\) cultures, D – \([C_6\text{mim}]\text{Cl}\) cultures. Numbers represent time points, 0 – 0h, 1 – 2h, 2 – 4h, 3 – 6h, 4 – 8h, 5 – 10h and 6 – 24h

The comparison of the responses of the three test organisms has revealed a number of differences between the three. The most striking difference is observed
in the cultures exposed to the ionic liquid [C₄mim] Cl. A direct comparison of the specific growth rates for the three test organisms can be seen in Table 9.5.

The extended lag time following the addition of the ionic liquid [C₄mim] Cl to the test organism *E. coli* MG1655 as compared to the two *P. putida* strains, suggests that the *P. putida* strains have a better ability to adapt to the addition of a potentially toxic solvent to the culture media than the *E. coli* MG1655 cultures.

Table 9.5 – Comparison of specific growth rates from the three test organisms tested with imidazolium chloride ionic liquids, specific growth rates are recorded as a percentage of the positive control cells

<table>
<thead>
<tr>
<th></th>
<th>Batch 1</th>
<th>Batch 2</th>
<th>Batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. coli</em> MG1655</td>
<td><em>P. putida</em> KT2440</td>
<td><em>P. putida</em> DOT-T1E</td>
</tr>
<tr>
<td>[C₂mim] Cl</td>
<td>79% ± 17</td>
<td>81% ± 7</td>
<td>85% ± 9</td>
</tr>
<tr>
<td>[C₄mim] Cl</td>
<td>16% ± 1</td>
<td>79% ± 13</td>
<td>70% ± 6</td>
</tr>
<tr>
<td>[C₆mim] Cl</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

From the comparison of the specific growth rates for the different test organisms it can be seen that whilst none of the test organisms are able to grow in the presence of the ionic liquid [C₆mim] Cl, the response of the test organisms to the ionic liquids [C₂mim] Cl and [C₄mim] Cl varies considerably.

9.4. Conclusions

Overall, the *P. putida* strains performed best when exposed to the ionic liquid series, with little to choose between the specific growth rates for the two strains.
Of the three test organisms the *E. coli* MG1655 cultures performed worst after exposure to the ionic liquid [C₄mim] Cl. Although these *E. coli* cultures in general performed well against the ionic liquid [C₂mim] Cl with specific growth rates for batches 1 and 2 giving specific growth rates of over 70%. However exposure to the ionic liquid [C₄mim] Cl, greatly reduces the specific growth rates of the cultures, with only one of the three batches achieving a specific growth rate of greater than 20%, this would agree with previously reported findings in which it was shown there was a significant difference in growth rates between [C₂mim]⁺ and [C₄mim]⁺ ionic liquids (Wells and Coombe, 2006).

These results suggest that the *P. putida* strains had an advantage over the *E. coli* cultures that allowed the *P. putida* cultures to adapt more efficiently and in a shorter space of time than the *P. putida* cultures.

As has been previously discussed it is believed that the toxicity of an ionic liquid is part caused by its ability to accumulate in the cell membrane in high concentrations causing disruption to the cell membrane. The disruption of the membrane (Cornmell *et al.*, 2008b) affects both the cells ability to generate ATP, through protein motive force (pmf), as well as the structural integrity of the cell. Ionic liquids with longer alkyl chains cause greater disruption to the membrane due to their ability to accumulate to higher levels within the membrane (Latala *et al.*, 2009). Accumulation of ionic liquid within the cell membrane has been reported to increase the fluidity of the cell membrane. To counter this increase in fluidity cells have a number of adaption mechanisms which can be utilized.

Some of these tolerance mechanisms have been reported across numerous species such as altering the degree of saturation of the membrane fatty acids and alteration of the composition of the phospholipid head groups to increase the degree of cardiolipin in the cell membrane, thereby reducing the fluidity of the cell membrane (Segura *et al.*, 1999), (Weber and deBont, 1996). However both of these tolerance mechanisms require the synthesis of new lipid molecules and therefore can not be
used to give immediate protection to the organism following a sudden addition of toxic solvent.

An additional mechanism of solvent tolerance that has been reported in *P. putida* strains is the alteration of the proportion of *cis* to *trans* fatty acids in the phospholipid membrane (Diefenbach et al., 1992), (Heipieper, 2003), (Heipieper et al., 1992). The *cis/trans* isomerisation process does not require the synthesis of new lipid and as such is a response mechanism which can be immediately deployed by the cell to counter the effects of solvent exposure (Junker and Ramos, 1999).

From the observations made from the specific growth rates (Table 9.5), it may be possible that the *cis/trans* isomerisation mechanism is the advantage which the *P. putida* cultures have as compared to the *E. coli* cultures following exposure to the ionic liquid [C₄mim] Cl. If the *P. putida* cultures are able to employ the *cis* to *trans* isomerisation mechanism this would allow them to adapt immediately to a sudden exposure to a toxic solvent. However for the *E. coli* cultures, new fatty acid synthesis would be required to counter the membrane solubilising impact of the toxic solvent. This new lipid synthesis would require both time and energy and as such may explain the extended lag time shown by the *E. coli* cultures following exposure to [C₄mim] Cl.

The results collected from this series of exposure experiments, show that FT-IR analysis of collected biomass samples can be separated based on the physiological changes within the cells, rather than simply detecting the presence or absence of an ionic liquid within a culture.

Additionally, the large differences observed between the specific growth rates of the two strains of *P. putida* and *E. coli* suggest that when selecting an organism for use with a particular ionic liquid it is essential to test the specific micro-organism against the chosen ionic liquid, rather than relying on general reports of toxicity responses for similar organisms.
10. Conclusion

10.1. Solvent Toxicity

The ability of microorganisms to withstand and adapt to the presence of toxic solvents is becoming an increasingly important area of research. As new ways are sought to lessen the impact of our actions on the environment the importance of microorganisms as both biocatalysts and bioremediation agents will continue to grow.

Currently the synthesis of many critical and high value products, such as chemicals and pharmaceutical intermediates is achieved through the use of synthetic chemistry and chemical catalysis. These processes are often complex and non-specific; producing unwanted side products, which in themselves may be environmentally damaging. In addition, they are also energy intensive when considering the amount of fossil fuel required to achieve the high temperatures and pressures required for many chemical catalysis processes.

The substitution of a biocatalyst for a chemical catalyst will have the double benefit of reducing the energy requirement of the process, as most biocatalysts operate at mild temperatures and atmospheric pressure, as well as producing a more specific process, including enantiomeric selectivity, in which the formation of undesirable side products can be largely avoided.

Not only can biocatalysts reduce fuel usage in catalytic processes they also have the potential to generate new types of re-newable fuels (Nicolaou et al., 2010). The use of carbon neutral biomass to produce biofuels include microbial diesel (Rude and Schirmer, 2009), bioethanol (Rao et al., 2007) and short chained alkanes and alkenes (Schirmer et al., 2010).

That microorganisms can provide greener, cleaner alternatives to many processes, including the generation of new fuel sources is becoming increasingly apparent. However, the use of microorganisms in these processes presents a set of operational considerations not encountered with the use of chemical catalysts and
fossil fuels. It is often the case that either the substrate or product of a microbial biotransformation is toxic to the microorganism, often at concentrations well below that which would need to be employed to make such biotransformation processes economically feasible (Nicolaou et al., 2010).

To begin to address these process limitations it is necessary to have both an understanding of the way in which exposure to toxic solvents affects the microorganism and a reliable way of observing when phenotypic changes to the microorganism are caused by solvent exposure.

The mechanisms of solvent toxicity have been widely investigated and reported in published literature (Ramos et al., 2002), (Heipieper, 2003), (Ramos et al., 1998), (Ramos et al., 2001), (Rojas et al., 2001), (Segura et al., 1999), (Segura et al., 2005), (Segura et al., 2003), (Sikkema et al., 1994), (Sikkema et al., 1995), (Junker and Ramos, 1999), (Nicolaou et al., 2010). The overall impact of a solvent on a microorganism is a combination of two factors; the toxicity of the solvent and the intrinsic resistance of the microorganism. A solvent will partition into the phospholipid cell membrane of a microorganism causing disruption to cellular processes, such as ATP generation which occur across the membrane as well as disruption to the structure of the membrane itself. The degree to which the solvent will partition into and accumulate in the membrane is determined by the lipophilicity of the solvent.

As important as the properties of the solvent are the properties of the microorganism. Most microorganisms have evolved mechanisms by which they are able to adapt to and reduce the impact of exposure to toxic solvents. These mechanisms include the up regulation of membrane efflux pumps (Aono, 1998), metabolism of the solvent (Mosqueda et al., 1999) and alteration to the physical properties of the cell membrane (Junker and Ramos, 1999), (Ramos, 2001), (Ramos et al., 2002), (Heipieper et al., 1992), (Heipieper et al., 2003), (von Wallbrunn et al., 2003).

The expression of these adaption mechanisms are reflected as phenotypic alterations to the cell. In order to determine whether or not microorganism are
able to adapt to the presence of solvent it is desirable to have a method by which
phenotypic cell differences can be monitored. In Chapters 3 and 4 the use of
specific growth rates in combination with FT-IR spectroscopy and cluster analysis
has been described as a method by which phenotypic alterations between different
culture conditions can be monitored.

In this instance exposure of the microorganisms E. coli MG1655 and P. putida
KT2440 to toluene has been monitored at three exposure concentrations over a
24h time course. For both E. coli MG1655 and P. putida KT2440 it has been possible
by the use of FT-IR spectroscopy combined with chemometrics to visualise
phenotypic difference between both the solvent concentration and the exposure
times of the test cultures.

