CHAPTER 1: INTRODUCTION

Ionic liquids (ILs) are low melting point salts composed entirely of ions, and many of them are liquids at room temperature. Any salt that melts without decomposing or vaporizing will usually yield an ionic liquid. Conversely, when an ionic liquid is cooled, it will often form an ionic solid — which may be either crystalline or glassy. Room temperature ionic liquids (RTILs), often referred to as 'designer solvents', have been the great focus of scientists in various fields since they can be tuned for specific applications. Different cation and anion combinations are possible to change the properties such as polarity, hydrophobicity and solvent miscibility behavior (Romero et. al., 2008).

The applications of RTILs are many, due to their unusual physical and chemical properties like high thermal stability, lack of inflammability, low volatility, chemical stability and excellent solubility with many organic compounds. Thus, these are considered to be emerging green solvents and potential alternatives to the classical volatile organic solvents, with the aim of facilitating sustainable chemistry. This green adjective is mainly attributed to their negligible vapor pressure, which avoids the loss of solvent to the atmosphere and decreases the worker exposure risk. Thus, room-temperature ionic liquids could provide environmentally friendly solvents for the industrial applications. Typical ILs consist of an organic cation with delocalized charges and a small inorganic anion, most often halogen anions weakly coordinating such as Cl, BF_4 or PF_6 (Walker and Bruce, 2004).

Ionic liquids have many applications, such as electrically conducting fluids (electrolytes) and catalyst enhancer. Numerous reports have revealed that many catalysts and reagents which were supported in the ionic liquid phase, resulted in enhanced reactivity and selectivity in various important reaction transformations (Sowmiah et. al., 2009). Although ionic liquids have many important industrial and commercial applications, the environmental fate and any potential toxicity issues for most ionic liquids are not known (Swatloski, 2003). The lack of readily accessible

ecotoxicity data is a problem when considering the employment of ionic liquids on a pilot or manufacturing scale (Wells and Coombe, 2006). The draining of contaminated process water represents the most likely release of ionic liquids into the environment. Thus, it is important to assess the hazard of ionic liquids towards aquatic compartment.

1.1 PROBLEM STATEMET

Hydroxyl functionalized ionic liquids are not yet thoroughly studied, but this type of ionic liquids have the nontoxic property and they are easily biodegradable. Incorporation of oxygen in the side chain of ionic liquids can enhance the hydrophilicity of ionic liquids. Hydroxyl functionalized ionic liquids are soluble in water and have the potential to accumulate in aquatic environments if released into natural waters such as spillage and effluents. Data on toxicity of hydroxyl functionalized ionic liquids towards aquatic lives are not available in the international literature. Therefore, a research needs to be carried out in order to determine the effect of hydroxyl group in imidazolium ionic liquids on its toxicity. This project will evaluate the acute toxicity of hydroxyl functionalized imidazolium ionic liquids to guppy fish (*Poecilia reticulata*). Guppy plays an important role in ecotoxicology as a prominent model vertebrate that are prone to contaminant exposure in aquatic systems because they live their whole life in water.

1.2 OBJECTIVES

The objectives of this study are:

1. To determine the effect of hydroxyl group in imidazolium ionic liquids on its toxicity.
2. To characterize hydroxyl functionalized ionic liquids that have been synthesized in the laboratory.
3. To determine and compare the effect of having hydroxyl group in cations and anions of imidazolium ionic liquids, and their impact on biological systems.

1.3 SCOPE OF WORK

Before beginning the test of toxicity, the properties of the environment water in the aquarium and properties of chemicals that will be used need to be determined. Then, four types of hydroxyl functionalized imidazolium ionic liquids will be synthesized. Next, the acute toxicity of these ILs for guppy will be assessed using OECD standard methods in Guideline 203. The number of dead fish will be recorded after 1, 12, 24, 48, 72 and 96 hours. Acute toxicity is expressed as the median lethal concentration (LC50). LC comprised in the range of LC1 to LC99 are also determined. The LC values and their 95% confidence limits will be determined by probit analysis using a computer software. Histopathological examination will be performed on control and on all dead fish

CHAPTER 2: LITERATURE REVIEW

The literature review is divided into subtopics to allow the readers to easily understand the contents of this chapter. In the below lines, several studies have been cited related to fate and effect of imidazolium ionic liquids on biological systems.

2.1 GREEN IONIC LIQUIDS

Ionic Liquids (ILs) are low-melting-point salts that have become increasingly attractive as green solvents for industrial applications. This green adjective is mainly attributed to their negligible vapor pressure, which avoids the loss of solvent to the atmosphere and decreases the worker exposure risk. Thus, room-temperature ionic liquids could provide environmentally friendly solvents for the chemical and pharmaceutical industries. An ionic liquid can be thought of as "designer" solvent because it is possible to design, or tailor, a solvent for a certain reaction. Therefore, many cation and anion combinations are possible, which will change the properties such as polarity, hydrophobicity and solvent miscibility behavior. Among these possibilities, the 1-alkyl-3-methylimidazolium is one of the most used cation because it is non-volatile, non-flammable, presents high thermal stability and is an excellent solvent for a wide range of inorganic and organic materials (Romero *et. al*., 2008).

2.2 CHARACTERISTICS OF IONIC LIQUIDS

Ionic liquids are often moderate to poor conductors of electricity, non-ionizing (e.g. non-polar), highly viscous and frequently exhibit a low vapor pressure. Typical IL consist of an organic cation with delocalized charges and a small inorganic anion, usually halogen anions such as chloride [Cl]-, tetrafluoroborate [BF_4]-, bis(trifyl)imide [NTf_2]- or hexafluorophosphate [PF_6]-. Their other properties are diverse: many have low combustibility, excellent thermal stability, wide liquid regions, and favorable solvating properties for a range of polar and non-polar compounds. Many classes of chemical reactions, such as Diels-Alder reactions and Friedel-Crafts reactions, can be

performed using ionic liquids as solvents. Recent work has shown that ionic liquids can serve as solvents for biocatalysis (Walker and Bruce, 2004).

The miscibility of ionic liquids with water or organic solvents varies with side chain lengths on the cation and with choice of anion. They can be functionalized to enhance hydrophilicity and miscibility of the IL in other chemicals. Because of their distinctive properties, ionic liquids are attracting increasing attention in many fields, including organic chemistry, electrochemistry, catalysis, physical chemistry, and engineering; see for instance magnetic ionic liquid.

2.3 STRUCTURAL FORMULA OF IMIDAZOLIUM IONIC LIQUIDS

There are four important classes of cations for ionic liquids (**Figure 2.1**). It is obvious that the variability of the side chains (R_1, R_2, R_3, R_4) in connection with the choice of different anions will result in a tremendous number of possible compounds. The main target class that we will concentrate on is imidazolium derivatives (**Figure 2.2**) of ionic liquids which exemplify our way of analysis (Jastorff *et. al.,* 2003).

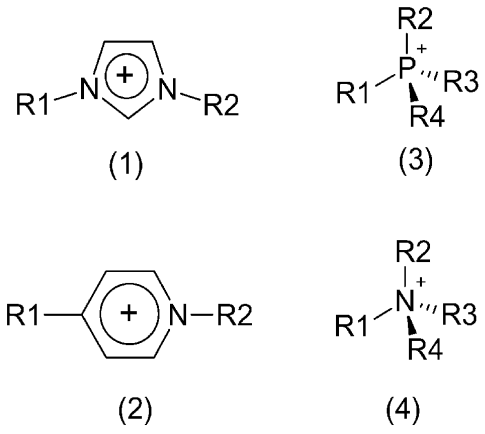


Figure 2.1: *Four important classes of cations for ionic liquids: (1) imidazolium-, (2) pyridinium-, (3) phosphonium-, (4) ammonium-class (Jastorff et. al., 2003).*

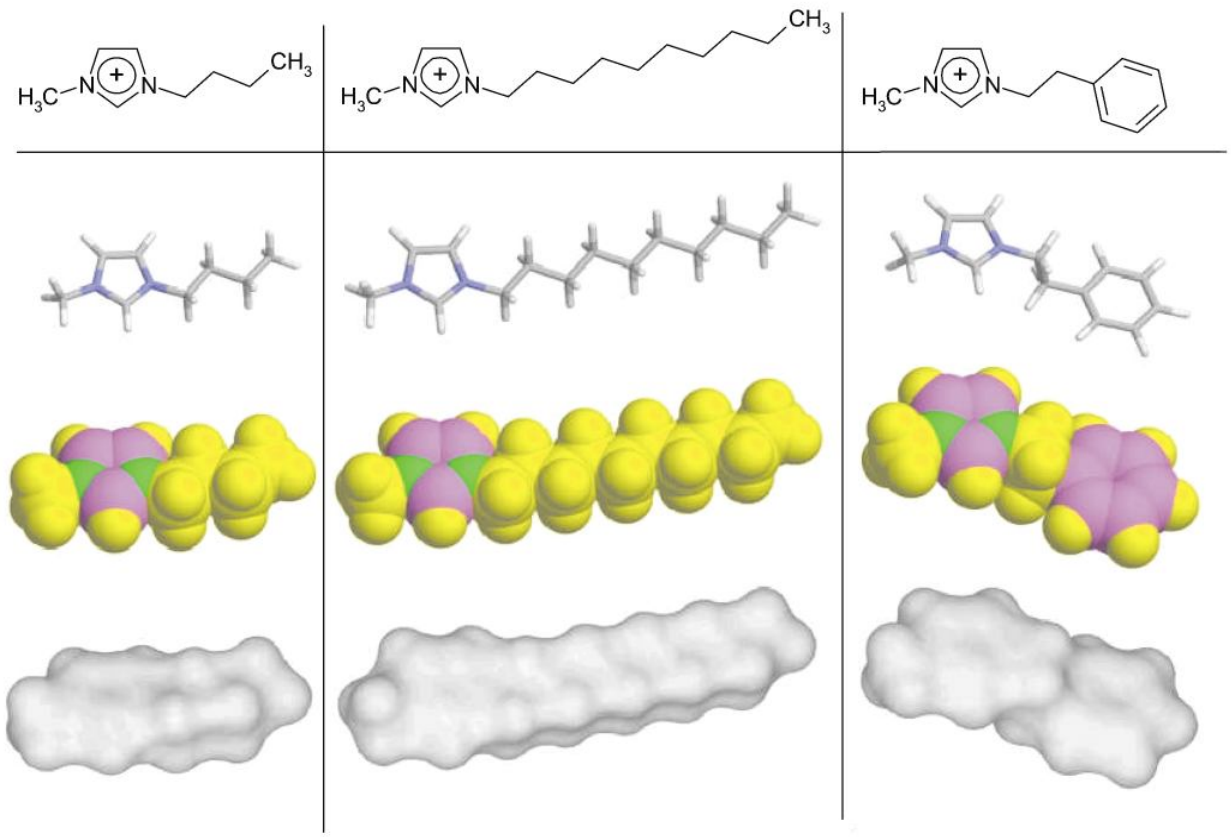


Figure 2.2: *Selection of three imidazolium cations: from left to right: bmim, dmim, 2-phenylethyl-mim. The top row shows their structural formulas. In the next three rows features of their 3-dimensional structures (optimized with MOPAC 2000, PM3 method) are shown: first row, stick models in CPK-coloration; second row, models in which the colours denote molecular interaction potential (yellow: hydrophobic interaction potential, green: positive charge, and violet: charge transfer potential); third row, water-accessible surface of the cations (Jastorff et. al., 2003).*

The central imidazolium ring is a delocalized aromatic system with high electron acceptor potential. Therefore, the nitrogen atoms are not able to form any hydrogen bonds. Elongation of side chain R_2 (C_4-chain, C_{10}-chain) leads to a continuous increase of flexibility implying more conformational freedom. The R_2-

residue of 2-phenylethyl-3-methylimidazolium chloride ([2-phenylethyl-mim][Cl]) contains an additional aromatic system. The phenyl ring shows a high electron density including electron donor potentials. Lipophilic parts within this molecule are the alkyl group and the phenyl structural element but some lipophilicity also resides in the aromatic imidazolium ring (Jastorff and colleagues, 2003).

All three selected compounds reveal the steric feature of a flat cation which results in flexibility and prevents direct and easy binding of polar compounds. Within our systematic algorithm the prediction of possible metabolites has to be considered. According to this theoretical approach several points of action can be identified. If these ionic molecules actually reach the cytochrome P_{450} system located in the endoplasmatic reticulum of any cell, they can be oxidized in different positions of the alkyl side chains. The resulting metabolites can further be broken down metabolically to biocompatible fatty acids and imidazole (for part of this proposed metabolism for the 1-butyl-3-methylimidazolium [bmim] cation) (see **Figure 2.3**) (Jastorff and colleagues, 2003).