In addition to this the microorganism P. putida KT2440 has also been cultured in the
presence of the solvents chlorobenzene and fluorobenzene. As with the toluene
exposed cultures, thorough a combination of specific growth rates, FT-IR
spectroscopy and clustering analysis it has been possible to visualise differences
between the phenotypes of the solvent exposed and positive control cultures.

The use of FT-IR spectroscopy as a means of monitoring phenotypic change in a
microbial cell culture has its limitations in that, due to the complexity of the spectra
collected, it is difficult to determine the exact cause of the phenotypic variation
between the culture. The use of techniques such as GC-MS or LC–MS would give
greater detail with regard to the alterations in the levels of metabolites. The use of
proteomics techniques such as 2D electrophoresis, MALDI-MS peptide mass
fingerprinting or mass spectrometry based protein quantification methods such as
ICAT or iTRAQ could be used to give information regarding the abundance and
identification of membrane proteins. Using a different approach, the use of
genomic screening methods could be used to identify the up and down regulation
of particular genes and associated those genomic changes with survival rates of the
test organism. However, the use of any, or all, of these methods requires sample
preparation, which is both complex and time consuming. By comparison, the
preparation of FT-IR samples is simple, time efficient and provides a good indication
of whether there has been a significant difference to the phenotype of the cell which would warrant further, in depth investigation using one or more of the methods described above.

The ability of a microorganism to withstand exposure to a toxic solvent is a vital property in both biocatalysis and bioremediation applications. However, there are a limited number of microorganisms which display intrinsic high levels of tolerance towards organic solvents, two such examples would be *P. putida* DOT-T1E (Ramos *et al.*, 1995) able to grow in the presence of 90% (v/v) toluene, and *P. putida* IH-2000 (Inoue and Horikoshi, 1989) able to grow in the presence of 50% (v/v) toluene. However, these microbial strains are exceptional in their ability to withstand such high concentrations of a toxin such as toluene. For most microorganisms the concentration of solvent which can be tolerated is far lower. This limitation drastically reduces the number of potential biocatalysts available, thereby, reducing the number of potential applications.

To counter this limitation attempts have been made to adapt specific bacterial cultures to the presence of toxic solvent. These attempts can follow one of two paths, firstly the genetic manipulation of the microorganism by insertion or over expression of specific beneficial gene sequences, or secondly by continuous culture of the microorganism in sub-lethal concentrations of the solvent. By culturing the microorganism in sub-lethal concentrations of the solvent continuous selective pressure is applied to the culture, so that those cells which show adaption to the solvent are able to divide and reproduce, whilst cells which show susceptibility to the solvent are gradually eliminated from the culture. This approach has been used to investigate the response of both *E. coli* and *P. putida* to long term exposure to toluene, salt and ethanol (Munoz *et al.*, 2009), (Arense *et al.*, 2010), (Goodarzi *et al.*, 2010).

Thus in order to assess the possibility of improving the tolerance of the test organisms *E. coli* MG1655 and *P. putida* KT2440 to toluene exposure, a three month continuous batch culture experiment was performed in which the two test organisms were repeatedly cultured in the presence of sub-lethal concentrations of
toluene, full experimental details of this work and its results are given in Chapter 4. At the end of the three month exposure period the phenotypic changes associated with each of the culture conditions i.e. toluene exposed and unexposed were assessed by means of FT-IR spectroscopy with cluster analysis and growth curves, recorded by means of optical density measurements. From the results collected it was seen that for both *E. coli* MG1655 and *P. putida* KT2440 samples which had been repeatedly cultured in a sub lethal concentration of toluene the final biomass for these samples was higher than that for the *E. coli* MG1655 and *P. putida* KT2440 which had been repeatedly cultured in the absence of toluene.

In addition to examination of the growth data, inspection of the FT-IR data also revealed phenotypic differences between the different culture conditions.

These results suggest that the continuous batch culture of microorganisms in sub lethal concentrations of a toxic solvent over a relatively short time period can positively influence the phenotype of the culture. These phenotypic adaptations suggest that continuous batch culturing in the way described, allow the culture to more easily adapt to a sudden addition of the same toxic solvent.

The results obtained from the work performed on solvent stress and adaption suggest that not only is adaption of microorganisms to sub lethal concentrations of solvent possible through the use of a continuous batch culture process but also that a combination of growth data, FT-IR spectroscopy with chemometrics are appropriate tools with which to observe phenotypic alterations in microorganism cultures during exposure to solvent.

**10.2. Proteomic Method Development**

As discussed above the exposure of microorganisms to solvents results in a number of phenotypic changes to the microorganism. These phenotypic changes are mostly associated with the cell membrane; one such change is the alteration in the amount and type of protein within the cell membrane. In order to detect and assess these changes it is necessary to develop a method which will quickly, accurately and
reproducibly show the alterations which are occurring within the cell membrane proteome.

The most common method of protein analysis is by the use of 2D gel electrophoresis. In this approach extracted proteins are separated both by their pI point and by their molecular weight. In order to produce clear, reproducible and good quality 2D gels which can be used for further analysis such as peptide mass fingerprinting (aided by MS), it is first necessary to develop a protein extraction method which is in itself both robust and reproducible.

To this end a number of different extraction methods were tested with the microorganism *P. putida* KT2440. Despite the many methods tested and variants thereon, it was not possible within the available time to develop a method which gave sufficiently good and reproducible results to allow for further analysis of the protein component of the *P. putida* KT2440 cultures.

As the effect of exposure of a microorganism to an ionic liquid is believed to be similar to the effect of solvent exposure, i.e. the ionic liquid will partition into and accumulate in the cell membrane, it was decided to proceed with the development of a method for the extraction of protein from cultures of *E. coli* MG1655, which had been exposed to a number of water immiscible ionic liquids over a 24h exposure period.

In this instance the method used for protein extraction (Molloy, 1998) appeared initially to give good 2D electrophoresis gels with a good number of resolved protein spots visible on all of the gels produced. However, as for the *P. putida* KT2440 cultures, repetition of the extraction and analysis process did not appear to give comparable results between the two batches.

Due to time limitations, additional analysis was not performed in this area, however the results obtained from the extraction of the *E. coli* cultures showed initially promising results which would be worthy of further investigation.
10.3. Ionic Liquid Toxicity

As has been described above the toxic nature of many conventional organic solvents towards potential biocatalysts has thus far limited the number of processes in which whole cell biocatalysts can be used. To improve this situation and allow for more widespread application of biological catalysts the use of catalytic systems in which ionic liquids would be used as replacements for conventional organic solvents have been discussed.

Ionic liquids are liquids composed entirely of ions at room temperature and have often been reported in the literature as ‘green’ solvents. This label has often been based solely on the negligible vapour pressure of these solvents at room temperature. Although this property of negligible vapour pressure will limit the loss of solvent to the atmosphere, thereby reducing both the risk of accidental release and operator exposure, it is not the only consideration which must be taken into account when assessing the potential of ionic liquids as replacements for organic solvents.

Toxicity screening of a number of ionic liquids has revealed that many are highly toxic to microbes- and aquatic organisms, as well as indicating that they are difficult to biodegrade and will accumulate to high concentrations within aquatic systems (Wang et al., 2011), (Bernot et al., 2005a), (Bernot et al., 2005b), (Cho et al., 2008), (Docherty and Kulpa, 2005), (Samori et al., 2011).

As such it is vital that the toxic potential of any ionic liquid under consideration as a replacement for a conventional solvent is thoroughly assessed. However, due to the large numbers of ionic liquid structures which are available, complete exhaustive toxicity testing against all structures is not a viable possibility both in terms of the cost and time commitments required. To this end a fast, reliable and simple to use initial screening system was required. The agar diffusion toxicity test has been used and adapted to screen a large number of ionic liquids with diverse cation and anion structures against five test organisms.
The majority of the screening work has been performed using the test organism *E. coli* MG1655 (Chapter 6). From a combination of data collected from the agar diffusion plates and specific growth rates calculated from OD measurements it has been possible to identify a number of toxic ionic liquid structures as well as a number of biocompatible ionic liquid structures.

In addition to screening *E. coli* MG1655 four strains of *P. putida* were also screened, of these strains one has been reported to be sensitive to the presence of toluene (KT2440) (Segura *et al.*, 2003), whilst a second has been reported to have a high level of intrinsic resistance to toluene exposure (DOT-T1E) (Ramos *et al.*, 1995). The third and fourth strains (DOT-T1E 18 and DOT-T1E PS28) are efflux pump knockout mutants of the highly toluene tolerant DOT-T1E strain (Chapter 7).

The screening of ionic liquids with this highly solvent tolerant strain (DOT-T1E) has not been previously reported in the literature. Despite the tolerance of the strain to high concentration of organic solvents, such as toluene, there did not appear to be any significant improvement in the specific growth rates or agar diffusion results of this strain as compared to either the toluene sensitive KT2440, the efflux pump knockout mutants or *E. coli* MG1655.

As with cultures exposed to conventional solvents it was of interest to determine whether the phenotypic differences in the cultures caused by exposure of the culture to ionic liquid could be visualised through the use of FT-IR spectroscopy and cluster analysis. To this end a series of batch cultures were prepared using the microorganisms *E. coli* MG1655, *P. putida* KT2440 and *P. putida* DOT-T1E and screened against a series of water miscible imidazolium chloride ionic liquids of increasing alkyl chain lengths (Chapter 9).