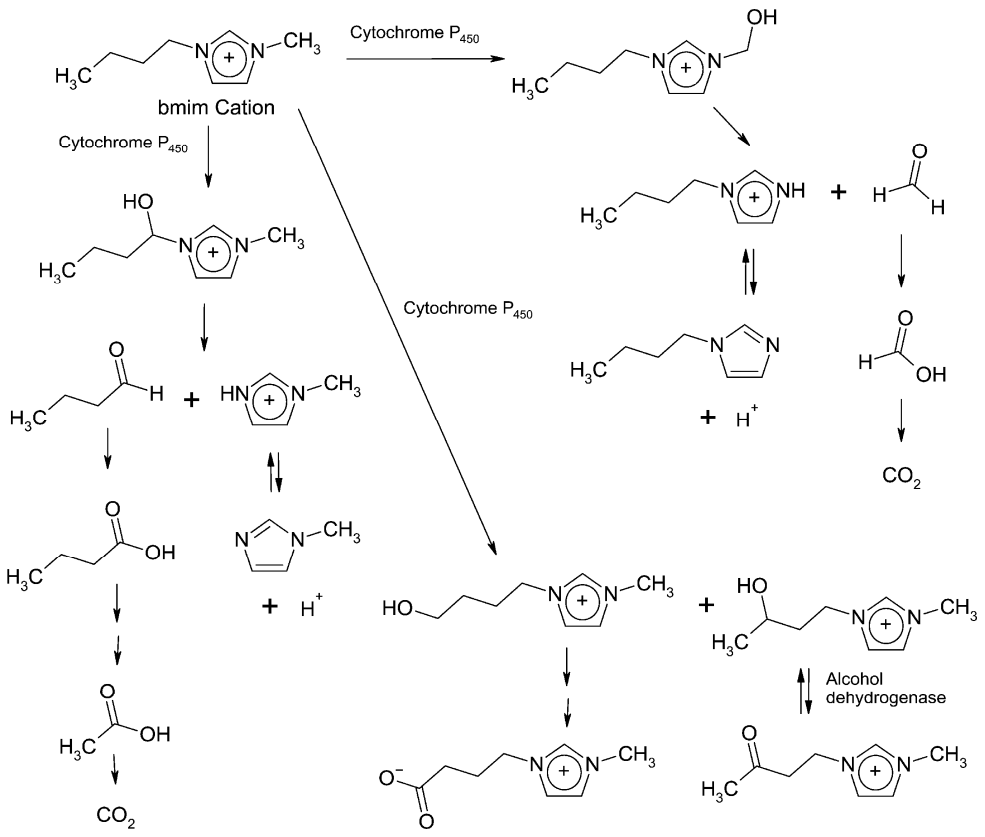


Figure 2.3: *Theoretically predicted metabolism scheme for [bmim] cation (Jastorff et. al., 2003).*

Of course it is possible that metabolites predicted theoretically may not be formed by nature. Nevertheless the prediction facilitates the analytical search for compounds while investigating the metabolic fate of ionic liquids in biological test systems. Such studies are under way within our cooperation network. Moreover, a computer supported expert system to predict metabolites of chemicals has been developed in collaboration with industry that might facilitate future work.

2.4 HYDROXYL-FUNCTIONALIZED IMIDAZOLIUM IONIC LIQUIDS

Ionic liquids can be functionalized to act as acids, bases or ligands. The interest in functionalized ionic liquids is growing because ionic liquids bearing ether, amino or alcohol functionalities have been shown to display special properties, including the ability to dissolve a larger amount of metal halide salts and to extract heavy metal ions from aqueous solutions. Adding a polar functional group on the cation will also form water-soluble ionic liquids. Salts whose imidazolium ring has a hydroxyl functionality are all soluble in water, even when the hydrophobic hexafluorophosphate anion is used (Mikami, 2005).

Holbrey et. al. (2003) had done a research where new ionic liquids containing (2-hydroxypropyl)-functionalized imidazolium cation have been synthesized by the atom-efficient, room temperature reaction of 1-methylimidazole with acid and propylene oxide; the acid providing the anionic component of the resultant ionic liquids. The incorporation of the secondary hydroxyl-functionality in the cation enhanced the water-miscibility and the IL is significantly more hydrophilic than the corresponding conventional 1,3-dialkylimidazolium systems. This hydrophilicity afforded by the secondary hydroxyl-group may be advantageous for stabilizing enzymatic catalyst systems in non-aqueous IL environments.

2.5 SYNTHESIS OF SECONDARY HYDROXYL-FUNCTIONALIZED IMIDAZOLIUM IONIC LIQUIDS

Holbrey et. al. (2003) had done the synthesis and characterization of new imidazolium-based ILs containing a secondary alcoholic functional group appended to the alkyl chain of the cation (refer **Figure 2.4**).

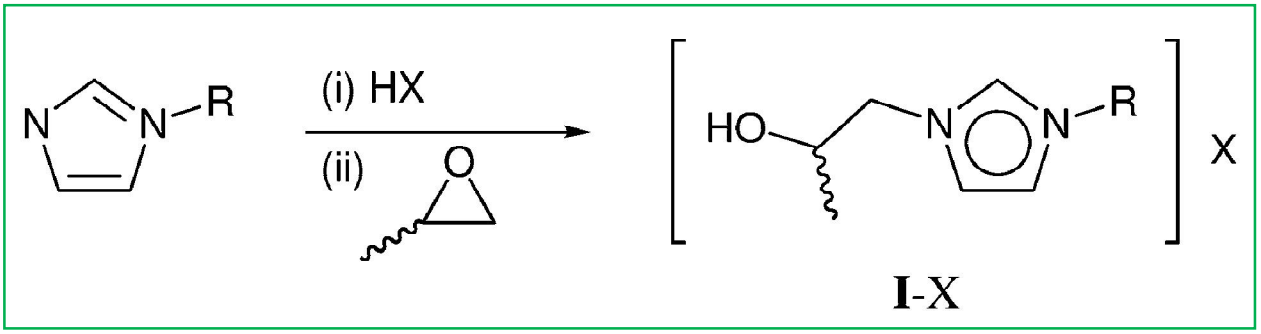


Figure 2.4: *Generalized synthesis of ILs containing 1-(2-hydroxypropyl)-3-methylimidazolium cations by reaction of 1-methylimidazole with acid and propylene oxide (R = CH_3; X = Cl, [NO_3], [PF_6], [NTf_2]) (Holbrey et. al., 2003).*

This 1-(2-hydroxypropyl)-3-methylimidazolium salt was prepared by protonating 1-methylimidazole with an acid, (example: HCl), followed by reaction with epoxide (example: propylene oxide). The reaction resulted in the quantitative formation of the corresponding ILs (I-Cl) containing the 1-(2-hydroxypropyl)-3-methylimidazolium cation. The choice of acid dictates the resultant anion of the IL prepared and so enables a range of ILs to be prepared with different anions in a clean, single-step process, without requiring further metathetical anion exchange steps (Holbrey *et. al*., 2003).

Holbrey and colleagues (2003) had also synthesized three other 1-(2-hydroxypropyl)-3-methylimidazolium salts which are I-[NO_3], I-[PF_6] and I-[NTf_2]. The following table shows the types of 1-(2-hydroxypropyl)-3-methylimidazolium salts synthesized and the reaction composition.

***Table 2.1**: Synthesized 1-(2-hydroxypropyl)-3-methylimidazolium salts and the respective reaction composition (Holbrey et. al., 2003).*

Salt	Base	Acid	Epoxide
1-(2-hydroxypropyl)-3-methylimidazolium chloride, (I-Cl)	1-methylimidazole	HCl	Propylene Oxide
1-(2-hydroxypropyl)-3-methylimidazolium nitrate, (I-[NO_3])	1-methylimidazole	HNO_3	Propylene Oxide
1-(2-hydroxypropyl)-3-methylimidazolium hexafluorophosphate, (I-[PF_6])	1-methylimidazole	HPF_6	Propylene Oxide
1-(2-hydroxypropyl)-3-methylimidazolium bis(trifyl)amide, (I-[NTf_2])	1-methylimidazole	$HNTf_2$	Propylene Oxide

Later, the reaction solvents (ethanol and water) and excess propylene oxide were removed to allow isolation of the ILs as clear, colorless liquids. Water content was determined for dried (and water-equilibrated I-[NTf_2]) samples by Karl–Fisher titration. **Table 2.2** shows the water content of the tested ionic liquids. All the ILs in **Table 2.2** were dried under high vacuum to yield moderately viscous fluids that varied in water content from 0.11 wt% (I-[NO3]) to 5.29 wt% (I-Cl). I-[PF6] was found to contain 2.22 wt% of water when dried (Holbrey et. al., 2003).

***Table 2.2**: Water content for I-Cl, I-[NO_3], I-[PF_6] and I-[NTf_2] (Holbrey et. al., 2003).*

Salt	Water content (wt%)	Density (g/mL)
I-Cl	5.29	1.15
I-[NO_3]	2.22	1.11
I-[PF_6]	0.95	1.57
I-[NTf_2]	0.11	1.17

From this finding, it can be concluded that the ILs prepared by Holbrey and co-workers (2003) are significantly more hydrophilic than corresponding conventional 1,3-dialkylimidazolium systems as a result of addition of the hydroxyl-function to the cation. Both I-Cl and I-[NO_3] were hydroscopic, absorbing water when exposed to a

moist atmosphere. Unusually, the hexafluorophosphate-containing IL, I-[PF_6], was also water soluble in contrast to most ILs containing $[PF_6]^-$ anions. The enhancement of water-miscibility by addition of the hydroxyl group can clearly be seen (Holbrey *et. al.*, 2003).

On the other hand, Yeon et. al. (2005) had synthesized 1-(2-Hydroxyethyl)-3-methyl imidazolium chloride in the laboratory by reacting 1-Methyl-imidazole (0.14 mol) with an excess of hydroxy-ethyl chloride (2-chloroethanol, 0.2 mol) in a round-bottom flask in a nitrogen atmosphere (70 ◦C, 48 h), using 200mL of acetonitrile as solvent. The molten salt of white crystalline solids was obtained by recrystallization under acetonitrile solvent in a freezer at −40 ◦C.

2.6 EFFECT OF IONIC LIQUIDS TO ENVIRONMENT

The development of ionic liquids as non-volatile solvents promises a group of solvents for synthesis whose dispersal into the environment should be more readily controlled and minimised. But how problematic are they if they do get into the environment? Although the information about physical, thermodynamic, kinetic or engineering data has been extended continuously, only little data with regard to the toxicity and ecotoxicity of ILs have been available until now. The ‘‘green character’’ of ILs has usually been justified with their negligible vapour pressure, but even if ILs do not evaporate and do not contribute to air pollution most of them are water soluble and might enter the environment by this path (e.g. accidental spills, effluents). To the best of our knowledge no data are available in the international literature on the acute toxicity of ILs on fish, the main living organism in freshwater (Pretti *et. al.*, 2006). Therefore, it is important to determine the further consequences and the environmental risk of the presence of ILs in wastewaters.

Most employed methods to evaluate the environmental risk of a substance in an aqueous media are those measuring their toxicity by using an inhibition assay. Different microorganism or enzymes have been used in this inhibition measurements, for example

the acute toxicity test which uses the *V. fischeri* (formerly *Photobacterium phosphoreum*) bioluminiscence inhibition assay being one of the most applied. This is a standard ecotoxicological bioassay in Europe (DIN EN ISO 11348). It is very rapid, cost-effective, and it is a widely accepted method for toxicity determination used extensively in the literature focusing on environmental issues. Up to now, the influence of some ionic liquids on organisms of the aquatic compartment has been shown for the luminescent bacteria Vibrio fischeri various green algae species, the crustacean Daphnia magna, the aquatic plant Lemna minor, the snail Physa acuta and the zebrafish Danio rerio (Stolte *et. al.*, 2007).

According to Jastorff et. al. (2003), although the number of publications concerning ionic liquids has increased substantially for over the past few decades, until now only few toxicological and ecotoxicological data are available for this group of chemicals. More information is needed to assess ionic liquids with regard to sustainability and to the principles of green chemistry. An adequate product design for this promising group of chemicals should consider not only technological needs but also toxicological and ecotoxicological risks.

2.7 CHEMICAL DEGRADATION OF IONIC LIQUIDS

Pham *et. al.* (2010) stated that ionic liquids can imposed a negative aspect for their treatment after usage prior to disposal because they possess excellent chemical and thermal stability. To assess the persistence of ILs in the environment as well as verify possibilities of their cleanup by chemical methods, several groups have focused their attention on oxidative and thermal degradation of ILs in aqueous media (Pham *et. al.*, 2010). Pioneering work in the field of oxidative degradation was done by Stepnowski and Zaleska (2005) and Morawski *et al.* (2005) who showed that the greatest degradation efficiency for imidazolium ILs was achieved with a combination of UV light and a catalytic oxidant such as hydrogen peroxide or titanium dioxide. The level of degradation was dependent on the alkyl chain length, Stepnowski and Zaleska (2005) indicated that lengthening the alkyl chain lowered the rate of IL degradation.

2.8 BIODEGRADABILITY OF IONIC LIQUIDS

In contrast to chemical degradation, which requires the assistance of a certain oxidant for catalysis, biodegradation is the microbial breakdown of chemical compounds. Biodegradation seems to be more environmentally friendly compared to chemical decomposition process. The initial attempt to examine the degradation potential of different IM14 cations combined with $[Br]^-$, $[BF_4]^-$, $[PF_6]^-$, $[N(CN)_2]^-$, $[(CF_3SO_2)_2N]^-$ and octylsulfate as the counter ion was done using the Sturm and Closed-Bottle test protocols by the group of Scammells. No compound showed significant degree of biodegradation with the exception of the octylsulfate-containing IL (Pham *et. al.*, 2010).

The studies of environmental fate and toxicity of ILs have shown that the ILs commonly used to date are toxic in nature and their toxicities vary considerably across organisms and trophic levels. In addition, the introduction of functional polar groups to the alkyl chain has been shown to reduce the toxicity of ILs and increase the biodegradation efficiency to some extent. This indicates the possibility of tailoring ILs by coupling suitable functional groups to their structure, which in turn leads to a more environmental friendly compound. The side chain length effect has been found to be consistent in all levels of biological complexity as well as different environmental compartments. Also, an increase in alkyl-chain length, or lipophilicity, was observed to be related to an increase in the rate of degradation as well as an increase in toxicity. This indicates a conflict of aims between minimizing the toxicity and maximizing the biodegradability of these neoteric solvents (Pham *et. al.*, 2010).

CHAPTER 3: METHODOLOGY

. This section will discuss the matters regarding research methodology of this project. It comprises of Research Methodology and Gantt Chart/Key Milestone.

3.1 RESEARCH METHODOLOGY

The research methodology is divided into three categories: Materials, Experimental Work and Theoretical Work.

3.1.1 Materials

a) Chemicals

The following chemicals are used to synthesize 1-(2-hydroxyethyl)-3-methyl imidazolium chloride [IM12OHCl], 1-(3-hydroxypropyl)-3-methyl imidazolium chloride [IM13OHCl], 1-(6-hydroxyhexyl)-3-methyl imidazolium chloride [IM16OHCl] and 1-(2-hydroxyethyl)-3-ethyl imidazolium chloride [IM22OHCl]. These chemicals are obtained by purchase from manufacturer.

i. 1- methylimidazole
ii. 1-ethylimidazole
iii. 2-chloroethanol
iv. 3-chloropropanol
v. 6-chlorohexanol
vi. Acetonitrile

b) Test Organism

Guppy (*Poecilia reticulata*) is used as the test organism in this project. It is a species of freshwater fish. They are suitable to live in lake water, pond water, river

water and tap water (unchlorinated). The guppy used in this project are bought from My Pets, a pet store in a nearby town.