From the FT-IR spectra of the biomass samples collected over the course of a 24h exposure experiment it was demonstrated that FT-IR spectroscopy could be used to visualise phenotypic changes in the culture due to the presence of ionic liquid.

Also investigated by the use of FT-IR spectroscopy and chemometric analysis was the response of the microorganism *E. coli* MG1655 to a set of three water
immiscible ionic liquids. As for the water miscible ionic liquids it was determined that phenotypic alterations to the test cultures could be visualised by means of FT-IR spectroscopy. For the water immiscible ionic liquids it was also noted that the trajectories of the ionic liquid exposed samples in the clustering plots were different, possibly suggesting that the phenotypic variation in the culture is dependent upon the structure of the water immiscible ionic liquid to which the culture is being exposed (Chapter 8).

10.4. Concluding Remarks

The possibility of decreasing the environmental impact of synthetic processes by the more wide spread use of biocatalysts in industrial processes is of great interest at the current time. In order for biocatalysts to become viable alternatives to current processes there are a number of obstacles which must be overcome. Chiefly, the susceptibility of many potentially useful biocatalysts to either the substrate or end product of a desired reaction.

To investigate this issue two different approaches have been pursued: first, the monitoring of phenotypic changes to a microorganism culture during solvent exposure and the adaption of a microorganism to withstand the presence of solvent through continuous batch culture. Secondly, the possibility of replacing conventional solvents with ionic liquids, as with the solvent exposed cultures the phenotypic changes to the culture during ionic liquid exposure was monitored and an ionic liquid toxicity screening process based on agar diffusion plates and specific growth rates was developed.

In order to assess the impact of toxicity on different micro-organisms the technique of FT-IR spectroscopy combined with multivariate statistics has been applied throughout this work. This technique has been used successfully to monitor the phenotypic changes occurring within a culture of micro-organisms. Although this technique has been applied with some success in the present work it is important to note the limitations of this approach, i.e. the collection of FT-IR spectra from biomass samples from whole cells produces FT-IR spectra and loading plots from
PCA are complex and difficult to interpret. In order to negate this problem in future work it may be beneficial to fractionate samples into cellular components before FT-IR analysis, this approach may have the effect of reducing the complexity of the collected spectra making the spectra easier to interpret in terms of biological significance.

Both of these approaches have both positive and negative aspects and it seems likely that future catalytic processes which make use of biological catalysts will utilise both highly resistant microorganisms and solvents such as ionic liquids, which offer the best possible combination of desirable properties in terms of both reaction rates and environmental impact.
11. Bibliography


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JASTORFF, B., STORMANN, R., RANKE, J., MOLTER, K., STOCK, F., OBERHEITMANN, B., HOFFMANN, W., HOFFMANN, J., NUCHTER, M., ONDRUSCHKA, B. & FILSER, J.


OECD.


OECD.


UFT/MERCK Ionic Liquids Biological Effects Database.


WALDEN, P. (1914) B ACAD SCI ST PETERSBOURG, 8, 405-422.


### 12. Appendix 1 Cation and Anion Structures

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-alkyl-3-methylimidazolium</td>
<td>[C$_R$mim]$^+$</td>
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<td>1-alkyl-1-methylpyrolidinium</td>
<td>[C$_R$mpyr]$^+$</td>
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<tr>
<td>1-methy-1-methylpiperidinium</td>
<td>[C$_R$mpip]$^+$</td>
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<td>tetraalkyphosphonium</td>
<td>[P$_{R1}R2R3R4]$^+$</td>
<td><img src="image4" alt="Structure" /></td>
</tr>
<tr>
<td>tetraalkylammonium</td>
<td>[N$_{R1}R2R3R4]$^+$</td>
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<tr>
<td>Name</td>
<td>Abbreviation</td>
<td>Structure</td>
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<tr>
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<td>--------------------</td>
</tr>
<tr>
<td>singly hydroxylated tetraalkylammonium</td>
<td>$[N_{R_1 \ R_2 \ R_3} \ OH]^+$</td>
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</tr>
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<td>bis(2-hydroxyethyl)dialkylammonium</td>
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<td>tris(2-hydroxyethyl)alkylammonium</td>
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<td>[(C₄O)₂PO₂]⁻</td>
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<td>Abbreviation</td>
<td>Structure</td>
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<tr>
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<td>([{^\text{C}_8\text{O}}_2\text{PO}_2}]^-)</td>
<td><img src="image1" alt="Structure" /></td>
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<tr>
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<td>([C_n\text{OSO}_3]^-)</td>
<td><img src="image2" alt="Structure" /></td>
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<tr>
<td>4-methoxyphenylsulfonate (tosylate)</td>
<td>[tosylate]^-</td>
<td><img src="image3" alt="Structure" /></td>
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<tr>
<td>1,4-bis(2-ethylethoxy)-1,4-dioxo-butane-2-sulfonate (docusate)</td>
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<td><img src="image4" alt="Structure" /></td>
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</table>

All structures kindly provided by Prof Gill Stephens, University of Nottingham
13. Appendix 2 - Published Literature Review

Accelerating the discovery of biocompatible ionic liquids

Nicola Wood and Gill Stephens

Received 9th November 2009, Accepted 11th December 2009
First published as an Advance Article on the web 12th January 2010
DOI: 10.1039/b9234286

High throughput screening is the first stage of determining the ecotoxicity of ionic liquids. The available methods are reviewed, and a critical analysis of the problems and pitfalls is presented.

Introduction

Ionic liquids have numerous applications in industrial processes. In general, ionic liquids present lower hazards or environmental risks than conventional solvents when used in “closed” processes, since they are non-volatile and are rarely flammable or explosive. However, ionic liquids are also candidates for use in consumer goods (e.g. batteries, textiles, household goods, cosmetics), in applications where accidental or deliberate environmental release is a possibility, and in applications where consumer perceptions of low environmental risk are crucial.

In such cases, a thorough understanding of potential health hazards and environmental impact is essential. The target is to identify safe, non-toxic, environmentally friendly ionic liquids by thorough ecotoxicological testing.

“Our danger is not too few, but too many options... to be puzzled by innumerable alternatives.”
Sir Richard Livingstone

Although Richard Livingstone was not describing ionic liquids, his statement is fitting. Ionic liquids have a tunable nature, meaning that the combination of a different cation and anion will produce a wholly new ionic liquid with its own unique structure and properties. This diversity presents the opportunity to engineer solvents with the specific properties required for a particular application. At the same time, this presents an unusual problem: there are so many possible structures that to synthesize, characterize and test them all would require a truly massive effort. At present, this level of analysis is not a possibility and so QSAR (quantitative structure property relationship) modeling of existing data coupled with high throughput screening of new ionic liquids must be relied upon to point the way towards useful ionic liquid structures.

That the chosen solvent should be fit for purpose is a given, but another set of criteria must be given at least equal importance. Finding ionic liquids which will have the smallest environmental impact must be a priority. The majority of the ionic liquid toxicity studies to date have focused on ionic liquids containing midazolam-based cations, and many of the structures tested have been found to be as toxic or, in some cases, more toxic than the conventional organic solvents which they are intended to replace. 1-3. Ionic liquids have the potential to make a big difference to the environmental impact.

Nicola Wood is a PhD student in the final year of her PhD program at the University of Manchester with Prof. Roy Gosling and Dr Gill Stephens. Her research is focused on toxicity screening of ionic liquids using microorganisms. Her research is funded by the BBSRC. She previously obtained a BSc qualification in Forensic Science from Anglia Ruskin University and an MSc qualification in Physical Methods of Bioanalysis and Post Genome Science from the University of Manchester.

Gill Stephens is an interdisciplinary biochemist, with a BSc in Biochemistry from Kent and a PhD from Warwick. In 1988, she moved to a lectureship in Chemical Engineering, UMIST (now University of Manchester), and she will move to a chair in Bioprocessing at the University of Nottingham in March 2010. Gill's group works on biosynthetic production of chemicals, especially from renewable feedstocks. In this context, ionic liquids often outperform conventional solvents for delivery of water-insoluble and/or toxic substrates, hence our interest in their biocompatibility.
of chemical manufacturing processes. The key to the realisation of this potential is to select the least toxic ionic liquid structures. This depends crucially on having reliable methods in place to identify these structures.

**Ecotoxicological test methods**

Industry standard tests and those prescribed by regulatory agencies (e.g., OECD) for the measurement of ecotoxicity include EC₅₀ and LC₅₀ measurements, using both unicellular and complex, multicellular organisms. The organisms are exposed to the test substance at various concentrations and the concentration required to give half the maximal effect or half maximal inhibition is calculated. Further detailed studies such as histological effects, mutagenicity and metabolic fate may also be required. Each test has to be done with a specific living organism. Different organisms respond differently to chemical toxicants, and different parameters are measured according to the type of test. Therefore, a number of complementary tests is needed to determine the likely environmental impact of any chemical. For this reason, a detailed test battery for ecotoxicity testing of ionic liquids has been developed. This test battery begins with an initial high throughput screen and, if the results are promising, further testing is done using a hierarchical system of tests, with increasing biological complexity. The test battery assesses ionic liquid toxicity using multidimensional risk analysis, by analysing five ecotoxicological risk factors. The risk factors identified are release, spatiotemporal range, bioaccumulation, biological activity and uncertainty.