3.1.2 Experimental work

The toxicity test is adapted from OECD (Organisation for Economic Co-operation and Development) Guidelines for Testing of Chemicals: Fish Acute Toxicity Test (Guideline 203).

i. Four types of hydroxyl functionalized imidazolium ionic liquids are synthesized by reacting alkylimidazole (1-methylimidazole, 1-ethylimidazole) with excess chloroalkanol (2-chloroethanol, 3-chloropropanol, 6-chlorohexanol) in a round bottom flask, using acetonitrile as solvent and by refluxing at 80°C
ii. The synthesized ionic liquids are characterized by IR, NMR (400 MHz, CD_3OD) and CHNS analysis.
iii. Six aquarium filled with 5L of lake water are prepared.
iv. The properties of the lake water (hardness, pH, alkalinity, dissolved oxygen concentration and temperature) are determined using proper equipment.
v. The water temperature is kept at 23 ±1 °C and aerated to restore the concentration of dissolved oxygen to at least 60% of its air saturation value.
vi. 10 fish are placed in each aquarium and the aquariums are kept under normal laboratory illumination with a daily photoperiod of 12 h.
(Note: No food are provided for the fish during the test)
vii. A limit test is performed at the concentration of 100 mg L^{-1} of ionic liquid before running the main toxicity test.
(Note: If no mortality occurs after 96 hours, this means that the LC50 value is greater than 100 mg L^{-1}. Therefore, no further testing will be done. If sublethal effects are observed, the observation will be recorded. When one fish in the test group dies, the test will be terminated and the remaining fish will be humanely killed.)
viii. The main test is performed and a full study is conducted based on OECD Guideline 203.

ix. The ionic liquid is directly dissolved in the rearing water at five concentrations (20.0, 40.0, 60.0, 80.0 and 100 mg L^{-1}).

x. The number of dead fish for each concentration is recorded after 1, 12, 24, 48, 72 and 96 hours.

xi. Acute toxicity is expressed as the median lethal concentration (LC50), that is the concentration in water which kills 50% of the test batch of fish within a continuous period of exposure of 96 hours.

xii. LC values comprised in the range of LC1 to LC99 are also determined. The LC values and their 95% confidence limits are determined by probit analysis using a computer software (USEPA Probit Analysis Program used for calculating EC values, version 1.5).

3.1.3 Tools and Equipment

The tools and equipment used in this project are:

i. A set of glassware for synthesis (condenser, round bottom flask, guard tube, etc.)

ii. Aquarium (Volume: 6 L)

iii. Petri dish

iv. Test tubes

v. Glass bottle

vi. Spatula

vii. Dropper

viii. Air pump – SOBO Aquarium Air Pump

ix. Dissolved oxygen meter – Mettler Toledo Portable DO Meter (Model MO128)

x. pH meter – Eutech Instruments pH 510 Bench Meter

xi. Coulometric Karl Fischer (Mettler Tolledo) titrator

xii. NMR spectrometer – Bruker Avance Spectrometer DPX 300

xiii. CHNS analyzer – Leco-CHNS-932 Analyzer

xiv. Densimeter - Anton Paar Vibrating Tube Densimeter Model DMA 5000)

3.1.4 Theoretical Work

A well-establish approach exists for quantifying lethal effects from data sets of concentration versus proportion of exposed individuals dying. The most common is the log normal model which involves log transformation of the concentration and then fitting of the data to the following model (Finney, 1971),

$$P = \frac{1}{\sigma\sqrt{2\pi}} \int_{-\infty}^{x_0} exp\left\{-\frac{(x-\mu)^2}{2\sigma^2}\right\} dx \qquad [1]$$

Where, P = the proportion expected to die, x_0 = the concentration for which predictions are being made, μ = the mean, and σ = the standard deviation.

Bliss (1943) suggested a slightly different response parameter, he defined the probit of P ('probit' = probability unit) as Y, where,

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{Y-5} exp\left\{-\frac{1}{2}u^2\right\} du \qquad [2]$$

Note that, by writing, $X = \mu + \sigma u$, and $Y=(X-\mu)/\sigma$, then the generalized probit function from which, we can get LC_{50} and confidence interval, it will be like as follows:

$$Y = 5 + \frac{1}{\sigma}(x - \mu) \qquad [3]$$

After results have been obtained for this full test, the percentage of mortality will be calculated and converted to probit using a table of probit (Refer **Table 3.1**).

Table 3.1: *Transformation of percentage to probits (Finney, D.J.,1971).*

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	**0.0**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**	**0.6**	**0.7**	**0.8**	**0.9**
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Next, a plot of percent mortality versus log10 concentration is constructed and will produce a sigmoid curve (Refer **Figure 3.1**), while a plot of probit versus log10 concentration will produce a straight line (Refer **Figure 3.2**). The concentration at which half of the population are killed is determined from the probit-concentration graph and denoted as LC50. The maximum concentration causing no mortality and minimum concentration causing 100 per cent mortality within the period of the test are also determined from the graph.

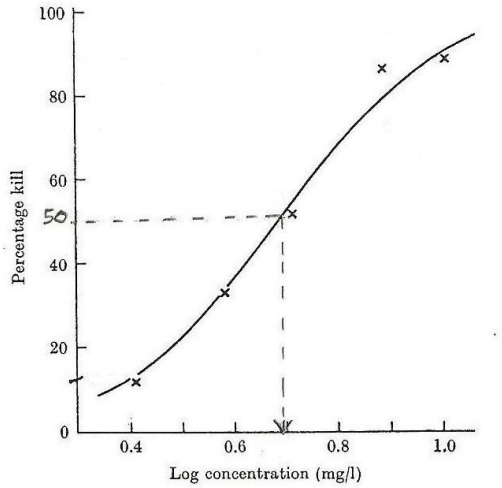


Figure 3.1: *Relation between percent mortality and log_{10} concentration (Finney, D.J.,1971).*

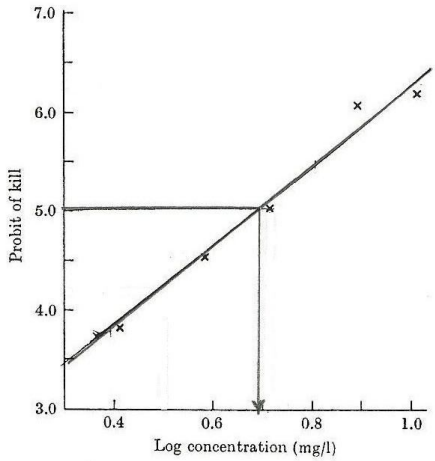


Figure 3.2: *Relation between probit and log_{10} concentration (Finney, D.J.,1971).*

From the probit-concentration graph, a linear equation called the probit regression line is developed to represent the relation between probit (Y) and log concentration (x) in the form of:

$$Y = a + Bx \qquad [4]$$

where, a = line intercept with y-axis

B= slope of the line

3.2 GANTT CHART/KEY MILESTONE

No.	Detail/ Week	1	2	3	4	5	6	7	8	9	10	11	12	16	19	20
1	Project Work: Study further detailed on the research scope and method							MID-SEMESTER BREAK								
2	Set up apparatus for acute toxicity test: Buying equipment, test organism and synthesizing test substance															
3	Run experimental and theoretical work, and analyzing data															
4	Submission of Progress Report 1															
5	Submission of Progress Report 2															
6	Poster Exhibition															
7	EDX															
8	Submission of Final Report (CD & softbound)															
9	Final Oral Presentation															
10	Submission of Project Dissertation (hardbound)															

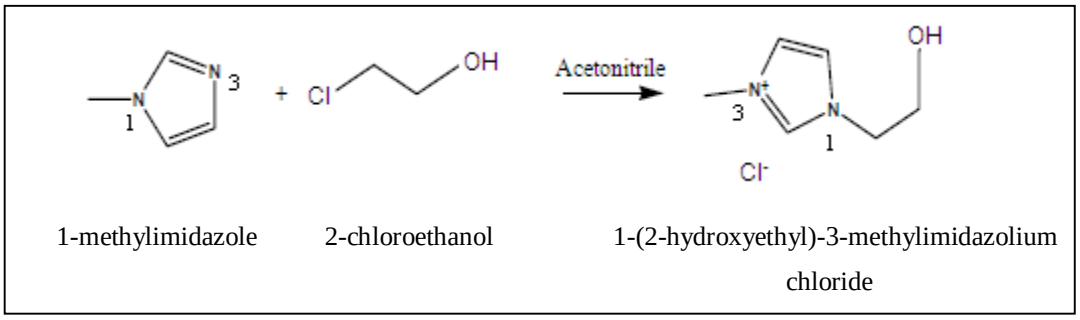
Figure 3.3: *Gantt Chart*

CHAPTER 4: RESULTS AND DISCUSSION

This section will display the results of the experiment done and discussions on the findings.

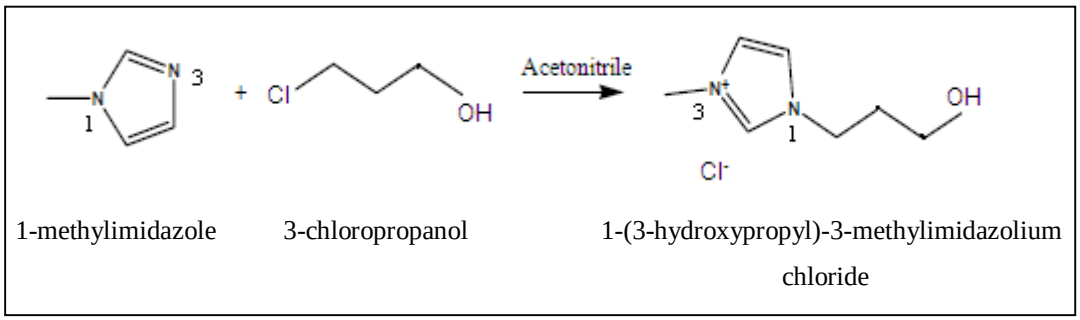
4.1 SYNTHESIZE OF IONIC LIQUIDS

Four types of hydroxyl functionalized imidazolium ionic liquids are synthesized by reacting alkylimidazole (1-methylimidazole, 1-ethylimidazole) with excess chloroalkanol (2-chloroethanol, 3-chloropropanol, 6-chlorohexanol) using acetonitrile as solvent. The mechanism and product of reaction are shown in **Figure 4.1**, **Figure 4.2**, **Figure 4.3** and **Figure 4.4**.



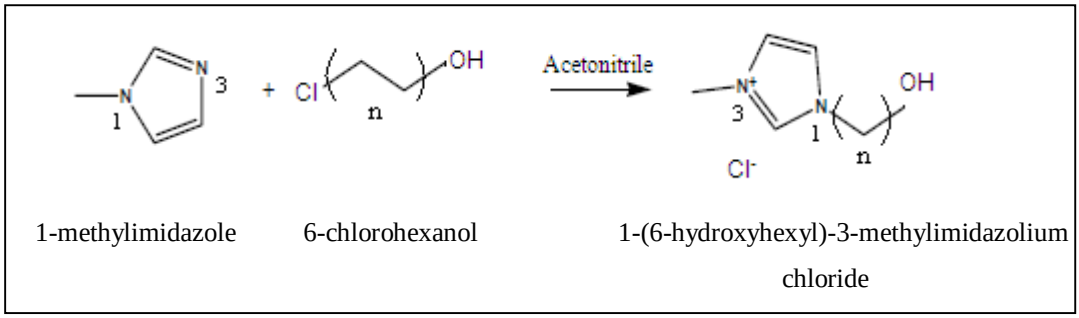
1-methylimidazole 2-chloroethanol 1-(2-hydroxyethyl)-3-methylimidazolium chloride

Figure 4.1: *Reaction of 1-methylimidazole with 2-chloroethanol*



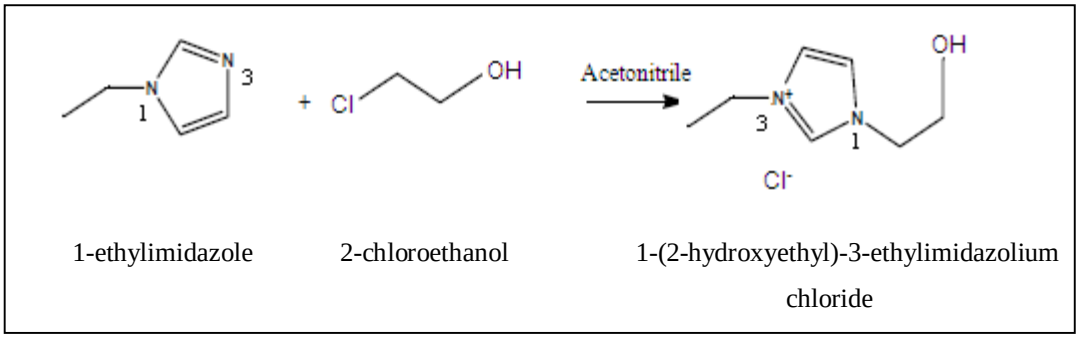
1-methylimidazole 3-chloropropanol 1-(3-hydroxypropyl)-3-methylimidazolium chloride

Figure 4.2: *Reaction of 1-methylimidazole with 3-chloropropanol*



1-methylimidazole 6-chlorohexanol 1-(6-hydroxyhexyl)-3-methylimidazolium chloride

Figure 4.3: *Reaction of 1-methylimidazole with 6-chlorohexanol, where n=6*



1-ethylimidazole 2-chloroethanol 1-(2-hydroxyethyl)-3-ethylimidazolium chloride

Figure 4.4: *Reaction of 1-ethylimidazole with 2-chloroethanol*

4.2 CHARACTERIZATION OF IONIC LIQUIDS

The ionic liquids synthesized are characterized by their water content, ^{1}H NMR spectra, elemental analysis and density. The water content of ionic liquids is measured by coulometric Karl Fischer (Mettler Tolledo) titrator. Ionic liquids are tested immediately after drying under high vacuum, and the water content is below 150 ppm for all samples. The results of ionic liquids characterization are shown in **Table 4.1**.