These tests provide accurate, reliable information, essential for defining the toxicity of ionic liquids which are potential candidates for use in industrial processes or consumer products. However, they are very time-consuming, costly and require significant operator expertise. Few companies or academic institutes have the financial resources or manpower to systematically test large numbers of ionic liquids using these methods. Therefore, it is important to choose the first stage screening step very carefully. The screen needs to be reliable enough to identify the most toxic ionic liquids so that they can be rejected from further studies, and it must be rapid so that throughput is maximised. In addition, it would be desirable to have tests that require simple equipment and can be done by non-experts. Ideally, the test would be suitable for research chemists to integrate within the characterisation protocols for new ionic liquids, so that preliminary indications of toxicity can be reported alongside the chemical data.

**Toxicity screening**

At present, a range of screening methods are available to test the toxicity of ionic liquids. The vast majority of these methods utilise microorganisms as their test subjects. By employing microorganisms rather than complex multicellular organisms, costs can be kept to a minimum, since microbiological media components are relatively inexpensive and the necessary equipment, such as incubators and Bunsen burners, are available in most laboratories. Commonly used toxicity tests include measurements of minimum inhibitory concentrations (MICs) or minimum biocidal concentrations (MBCs). To measure MICs, the microorganisms are grown with the test chemical at a wide range of concentrations, either in broth or agar cultures. The MIC value is the lowest concentration of the test chemical which produces no visible bacterial growth in an overnight culture. MBCs are measured by exposing the organism to the test chemical at different concentrations and then determining the lowest concentration at which there are no survivors, measured by the absence of colony formation on agar plates. The tests can be combined, and can be used as effective measures of ionic liquid toxicity and may also be used in the evaluation of the susceptibility of biofilms to ionic liquids. However, considerate microbiological expertise is needed to prepare the test cultures, the media and the dilution series of the test chemical under sterile conditions.

Further quantitative information about toxicity can be obtained by measuring inhibition of microbial growth rates in the presence of the test chemical. This allows rapid determination of EC₅₀ values. Growth rates can be measured either in a high throughput screening system such as a plate reader, or in flask cultures. The main drawbacks with these methods are that plate readers are expensive, whereas aseptic sampling is crucial to measure growth in flasks. Furthermore, flask cultures require significant quantities of ionic liquid for testing. Both methods require careful preparation of inocula and culture media, and, therefore, require significant expertise in microbiology.

Further information regarding toxicity of ionic liquids can be gleaned through measuring effects on cell viability. The traditional method is to measure viable counts. Viable counts are measured by growing a culture, making a range of dilutions, and then spreading small samples over the surface of agar plates, making an appropriate number of replicates. This is also a relatively complex procedure and requires microbiological expertise. Commercially available cell viability test kits (e.g., LIVE/DEAD BacLight bacterial viability kits) are also available. These kits measure microbial cell viability by measuring membrane integrity or cellular ATP content. Although these kits use smaller volumes of ionic liquid, they still require growth of cultures and preparation of samples for analysis. In addition, the kits and reagents are often expensive and specialised equipment is required to read the assay results. Although these test kits provide faster experimental throughput than viable counts, there has been some literature report to suggest that changes in cell morphology caused by exposure to ionic liquids can alter the readouts from a cell membrane integrity assay.

Recently, the Agar diffusion test (or Kirby-Bauer test) has been adapted to measure ionic liquid toxicity. This method is widely used in clinical laboratories to determine antibiotic susceptibility of microorganisms. The method is simple, inexpensive, requires no specialised equipment and uses only a small volume of ionic liquid. In this method, the ionic liquid is added to a sterilised filter paper disc. The filter paper is then transferred, aseptically, to a prepared lawn of microbial cells and incubated overnight. If a clear zone is formed around the filter paper, this indicates that the ionic liquid is inhibitory. The size of the inhibition zone can be measured to give an
indication of the degree of toxicity. Conversely, confluent growth around the filter indicates that the ionic liquid is not toxic to the test organism.

The great advantage of this test is that the state of the inoculum is not critical, so it would be possible to use commercially available cultures of live bacteria (e.g. from monoculture). Pre-conjugation plates. Similarly, pre-sterilized, poured agar plates are also available, so the only components needing sterilization are the filter paper discs. The other apparatus can either be bought pre-sterilized, or can be sterilized by heating with a Bunsen burner. Thus, the test is simple, cheap, requires little preparation and only basic microbiological skills are needed.

The agar diffusion method has previously been used to screen a range of ionic liquids against both Gram positive and Gram negative bacteria. Although the test can be used reliably to identify highly inhibitory ionic liquids, it is more difficult to rank the least inhibitory structures. Therefore, the method is intended to be used only as a preliminary screen, and should never be used as a substitute for proper ecotoxicity testing. We recommend that the test should be used to select the least inhibitory ionic liquids for further testing and to reject the most toxic structures. Detailed ecotoxicological testing should then be undertaken to confirm the results, and select the best ionic liquids for the desired end use.

Problems and pitfalls

Of most concern when assessing the results of any test for ionic liquid toxicity is our lack of understanding of the way in which the ionic liquid interacts with the culture media and any test results. Ionic liquids are salts, and may therefore react with the ionic components of the culture medium, resulting in pH changes, formation of precipitates, etc. Such interactions would affect measurements of microbial growth rates, and in extreme cases may affect viability tests. Similarly, these interactions may affect the agar diffusion test, since it relies on diffusion of water miscible ionic liquids through the medium to create a concentration gradient. Interactions between medium components and the ionic liquid may influence the diffusion rate or may affect the stability of the resulting concentration gradient. A further problem is that ionic liquids may be chemically reactive or may act catalytically to accelerate changes in the composition of the culture media or materials used to prepare the assays. For example, the interaction between certain ionic liquids and cellulose has been widely reported, and this may have an influence on the outcome of the agar diffusion test, in which cellulose filter papers are used. Agar itself is a carbohydrate, so it is possible that some ionic liquids may interact with agar used in MIC/MBC tests, viable counts or agar diffusion tests. In addition, the phase behaviour of ionic liquids may also interfere with analysis of results when using spectrophotometric methods to measure cell growth or viability (e.g. in biaxial systems, or when emulsions, gels or pastes are formed). Therefore, cultures and test assay mixtures must be inspected visually for any changes in colour, opacity, physical state etc., and the pH should also be measured, to ensure that potential sources of error are assessed and taken into account when interpreting the results.

The need for hierarchical screening

No single test system should be used in isolation to determine ionic liquid toxicity. Thus, high throughput toxicity screening should not be used as the first stage, simply to shortlist the least toxic structures for detailed ecotoxicological testing. Nevertheless, the ability to narrow the field of candidate ionic liquids is becoming ever more important, especially since the introduction of the European Union Regulation regarding the Registration, Evaluation and Authorisation of Chemicals (REACH) in 2007. This legislation is applicable to chemicals on the European market manufactured in quantities of more than 1 tonne per annum. The legislation lays the onus on the manufacturer to provide appropriate safety data regarding their product and failures to provide such data will mean that the product can no longer be manufactured or supplied legally.

At such a level, it is important from a cost perspective for manufacturers to test only the strongest potential candidates for future manufacturing in REACH standards.

We have been surprised by the numbers of non-inhibitory ionic liquids that we have been discovering using the agar diffusion test (ref 36 and unpublished data). This suggests that it would be sensible to use a further level of high throughput testing to narrow the field of candidate ionic liquids as much as possible before embarking on detailed regulatory tests or the ecotoxicological test battery, since detailed testing generates a large workload. Therefore, we suggest the development of a work flow using a sequence of simple toxicity tests to enable rapid, inexpensive identification of the best structures. At the first testing stage, the most toxic structures can be eliminated from further investigation, e.g. using the agar diffusion method. At the next stage, the remaining candidate structures can be investigated using either MIC/MBC tests or growth rate measurements and EIO calculations. By eliminating the structures which perform poorly in these tests, a small set of ionic liquids can be identified for in-depth ecotoxicological analysis. By following a step-wise process of ecotoxicity screening, potentially useful, biocompatible ionic liquids can be identified quickly, simply and at relatively little expense.

Data representation

Although data collected from individual toxicity tests can be considered separately, to get the most accurate toxicity profile for a particular ionic liquid, the data collected from the various toxicological tests should be considered together. However, the data will cover a wide range of disparate biological systems and will be in a variety of formats. Therefore, visualization, integration and interpretation of data from different test systems must be considered carefully.

One possibility for the integration of data from different tests was suggested by Tom Welton at the recent BAT112 meeting (Dochema, Frankfurt, 2009). We wish to commend the implementation of the method. The idea is that the data from each test is given a score (e.g. 1-3, ranging from 1 for non-toxic to 3 for very toxic). The scores from the different toxicity tests are then combined to give an overall score for the ionic liquid structure. Depending on the desired criteria, boundaries can then be set to select potentially biocompatible
ionic liquids for further testing or to rule out ionic liquids which are too toxic to warrant further investigation.

We wish to make a further suggestion to represent the scores visually, using a traffic light system, where a score of 1 (non-toxic) is green, 2 (intermediate toxicity) is amber and 3 (toxic) is red. The ionic liquids can then be colour coded according to their toxicity and arranged according to their structure i.e. by anion class with increasing alkyl chain length, and by the type of anion. The data can then be arranged as a matrix or as a dendrogram (Fig. 1). This makes it very easy to visualise the data, so that toxicity trends can be identified and interpreted. In this way, the data collected from simple, cost effective toxicity tests can be used to identify possible future synthetic targets.

Conclusion

The need for simple, cost effective, high throughput screening methods to determine the toxicity of ionic liquids is clear, given the huge number of potential structures available. Any attempt to screen all structures using the industry standard ecotoxicity tests would be prohibitively costly, laborious, and time consuming.

The agar diffusion test gives a fast, cost-effective and simple initial screening method to shortlist ionic liquid structures which are sufficiently non-inhibitory to warrant further in-depth investigation. The selected ionic liquids should then be screened again by a different high throughput test, to further refine the shortlist. In this way, only a relatively small number of the least toxic ionic liquids will need to be assessed via expensive and time-consuming ecotoxicological profiling.

*Make things as simple as possible, but no simpler*
Albert Einstein

Acknowledgements

This work was funded by the Biotechnology and Biological Sciences Research Council via a studentship awarded to Nicola Wood and a Research Development Fellowship to Gill Stephens.

References


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*Phys. Chem. Chem. Phys.*, 2010, 12, 1670-1674 | 1673
Screening ionic liquids for use in biotransformations with whole microbial cells†

Nicola Wood, Jamie L. Ferguson, H. Q. Nimal Gunaratne, Kenneth R. Seddon, Royston Goodacre and Gillian M. Stephens*

Received 20th September 2010, Accepted 20th April 2011
DOI: 10.1039/c0gc00579g

A wide range of ionic liquids, both water miscible and water immiscible, containing a diverse set of cations and anions, were screened for toxicity towards Escherichia coli K-12, using both Agar Diffusion tests and growth inhibition tests in liquid cultures. The data provide preliminary rules to enable the design of non-toxic ionic liquids for use in biocatalytic processes.

Introduction

Biocatalytic processing provides an increasingly attractive option for highly selective, atom efficient production of high value chemicals in short reaction sequences. Furthermore, microbial fermentations enable production of biofuels and value-added chemicals from renewable feedstocks, using natural or engineered metabolic pathways. Unfortunately, biotransformation products and/or substrates are frequently toxic towards microbial biocatalysts, and this may restrict productivity, making the processes uneconomic. The problem can often be solved by using a biphasic biotransformation process, where the toxic material is extracted into a water-immiscible solvent, to minimise contact with the cells in the aqueous phase. Unfortunately, polar conventional solvents are, themselves, toxic. Therefore, in situ extraction is only possible when there is a serendipitous match between the physical properties of the material to be extracted and the available, non-polar biocompatible solvents. For this reason, there is considerable interest in using ionic liquids as alternative extraction media in whole cell biotransformations.

There have been some notable successes. For example, biphase systems containing water-immiscible ionic liquids provided significant improvements in productivity compared with conventional solvents for reduct biotransformations using whole cells. Even water-miscible ionic liquids can provide significant increases in product yields compared with conventional solvents when used as additives. Therefore, ionic liquids are extremely promising co-solvents for use in whole cell biotransformations.

To date, relatively few ionic liquids have been tested for use in microbial processes. The beauty of ionic liquids is the ability to vary the structure and, thus, tune the physical properties of the solvent to match the specific requirements of the process (e.g. efficient extraction of a biotransformation product). Therefore, it would be desirable to identify a wider range of biocompatible types.

We studied the toxicity of over ninety ionic liquids from diverse structural classes towards Escherichia coli. E. coli is a good choice because it is used very frequently as a host strain for expression of industrial enzymes and for metabolic engineering. Therefore, our results can be implemented rapidly in industrial biocatalysis. We screened ionic liquids based on imidazolium, pyridinium, quaternary ammonium, alkylammonium, and quaternary phosphonium cations (Table 1), combined with a diverse range of anions. Whole cell biotransformations depend on having live, functional cells to ensure efficient cofactor recycling, enzyme functionality, substrate uptake, etc. The simplest, fastest indicator of cell functionality is to measure growth of the cells. This also ensures that the results are general, and can be applied to any biocatalytic reaction. Therefore, we used our high throughput, agar diffusion test to test for growth inhibition, backed up with high throughput measurement of growth rates in the presence of the ionic liquids. Most importantly, we have extended the screening method to allow testing of water-immiscible ionic liquids, thus enabling selection of solvents for biphase biotransformations. Therefore, preliminary rules are now available to guide the selection of non-toxic ionic liquids for use in whole cell biotransformations, together with strategies to design a wider range of new, biocompatible ionic liquids.
Table 1  Cations used in this study

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<th>Abbreviation</th>
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Table 2  Neosulfate anions used in this study

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Table 3  Sulfate and sulfonate anions used in this study

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Experimental

Microorganism and growth

E. coli MG1655 was obtained from Simon Andrews (Reading University, UK) and was maintained on LB agar. Inocula were grown from a single colony in either LB or MSX medium at 37 °C with shaking at 200 rpm. Agar No. 2 was purchased from Lab M, and yeast extract was purchased from Foremost. All other chemicals and media components were purchased from Sigma–Aldrich.

High throughput agar diffusion test for ionic liquid toxicity

The agar diffusion test developed previously was used to screen ionic liquids (Tables 1–3), except that the lawn of E. coli MG1655 was prepared by spreading a sample from an overnight culture (100 μl) on an LB agar plate. The ionic liquids were added to sterile, pre-weighed filter paper discs as before, except that the discs were 6 mm in diameter and were transferred onto LB agar plates. In some cases, ionic solids were tested to obtain further insight into the structure-toxicity relationship. These salts were weighed, and sterile distilled water was added drop-wise until the
salt dissolved. An aliquot (5 μl) of the salt solution was added to the filter paper disc. The weights absorbed were in the range 0.004–0.0125 g of dissolved ionic solids added to the filter paper. All plates were incubated overnight in static incubator at 37 °C, and the radius of the inhibition zone around the filter paper was recorded as before. Each ionic liquid was tested in triplicate and inhibition zones quoted are the average of the three replicates, unless otherwise stated.

**Ionic liquid toxicity testing in liquid media**

Growth rates were measured in LB and MSX media in microculture (200 μl) in a Bioscreen C incubator/plate reader (Thermo Labsystems, Franklin, MA) in the presence and absence of ionic liquids. It should be noted that the plate reader used previously was unsuitable for use with water-immiscible materials. To decrease the viscosity, water miscible ionic liquids were weighed and mixed with sterile, distilled water to produce a stock solution (95% v/v). Viscous ionic liquids or ionic liquids with unusual phase behaviour (e.g., with the 1,4-bis(2-ethylhexyl)-1,4-dioxobutane-3-sulfonate [AOT] anion) were also prepared in the same way, but in this case a suspension was made. The stock solutions were vortexed before adding an aliquot (4 μl) to the microcultures to a final concentration of 2% (v/v). Non-viscous water-immiscible ionic liquids (e.g., [NTf₂]⁺ salts) were added directly to the cultures by volumetric measurement to produce a biphasic system (2% v/v). The cultures were incubated with a sample (10 μl) from an overnight culture of *E. coli* MG1655 grown in the same medium. The plates were incubated for 24 h at 37 °C with continuous, mild shaking.

The OD measurement for each well was recorded automatically every 10 min, using a wideband filter. Growth curves were produced using Microsoft Excel (Microsoft Office 2003). Specific growth rates (μ, h⁻¹) were calculated by selecting two points at time t₀ and t₁ in the exponential growth phase and applying the equation:

\[ \mu = \frac{\ln(OD_{t_1}) - \ln(OD_{t_0})}{t_1 - t_0} \]

Specific growth rates are the mean of three replicates, unless otherwise stated, and are reported as a percentage of the growth rate in control cultures grown without ionic liquids.

**Syntheses of ionic liquids**

The methods used to synthesise the ionic liquids are described in the ESI.†

**Results**

More than ninety ionic liquids were screened for biocompatibility with *E. coli* MG1655, using both the agar diffusion test and measurements of growth rates in the presence and absence of ionic liquids. Growth curves were generated in both LB and MSX culture media, which contain entirely different chemical components. Thus, LB contains yeast extract, peptone and glucose, whereas MSX is a defined salt solution, with glucose as the sole carbon and energy source. This made it possible to control for potential interactions between the ionic liquids and medium components or potential changes in ionic liquid toxicity with different physiological states of the cells. The effect of the ionic liquids on pH was checked in cultures and unincubated controls using universal indicator (20 μl) added before and after growth. In general, effects on pH were small (<0.5 pH units) except where stated in the text. The results are presented according to the anions tested.

**Halides**

Initially, a range of 1-alkyl-3-methylimidazolium halide ionic liquids was screened for toxicity, aiming to benchmark the behaviour of *E. coli* against previous studies with other living organisms (Table 4). The [C₅mim]⁺ and [C₆mim]⁺ chlorides and bromides did not produce inhibition zones in the agar diffusion test, but inhibition zones were produced with increasing radii when the alkyl chain was increased to hexyl or octyl. The specific growth rates varied between the LB and MSX media, suggesting possible interactions between the ionic liquid and the medium, or medium-dependent variations in cell physiology, which changed the susceptibility to ionic liquid toxicity. However, the overall trend was that growth was inhibited progressively as the length of the 1-alkyl chain increased. Similar relationships between increasing length of the alkyl chain/tropophility of the imidazolium cation and the increasing toxicity of the ionic liquid have been widely reported,19 suggesting that the response of *E. coli* MG1655 to imidazolium halides is similar to other living organisms.

We also wish to note that the halides were more toxic than the chlorides and bromides, possibly because iodide is the most easily oxidised. The large errors associated with the iodide salts may have been due to the black colouration caused by photosensitised oxidation of the iodide anion.