All ^{1}H NMR spectra are recorded on a Bruker Avance spectrometer DPX 300 at 27°C, using deuterated methanol as solvent. The carbon, hydrogen, nitrogen and sulfur percentages in the ionic liquids are analyzed according to the approved method ASTM D-5291 by employing Leco-CHNS-932 analyzer. The solid samples of less than 2 mg each are covered in silver capsule and then analyzed while the liquid ones are analyzed in silver capsules containing sorbit pad.

Density is measured with an Anton Paar vibrating tube densimeter, model DMA 5000, operating at atmosphere and within the temperature range 303 to 353 K. The internal calibration of the instrument is confirmed by measuring the densities of atmospheric air and doubly distilled water, according to the recommendations of the manufacturer. All the ionic liquid samples are degassed under vacuum and moderate temperature conditions for periods longer than 24 h, and stored in sealed vials prior to injection in the densimeter.

***Table 4.1**: Results of ionic liquids characterization*

Ionic Liquid (MW)	^{1}H-NMR	Elemental Analysis (%)	Density (g/cm^3)
1-(2-hydroxyethyl)-3-methylimidazolium chloride (162.5)	3.90 (t, 2H, NCH_2), 3.98 (s, 3H, CH_3), 4.35 (t, 2H, OCH_2), 7.62 (s, 1H, CH), 7.68 (s, 1H, CH), 9.01 (s, 1H, 2-CH)	C 44.53 H 7.03 N 17.11	1.23
1-(3-hydroxypropyl)-3-methylimidazolium chloride (176.5)	2.12 (p, 2H, CH_2), 3.62 (t, 2H, OCH_2), 3.99 (s, 3H, CH_3), 4.39 (t, 2H, NCH_2), 7.64 (s, 1H, CH), 7.71 (s, 1H, CH), 9.06 (s, 1H, 2-CH)	C 48.12 H 7.64 N 15.63	1.19
1-(6-hydroxyhexyl)-3-methylimidazolium chloride (218.5)	1.42 (m, 2H, CH_2), 1.57 (m, 2H, CH_2), 1.94 (m, 2H, CH_2), 3.55 (t, 2H, NCH_2), 3.98 (s, 3H, CH_3), 4.27 (t, 2H, OCH_2), 7.63 (s, 1H, CH), 7.70 (s, 1H, CH), 9.06 (s, 1H, 2-CH)	C 55.47 H 8.49 N 12.55	1.12
1-(2-hydroxyethyl)-3-ethylimidazolium chloride (204.5)	1.01 (t, 3H, CH_3), 1.41 (m, 2H, CH_2), 1.91 (m, 2H, CH_2), 3.91 (t, 2H, OCH_2), 4.29 (t, 2H, NCH_2), 4.36 (t, 2H, NCH_2), 7.70 (s, 1H, CH), 7.72 (s, 1H, CH), 9.10 (s, 1H, 2-CH)	C 52.16 H 8.97 N 12.98	1.13

4.3 LIMIT TEST

The limit test has been done using 1-(2-hydroxyethyl)-3-methylimidazolium chloride [IM12OHCl], 1-(3-hydroxypropyl)-3-methylimidazolium chloride [IM13OHCl], 1-(6-hydroxyhexyl)-3-methylimidazolium chloride [IM16OHCl] and 1-(2-hydroxyethyl)-3-ethylimidazolium chloride [IM22OHCl]. In this limit test, 500 mg of each of these ionic liquids were directly dissolved into the water in each aquarium. The test is run for 96 hours. Observations during the limit test are recorded in **Table 4.2**. After 72 hours, 2 fish died in the aquarium that contains IM22OHCl solution, while fish in other ionic liquid solutions are still alive. Therefore, a full toxicity test is run using IM22OHCl.

Table 4.2: *Observations after 24, 48, 72 and 96 hours of limit test*

Hour	Tank No.	Ionic liquid	No. of dead fish
1	1	None (control)	0
	2	[IM12OHCl]	0
	3	[IM13OHCl]	0
	4	[IM16OHCl]	0
	5	[IM22OHCl]	0
24	1	None (control)	0
	2	[IM12OHCl]	0
	3	[IM13OHCl]	0
	4	[IM16OHCl]	0
	5	[IM22OHCl]	0
48	1	None (control)	0
	2	[IM12OHCl]	0
	3	[IM13OHCl]	0
	4	[IM16OHCl]	0
	5	[IM22OHCl]	0
72	1	None (control)	0
	2	[IM12OHCl]	0
	3	[IM13OHCl]	0
	4	[IM16OHCl]	0
	5	[IM22OHCl]	0
96	1	None (control)	0
	2	[IM12OHCl]	0
	3	[IM13OHCl]	0
	4	[IM16OHCl]	0
	5	[IM22OHCl]	2

4.4 FULL OECD TOXICITY TEST (FIRST RUN)

In the first full test, IM22OHCl is directly dissolved in the rearing water at five concentrations (1.25, 2.5, 5.0, 10.0 and 20.0 mg L^{-1}). The number of dead fish for each concentration is recorded after 1, 12, 24, 48, 72 and 96 hours. Observations are recorded in **Table 4.3**.

Table 4.3: *Observations after 24, 48, 72 and 96 hours of first full toxicity test using1-(2-hydroxyethyl)-3-ethylimidazolium chloride*

Period (hours)	Ionic liquid concentration (mg/L)	Total number of dead fish	Observation on fish behavior	Oxygen concentration (%)	Temperature of water (°C)	pH of water
24	0	0	Fish movements are normal, but their abdomen are becoming a bit bigger	83.5	24.0	7.04
	1.25	0	Normal	86.2	24.0	7.05
	2.5	0	Normal	86.1	23.9	7.06
	5.0	0	Normal	84.3	23.8	7.08
	10.0	0	Normal	83.6	23.8	7.10
	20.0	0	Normal	82.5	23.8	7.12
48	0	0	Fish movements are normal, but their abdomen are becoming a bit bigger	83.4	23.7	7.05
	1.25	0	Normal	85.2	23.7	7.07
	2.5	0	Normal	85.0	23.8	7.09
	5.0	0	Normal	83.2	23.5	7.10
	10.0	0	Normal	85.6	23.5	7.11
	20.0	0	Normal	82.5	23.2	7.12
72	0	0	Fish movements are normal, but their abdomen are becoming a bit bigger	80.5	23.5	7.03
	1.25	0	Fish movements are normal, but their abdomen are becoming a bit bigger	81.1	23.4	7.07