A range of other haloid salts was also tested, in which the imidazolium cation was replaced with pyridinium and piperidinium and tetraalkyl ammonium cations (Table 5). As with mammalian cells,19 it was found that the toxicity of pyridinium and piperidinium halides towards *E. coli* increased with increasing alkyl chain length. Similarly, there was a reasonable correlation.

---

Table 4  Effect of imidazolium halides on growth of *E. coli*  

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition zone (mm)</th>
<th>μ% in MSX</th>
<th>μ% in LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₅mimCl⁺</td>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>C₅mimBr⁻</td>
<td>0.5 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₅mimI⁻</td>
<td>0.8 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₅mimF⁻</td>
<td>1.1 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₆mimCl⁺</td>
<td>0</td>
<td>90 ± 1.38</td>
<td>73 ± 0.82</td>
</tr>
<tr>
<td>C₆mimBr⁻</td>
<td>0</td>
<td>56 ± 5.56</td>
<td>77 ± 4.75</td>
</tr>
<tr>
<td>C₆mimI⁻</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₆mimF⁻</td>
<td>0.9 ± 0.15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₇mimCl⁺</td>
<td>0.37 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₇mimBr⁻</td>
<td>1.0 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₇mimI⁻</td>
<td>0.6 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. – data not available. Data are the means of 3 replicates and the standard deviations are shown. All of the ionic liquids were water miscible. 
Table 5 Effect of pyrrolidinium, piperidinium and quaternary ammonium halides on growth of E. coli

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition zone/cm</th>
<th>μ% in MSX</th>
<th>μ% in LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₅mepy][Br]</td>
<td>0.08 ± 0.03</td>
<td>91 ± 6.60</td>
<td>99 ± 2.28</td>
</tr>
<tr>
<td>[C₅mepy][I]</td>
<td>0</td>
<td>0</td>
<td>531 ± 1.36</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.16 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][Cl]</td>
<td>0.05</td>
<td>83 ± 5.69</td>
<td>70 ± 3.35</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.2 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.5 ± 0.06</td>
<td>88 ± 3.73</td>
<td>94 ± 2.49</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.9 ± 0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.6 ± 0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.3 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.1 ± 0.06</td>
<td>110 ± 0.53</td>
<td>93 ± 1.41</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.1 ± 0.1</td>
<td>104 ± 7.00</td>
<td>122 ± 2.57</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0</td>
<td>104 ± 1.71</td>
<td>96 ± 0.74</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. Data are the means of 3 replicates and the standard deviations are shown. All were water miscible solvents except - liquid, - water immiscible.

Table 6 Effect of saccharinate and alkanonic acid salts on growth of E. coli

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition zone/cm</th>
<th>μ% in MSX</th>
<th>μ% in LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₅mepy][Br]</td>
<td>0.5 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][I]</td>
<td>0.7 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.8 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.07 ± 0.06</td>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.06 ± 0.05</td>
<td>27 ± 6.07</td>
<td>64 ± 4.25</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.9 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.6 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.1 ± 0.06</td>
<td>50 ± 2.81*</td>
<td>23 ± 2.83</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.3 ± 0.06</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.3 ± 0.12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[C₅mepy][PF₆]</td>
<td>0.1 ± 0.12</td>
<td>67 ± 13.87</td>
<td>86 ± 5.84</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. - data not available. Data are the means of 3 replicates and the standard deviations are shown. All were water miscible liquids, except - liquid, - water immiscible. * - halo effect, † - sheen-free synthesis. ‡ Only 2 replicates used.

between the agar diffusion results for the imidazolium and the corresponding pyrrolidinium and piperidinium ionic liquid with the same alkyl chain length.

As in earlier studies, increasing the alkyl chain length of tetraalkylammonium cations produced toxic ionic liquids. Thus, [N₄₄⁺][Br] produced less than 12% inhibition of the growth rate (although there was an inhibition zone on agar diffusion tests) whereas the halides of [N₂₄⁺][Br] and [N₄₄⁺][Br] were toxic.

When one or more of the alkyl chains was replaced with an alkanol group, the ionic liquids were much less toxic. However, it should be noted that the majority of the quaternary ammonium salts contained relatively long chain substituents (e.g. octyl), whereas the ethanolate and propanolate salts contained shorter alkyl substituents, which made them less toxic in any case (see above). Introduction of single hydroxyl groups into quaternary ammonium cations also decreases inhibition of the enzyme, acetylcholinesterase, by one order of magnitude⁴⁴ and decreases cytotoxicity.⁴⁶

In general, the initial data correlate well with literature already published, thus providing a good indication that the agar diffusion method can be used to screen a range of structurally diverse ionic liquids. However, it is worth noting that there were some discrepancies between the agar diffusion test and the growth rate studies. Thus, inhibition zones were observed for [C₅mepy][Br] and [C₅mepy][I], whereas these ionic liquids had little effect on growth rates (< 30% inhibition). Similarly, [C₅mepy][PF₆] did not produce an inhibition zone, even though growth was inhibited completely in MSX, with 47% inhibition in LB. Similar discrepancies were also observed for [N₄₄⁺][Br] and [N₄₄⁺][I], which suggests that the agar diffusion test may be less reliable for quaternary ammonium halides than for imidazolium halides.

The acquired data suggest a number of possibilities for further investigation. Of the cyclic and acyclic quaternary ammonium halides tested, those with short alkyl chains were preferable, and introducing a hydroxyl group into one or more of the alkyl substituents tended to produce non-toxic salts. Furthermore, both bromide and chloride anions produced reasonably biocompatible ionic liquids when combined with short chain cations, whereas the iodides were invariably toxic. It should be noted that the quaternary ammonium and alkanolamine salts form an extremely large group, and there is considerable scope to discover new variants of the non-toxic structures.

Saccharinates

Saccharin-based ionic liquids have been suggested as a good starting point to produce non-toxic ionic liquids, as saccharin (sodium saccharinate) is non-toxic and has already been approved for human consumption and is often used as a non-nutritive sweetener.⁴⁸ Therefore, we tested a range of imidazolium and quaternary ammonium saccharinates for toxicity. Unfortunately, none allowed growth of E. coli (Table 6). It should be noted however, that the cations tested were also toxic when combined with halide anions (Table 4 and 5). Thus, our findings confirm that the toxicity of ionic liquid is dominated by the toxicity of the most toxic component, in this case the cation.⁴⁸

An additional point of note is the apparent alteration of pH produced by the saccharinate ionic liquids. The saccharinate ionic liquids produced a drop in the pH value from pH 7.5 to pH 5.0 after the addition of the ionic liquid to the medium. Even with increased buffering capacity in the medium,⁴⁹ the saccharinate ionic liquids were the only ones to produce such a large shift in the pH value, and this, rather than inherent toxicity, may account for the observed growth inhibition.

Alkanolates and lactates

Recently, there has also been a lot of interest in producing ionic liquids from naturally-occurring anions (e.g. alkanolates, lactate)⁴⁹ and cations (e.g. cholinium, [N₄₄⁺][Br]),⁵⁰ since the component ions are well known to be biodegradable. However,
the group 1 and 2 metal salts of alkanoates and lactate are widely used as food preservatives (as are the free acids), and are known to be toxic towards E. coli. Therefore, we wished to analyse their effects on E. coli when combined with other cations, to make ionic liquids.

In initial tests, we found that the imidazolium and pyrrolidinium lactates tested were toxic. The lactate-containing ionic liquids originally tested were synthesised from silver salts and concern was raised that the toxicity of the ionic liquids may be the result of silver contamination rather than their inherent toxicity. To test this, [C₅mim][lactate] was synthesised using a silver-free synthesis, and compared with the same ionic liquid made from the silver salt (Table 6). The silver-free ionic liquid was much less toxic, confirming that it is important to use a silver-free synthesis to produce lactate-based ionic liquids. Indeed it emphasises that knowledge of the samples’ synthetic history is important in reporting toxicological data, and this is not always available for commercially aquired samples.

Although ethanoic acid and its corresponding sodium salt are known to be toxic for E. coli, cholium ethanoate was non-toxic. By contrast, [C₅mim][C₂O₄] caused significant growth inhibition (Table 6), but growth was, nevertheless, observed in both MSX and LB. [C₅mim][C₂O₄] was completely inhibitory, like [C₅mim][Cl] (Tables 5 and 6), perhaps unsurprising given the general toxicity of ionic liquids with oxyt substitutions (see also Table 4). This provides further confirmation that the toxicity of an ionic liquid is dominated by its most toxic component. Thus, like saccharinate salts, the toxicities of ethanoate salts are dominated by the toxicity of the cations.

We also investigated the effect of increasing the length of the alkanoate ion from 2 to 6 carbon atoms. Again, the toxicity of the sodium and potassium salts towards microorganisms has been investigated intensively, since the acids and their salts are used extensively as food preservatives. However, the higher homologues of the cholium alkanoates were relatively non-toxic, although the growth rates in MSX and LB decreased when the alkyl chain length of the anion was increased.

Sulfates and dimethyl phosphates

Alkyl sulfate salts are widely used as surfactants, and are relatively cheap. The derivatives with long alkyl chains disrupt bacterial cell membranes at high concentrations, and the toxicity varies with the type of metal cation. Therefore, we wished to analyse the toxicity of alkyl sulfate salts with organic cations. In a previous study of ionic liquid toxicity towards the anaerobe Clostridium butyricum, alkyl sulfate salts were identified as non-toxic, and alkyl sulfates and tolylates are also relatively non-toxic towards mammalian cells. Therefore, we studied the toxicity of a much wider range of alkyl sulfates and tolylates towards E. coli (Table 7). In general, there were some discrepancies between the results of the agar diffusion tests and the growth rate measurements. The inhibition zones observed in the agar diffusion tests had large standard deviations. This is difficult to explain, although it is possible that the ionic liquids interacted with the cellulose filter paper, thus altering their diffusion through the agar medium, or alternatively did not intrinsically diffuse freely through the agar medium. Furthermore, there was a rather poor correlation between the mean size of the inhibition zones and the growth rates observed in MSX and LB media. For this reason, the agar diffusion test is rather unreliable for testing the toxicity of alkyl sulfonic ionic liquids for E. coli, although better results were obtained with C. butyricum. Thus, we base our conclusions primarily on the growth rate data, rather than the agar diffusion data.

[C₅mim][C₅O₄], [C₅mim][C₆O₄], and [C₅mim][tosylate] were relatively non-toxic for E. coli. Furthermore, increasing the alkyl sulfate chain length had little effect. Although there was an inhibition zone with [C₅mim][C₅O₄], growth was only inhibited by 31% and 23% in MSX and LB, respectively. The errors were large for the latter cultures, possibly due to light scattering caused by the ionic liquid. The [C₅mim]+ cation is much less toxic than the cations used previously with the oxyt sulfate anion, which further emphasises the importance of balancing the toxicity of both the anion and cation when designing non-inhibitory ionic liquids. Introducing ether linkages into the alkyl chain ([C₅mim][C₅(OC)₃][O₄]) produced an ionic liquid which caused little growth inhibition and did not produce an inhibition zone in the agar diffusion test. [C₅mim][C₅O₄] and [C₅mim][C₆O₄] were slightly more toxic than the equivalent [C₅mim]+ salts. Including an extra carbon atom in the anion (rather than the cation; [C₅mim][C₆O₄]) had little effect on toxicity compared with ethyl sulfate. Branching had very little effect also ([C₅mim][C₅(OC)₃][O₄]), but increasing the chain length to isobutyl ([(C₅mim)][C₅(OOISO)] increased

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition zone/cm</th>
<th>μ%/μ in MSX</th>
<th>μ%/μ in LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>[C₅mim][C₅O₄]</td>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.17 ± 0.15</td>
<td>93 ± 3.44</td>
<td>147 ± 6.76</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0</td>
<td>68 ± 2.09</td>
<td>77 ± 15.99</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.01 ± 0.03</td>
<td>101 ± 7.69</td>
<td>111 ± 6.94</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.01 ± 0.06</td>
<td>82 ± 1.57</td>
<td>79 ± 3.33</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.07 ± 0.06</td>
<td>74 ± 2.07</td>
<td>70 ± 1.84</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.04 ± 0.05</td>
<td>73 ± 2.16</td>
<td>76 ± 0.62</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.02 ± 0.02</td>
<td>78 ± 2.56</td>
<td>81 ± 0.39</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.01 ± 0.01</td>
<td>65 ± 0.69</td>
<td>63 ± 2.02</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.00 ± 0.00</td>
<td>1 ± 0.00</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.02 ± 0.02</td>
<td>85 ± 1.93</td>
<td>91 ± 6.30</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.3 ± 0.21</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.2 ± 0.12</td>
<td>89 ± 1.79</td>
<td>46 ± 1.39</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.8 ± 0.1</td>
<td>94 ± 1.54</td>
<td>139 ± 11.43</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.6 ± 0.1</td>
<td>85 ± 1.33</td>
<td>46 ± 1.32</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.6 ± 0.1</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.05 ± 0.06</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.1 ± 0.06</td>
<td>0 ± 0.00</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.03 ± 0.03</td>
<td>97 ± 4.71</td>
<td>89 ± 0.73</td>
</tr>
<tr>
<td>[C₅mim][C₆O₄]</td>
<td>0.02 ± 0.01</td>
<td>102 ± 0.71</td>
<td>111 ± 1.28</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (μ) were measured in MSX or LB medium in the presence of ionic liquids (% v/v) and expressed as a percentage of the growth rate in control cultures without ionic liquid. n.d. = data not available. Data are the means of 3 replicates and the standard deviations are shown. All were water miscible liquids, except 1 - water immiscible or 2 - solid.
the toxicity slightly. Ehter linkages ([C\text{mim}][\text{CO}_3\text{O}_5\text{SO}_3]) increased the toxicity slightly compared with propyl sulfate or isopropyl sulfate, but the toxicity of [C\text{mim}][\text{CO}_3\text{O}_5\text{SO}_3] was lower than the equivalent isobutyl sulfate salt.

Like the respective [C\text{mim}][\text{NTf}_2] and [C\text{mim}] salts, [\text{C}_\text{mpyr}][\text{CO}_3\text{O}_5\text{SO}_3] was relatively non-toxic, but [\text{C}_\text{mpyr}][\text{CO}_3\text{O}_3\text{SO}_3] and [N\text{H}_\text{et}][\text{CO}_3\text{O}_5\text{SO}_3] were toxic. This may reflect the length of the alkyl chains on the cations. Indeed, [N\text{H}_\text{et}][\text{CO}_3\text{O}_5\text{SO}_3] was non-toxic and the alkyl sulfate could be extended to [\text{CO}_3\text{OSO}_3] without affecting the toxicity. On the other hand, all of the alkyl sulfate salts of [N\text{H}_\text{et}][\text{NTf}_2] and [N\text{H}_\text{et}][\text{NTf}_2] were toxic, as was the sulfonate salt, [N\text{H}_\text{et}][\text{ESO}_3].

The effect of including alcohol groups in the cationic side chains was investigated. [N\text{H}_\text{et}][\text{CO}_3\text{O}_5\text{SO}_3] and [N\text{H}_\text{et}][\text{CO}_3\text{O}_3\text{SO}_3] caused slight growth inhibition, but [N\text{H}_\text{et}][\text{CO}_3\text{O}_5\text{SO}_3] was non-toxic. [N\text{H}_\text{et}][\text{CO}_3\text{O}_3\text{SO}_3] was also non-toxic. Therefore, increasing the number of hydroxyl groups or ether linkages appears to make the ionic liquid less toxic. This supports previous observations that the introduction of a single hydroxyl group, or ether or ester linkages into the cation can result in less toxic ionic liquids, suggesting that the presence of oxygen atoms in the side chains is generally beneficial.

Overall, cations with long alkyl chains were toxic when combined with alkyl sulfates, but the short chain homologues and variants which included ether or hydroxyl groups were less toxic or non-toxic. The structure of the alkyl sulfate could be varied widely, to include branching or ether groups, without affecting toxicity to a significant extent, but without some evidence that toxicity increased as the alkyl chain length increased.

Thus, the alkyl sulfate anion appears to be very promising for the production of biocompatible ionic liquids. It is worth noting that the octyl sulfate anion is readily biodegradable, but this is unlikely to affect the biodegradability of the cation: an ionic liquid is only as biodegradable as its least biodegradable component. The findings from this screening exercise suggest that further investigation of both short-chained sulfate and various sulfonate anions may be beneficial in the search for biocompatible and biodegradable ionic liquids.

Finally, the dimethyl phosphates, [C\text{mpyr}][\text{C}_3\text{O}_3\text{PO}_3] and [N\text{H}_\text{et}][\text{C}_3\text{O}_3\text{PO}_3] were tested. On agar, [C\text{mpyr}][\text{C}_3\text{O}_3\text{PO}_3] produced a small inhibition zone in the agar diffusion test, but the variation between replicates was very large. However, the growth rate measurements in LB and MSX media showed that the ionic liquid was relatively non-inhibitory for E. coli. [N\text{H}_\text{et}][\text{C}_3\text{O}_3\text{PO}_3] was non-inhibitory. Therefore, dimethyl phosphates are potentially promising as non-toxic ionic liquids.

### Water Immiscible Ionic Liquids

The agar diffusion test was originally applied to test the toxicity of water-immiscible ionic liquids. However, we wished to examine the potential to use the test to study the toxicity of water-immiscible salts. In this study, we have avoided the use of bis(trifluoromethyl)sulfonamide (or bistriflimide, [\text{NTf}_2]) anion, since they are extremely insoluble in water and have low viscosities. We also studied the toxicity of 1,4-bis(2-ethylhexoxy)-1,4-dioxo-butan-2-sulfonate (or docusate, [\text{AOT}]) salt and bis(2,4,4-trimethylpentyl)phosphonate ([\text{C}_3\text{O}_3\text{PO}_3]) salts.

For hydrophobic ionic liquids, the agar diffusion test is no longer a simple diffusion test, because the ionic liquids will not dissolve in the aqueous agar medium to a significant extent. Instead, we expected that hydrophobic ionic liquids would be transported over the surface of the agar as a film of oil. However, we also expected additional complexity. Firstly, the ionic liquids might differ in their ability to wet the filter paper disc (which is made of cellulose), and this would affect delivery of the ionic liquid to the cultures, both in terms of quantity and transport onto the surface of the agar. However, all of the ionic liquids used here were able to wet the filters. Secondly, the ionic liquids might interact with components of the culture medium, and this might hinder their smooth transport across the surface. Fortunately, the agar diffusion test can be easily bench-marked against growth rate measurements, since the presence of water-immiscible solvents does not usually affect the accuracy of growth rate measurements to a significant extent. For the growth rate measurements, we used the ionic liquids above the solubility limit, to obtain a true biphasic system, as required for biotransformations using whole cells. However, we did encounter problems with docusate salts (table 8).

Despite these concerns, the agar diffusion test provided a surprisingly good indication of the toxicity of hydrophobic ionic liquids when compared with growth rate measurements (Table 8). However, these ionic liquids produced very large inhibition zones compared with water-immiscible ionic liquids. As noted above, this might be due to transport of a liquid film over the agar surface rather than diffusion through the gel itself.

The imidazolium-based bistriflimide salts caused complete inhibition of growth in liquid culture and produced some of the largest inhibition zones of any of the ionic liquids tested. This confirms earlier studies which showed that [C\text{mim}][\text{NTf}_2] is highly toxic and affect membrane integrity. Our study also demonstrates that extending the alkyl chain to
Table 9 Effect of docucuqate, bis(2,4,4-trimethylpentyl)phosphate and tetrabutylammonium bromide on growth of E. coli

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>Inhibition zone/cm</th>
<th>%/m in MSX</th>
<th>%/m in LB</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₅mim[AOT]</td>
<td>0</td>
<td>57 ± 13.68</td>
<td>84 ± 34.18</td>
</tr>
<tr>
<td>C₆mim[AOT]</td>
<td>0</td>
<td>88 ± 8.01</td>
<td>33 ± 37.95</td>
</tr>
<tr>
<td>C₇mim[AOT]</td>
<td>0</td>
<td>57 ± 6.39</td>
<td>198 ± 7.80</td>
</tr>
<tr>
<td>C₆mim[AOT][Br]</td>
<td>0</td>
<td>62 ± 6.34</td>
<td>66 ± 7.94</td>
</tr>
<tr>
<td>C₆mim[AOT][I]</td>
<td>0</td>
<td>7.6 ± 2.51</td>
<td>37 ± 7.03</td>
</tr>
<tr>
<td>P[N₄][AOT]</td>
<td>0</td>
<td>97 ± 1.75</td>
<td>94 ± 1.07</td>
</tr>
<tr>
<td>P[N₄][AOT]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C₅mim[PC₆(OH)₃]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N₃[PC₆(OH)₃]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N₃[N₄][PC₆(OH)₃]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N₃[N₄][bis(tritylammonium)]</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Inhibition zones were measured using the agar diffusion test, and growth rates (%) were measured in MSX or LB media in the presence of toxic liquids (2% v/v) and expressed as a percentage of the growth rate in control cultures without toxic liquid. Data are the means of 3 replicates and the standard deviations are shown. All of the salts were liquid at room temperature (except * - solid), and were water immiscible. * - only two replicates available.

Octyl also produces a toxic liquid. This suggests that the toxicity of [C₅mim][Bis] is attributed to the anion, since [C₅mim][X] (X = halide or alkyl sulfate) were relatively non-toxic.

The water-miscible quaternary ammonium salts tended to become more toxic as the alkyl chain length in the cation increased (Tables 5 and 7). However, the opposite trend was observed with the equivalent, water-immiscible bis trifluoromethylsulfonic acid. Thus, short-chain tetraalkylammonium bis trifluoromethylsulfonic acid was toxic, whereas their long chain equivalents were not (Table 8). This trend seems to be analogous to the toxicity of alkylbenzenes, where short chain alkylbenzenes are toxic but long chain alkylbenzenes are not. Furthermore, addition of a hydroxyl group produced an inhibitory effect on growth (Table 8), whereas this was not observed with the equivalent, water-immiscible bis trifluoromethylsulfonic acid. (Table 5). It appears that when combined with a hydrophobic, toxic anion, the more hydrophobic the cation, the more the anion will be "pulled" into aqueous solution. Thus, a small, hydrophobic cation probably increases the effective concentration of the bis trifluoromethylsulfonic acid in the agar, or in the liquid media, making the toxic liquid more toxic.

The dicarboxylic acid salts, [C₅mim][C₆(OH)₃]PO₄, [N₃][C₆(OH)₃]PO₄ and [N₃][C₆(OH)₃]PO₄ were also added. They all produced large inhibition zones and all inhibited growth in MSX and LB (Table 9). Therefore, we conclude that they are toxic.

Although the agar diffusion test appeared to be a reliable qualitative method to screen hydrophobic toxic liquids for toxicity, it was difficult to obtain an accurate, quantitative comparison with growth rates in aqueous media, when the water-immiscible toxic liquids exhibited unusual phase behaviour. In particular, hydrophobic toxic liquids containing the doceuqate (AOT) or tetrabutylammonium bromide showed a strong tendency to form gels or pastes when added to water. This caused light scattering, and the resulting interference with the OD readings produced large errors. For example, the growth curve obtained in the presence of [N₃][AOT] had large error bars and the starting OD readings were also high due to the light scattering (Fig. 1; the growth curve in the presence of the water-miscible ionic liquid, [N₃][Br], is shown for comparison). Therefore, the growth rates calculated must be regarded as estimates, and should be used as an indication of the presence or absence of growth, rather than as an absolute quantitative measure.

Fig. 1 Effect of pH-forming ionic liquids on growth of E. coli. E. coli was grown in MSX medium with (●) and without (■) ionic liquid (2% v/v). A, [N₃][Br] R, [N₃][AOT]. Uninoculated controls with (●) and without (■) ionic liquid control are also presented. Means of three replicates are shown and error bars are standard deviations.

In a number of cases, there were discrepancies between the agar diffusion test and the growth tests. Thus, [C₅mim][AOT] produced a large inhibition zone even though there was significant growth in LB and MSX. By contrast, the large inhibition zone produced with [C₅mim][AOT] correlated with growth inhibition in liquid media. [N₃][AOT] and [N₃][Br] also caused inhibition zones, even though there was growth in MSX and LB. Therefore, it is difficult to get reliable quantitative estimates of the toxicity of pyridinium and quaternary ammonium doceuqate salts using the agar diffusion test, and other, complementary methods will be needed to obtain robust measurements.

Observations on the agar diffusion test

In order to screen large numbers of ionic liquids for toxicity to a particular micro-organism, a simple and rapid method is required. The agar diffusion test is suitable, provided that the limitations of the methodology are appreciated. The agar screening method requires only small volumes of ionic liquids and generally available laboratory equipment. However, as discussed previously, a major limitation of the method is in the classification of ionic liquids which produce small diffusion
zones. Although reliable for identification of biocompatible or highly toxic ionic liquids against microorganisms, those which fall between the two extremes can be difficult to quantify. In some cases, the ionic liquids appeared to interact with the filter papers or the culture medium, possibly leading to anomalous results. For example, some of the ionic liquids did not diffuse evenly across the agar plates, and thus produced non-circular inhibition zones, leading to possible measurement errors (Fig. 2).

Fig. 2  Asymmetric inhibition zones in the agar diffusion test. An agar diffusion test was done using [N_{11+}][ClO_4]. The photograph shows that the inhibition zone was asymmetric and showed a variable zone of clearing, rather than forming a uniform circle around the filter paper.

Additionally some of the tested ionic liquids caused the filter paper to turn brown (see, for example, Fig. 3), suggesting a chemical reaction, whilst others interacted with the culture medium, causing a “halo” or a precipitate to appear around the inhibition zone. It should also be noted that there may be problems when the ionic liquid is not absorbed by the filter paper, and care should be exercised when interpreting the results of studies with water-immiscible ionic liquids. Therefore, it is very important to record the appearance of unusual results, and where appropriate to verify the results by testing for growth in liquid media to ensure that potentially useful ionic liquids are not overlooked. In any case, it must be remembered that the methodology should be used as a first-stage, high throughput screen to identify the most promising ionic liquids. It should never be deployed as a substitute for detailed toxicology testing.  

Conclusion

By using a combination of agar diffusion testing and growth rate measurements, we have identified a large number of relatively non-toxic ionic liquids. In general, imidazolium salts with short alkyl chains were relatively non-toxic, especially when mixed with alkyl sulfate anions. Methylypyridinium salts were also very promising, whereas water-miscible quaternary ammonium salts were generally toxic. However, there was evidence that decreasing the alkyl chain length decreased their toxicity.

Fig. 3  Formation of precipitates and colour changes in the agar diffusion test. Photographs of the inhibition zones from agar diffusion tests: (a) white precipitate around the filter paper disc in the presence of [C_{12}mim][AOT], (b) colour change in the presence of [C_{12}mim][taclate] prepared using the ather synthesis.

Overall, the most promising cation class appeared to be the alkanolammonium salts, since these were generally non-toxic. In terms of the anions, alkyl sulfates and their derivatives tended to produce non-toxic water-miscible ionic liquids, whilst dodecylsulfates produced non-toxic, water-immiscible ionic liquids, albeit with challenging phase behaviour. Interestingly, bis(trifluoromethylsulfonyl)imide tended to make longer chain quaternary ammonium salts non-toxic, whereas other cation classes became more toxic compared with their water miscible counterparts. A small number of dimethyl phosphates was also tested, and tended to be non-toxic.

In conclusion, this study has revealed some very promising toxicity trends which will form a basis for rational design of new, non-toxic ionic liquids. Furthermore, we have already discovered a large number of ionic liquids with very low toxicity towards E. coli. We are now deploying these ionic liquids in biotransformations to produce fuels and chemicals.
Acknowledgements
This work was funded by the UK Biotechnology and Biological Sciences Research Council, via a studentship awarded to Nicola Wood and a Research Development Fellowship to Gill Stephens, and by QUILL and the Technology Strategy Board’s Collaborative Research and Development programme.

References