	2.5	0	Normal	81.4	23.4	7.09
	5.0	0	Normal	80.3	23.3	7.12
	10.0	0	Normal	80.5	23.3	7.13
	20.0	0	Normal	81.1	23.2	7.14
96	0	0	Fish movements are normal, but their abdomen are becoming a bit bigger	73.3	23.5	7.08
	1.25	0	Fish movements are normal, but their abdomen are becoming a bit bigger	73.8	23.4	7.09
	2.5	0	Normal	72.1	23.4	7.10
	5.0	0	Normal	79.8	23.3	7.11
	10.0	0	Normal	76.6	23.3	7.12
	20.0	0	Normal	78.2	23.2	7.13

From observations recorded in **Table 4.3**, no fish had died after 96 hours of running the full toxicity test, which is an unexpected result. Therefore, the plot of percent mortality versus log10 concentration and a plot of probit versus log10 concentration cannot be constructed. It is expected that mortality will start to occur at a concentration which will be considered as the lethal concentration that will bring harm to the fish. This result might be because of the concentrations of ionic liquid used are too low. In the limit test, the concentration used is 100 mg/L, while the concentrations used in the first full test are lower than 100 mg/L. This means, the lethal concentration might be between 20 mg/L and 100 mg/L. Therefore, another full test needs to be conducted using IM22OHCl at concentrations of 20, 40, 60, 80 and 100 mg/L.

4.5 FULL OECD TOXICITY TEST (SECOND RUN)

In the second full test, IM22OHCl is directly dissolved in the rearing water at five concentrations (20.0, 40.0, 60.0, 80.0 and 100 mg L^{-1}). The number of dead fish in the aquarium for each concentration is recorded after 1, 12, 24, 48, 72 and 96 hours. Observations are recorded in **Table 4.4**.

Table 4.4: *Observations after 24, 48, 72 and 96 hours of second full toxicity test using1-(2-hydroxyethyl)-3-ethylimidazolium chloride*

Period (hours)	Ionic liquid concentration (mg/L)	Total number of dead fish	Observation on fish behavior	Oxygen concentration (%)	Temperature of water (°C)	pH of water
24	0.00	0	Normal	90.0	24.2	7.03
	20.0	0	Normal	91.0	24.2	7.05
	40.0	0	Normal	89.1	24.2	7.06
	60.0	0	Normal	84.5	24.2	7.08
	80.0	0	Normal	88.7	24.2	7.11
	100.0	0	Normal	89.4	24.1	7.12
48	0.00	0	Normal	91.0	23.4	7.05
	20.0	0	Normal	93.7	22.4	7.07
	40.0	0	Normal	90.5	22.5	7.09
	60.0	0	Normal	91.4	2.5	7.10
	80.0	0	Normal	91.9	22.7	7.12
	100.0	0	Normal	90.5	22.7	7.13
72	0.00	0	Normal	91.2	23.4	7.02
	20.0	0	Normal	91.4	23.4	7.08
	40.0	0	Normal	91.5	23.0	7.11
	60.0	0	Normal	91.3	23.0	7.13
	80.0	0	Normal	91.0	23.0	7.14
	100.0	0	Normal	90.2	23.0	7.14
96	0.00	0	Normal	91.0	23.8	7.07
	20.0	0	Normal	88.8	23.9	7.09
	40.0	0	Normal	87.3	24.0	7.10
	60.0	0	Normal	89.0	23.3	7.11
	80.0	0	Normal	91.5	23.4	7.12
	100.0	0	Normal	90.3	23.4	7.13

4.6 DISCUSSION

4.6.1 Limit Test

From observations recorded in **Table 4.2**, early conclusion made is that IM22OHCl is moderately toxic because two fish died in this solution. Meanwhile, IM12OHCl, IM13OHCl and IM16OHCl are all non-toxic to freshwater fish because 100 mg/L of these ionic liquids cannot kill any fish. According to OECD Guideline 203 (1992), if no mortality occurs after 96 hours of the limit test, this means that the LC50 value of that particular ionic liquid is greater than 100 mg/L, meaning that it is non-toxic. Therefore, further tests are only done using IM22OHCl.

4.6.2 Full OECD Toxicity Test

From observations recorded in **Table 4.3** and **Table 4.4**, it is found that no fish had died after 96 hours of running the full toxicity test using IM22OHCl for both first and second run. During the first toxicity test, it is assumed that the concentration of ionic liquids used are too low, where the lethal concentration might be between 20 mg/L and 100 mg/L. Therefore, another test is conducted using IM22OHCl at these concentrations. However, the result of the second toxicity test is also the same with the first test. This means that the assumption of the concentration of ionic liquids used in the first full toxicity test are too low is not true. The only relevant conclusion that can be made from the results is, the lethal concentration of IM22OHCl is higher than 100 mg/L. This means that IM22OHCl is non-toxic to freshwater fish.

The results of the full toxicity test have proven that the result of limit test is wrong. Several factors that might cause this mistake are:

i. The fish died because of infection by a disease or bacteria in the water.
ii. The water contains minerals or dissolved materials that are not suitable with the fish.

Since no fish died during the experiment, the plot of percent mortality versus $\log_{10}$ concentration and a plot of probit versus $\log_{10}$ concentration cannot be constructed. Same goes with the value of LC_{50} of all four ionic liquids tested cannot be determined.

4.6.3 Water Solubility of Hydroxyl-functionalized Ionic Liquids

Throughout the experimental work carried out in this project, it is observed that all the ionic liquids used are soluble in water. This observation is in agreement with recent published papers dealing with hydroxyl-functionalized ionic liquids solubility in water (Holbrey *et. al*., 2003).

4.6.4 Challenges Faced During The Project

During the commencement of this project, various of challenges have to be faced in order to arrive to the final conclusion. For example, the author had faced difficulties during the early stage of rearing the fish in the laboratory. At first, the author and colleagues used dechlorinated tap water to rear the fish in the aquarium. However, it is noticed that the fish is not suitable to live in tap water because a lot of fish died after a few days of holding the fish in the aquarium. This might be due to high chlorine concentration in the tap water. This has resulted in wasting of time and money because the fish died with no benefit to the research. Therefore, lake water is used to run the experiment because lake water contains natural minerals that are close to the original environment of the fish habitat.

Other than that, the limit test and full toxicity test are time-consuming. A total of 12 days are needed in order to complete each of the limit and full toxicity test, that is seven days for holding the fish in the new rearing water, and five days for running the toxicity test (from the time of dissolving the ionic liquids into the water until the time for recording the final observation). Considering a few failure of tests due to unsuitable type of water used in the early stage of this project, a lot of days had been spent in the laboratory in order to arrive to the final results.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

The syntheses of hydroxyl imidazolium ionic liquids are confirmed by the NMR data and elemental analysis, and the results of the characterization are as shown in **Table 4.1**. Based on the result of full toxicity tests done, it is proven that hydroxyl-functionalized imidazolium ionic liquids are all soluble in water. Two fish died in lake water containing IM22OHCl during the limit test, therefore a full toxicity test is run using IM22OHCl. After two consecutive full toxicity tests have been done using IM22OHCl, it is observed that no fish died during the test. Therefore, it is concluded that IM22OHCl together with IM12OHCl, IM13OHCl and IM16OHCl are non-toxic to freshwater fish. Considering that all the ionic liquids used have a hydroxyl functional group in cation, it is not possible to compare the effect of having hydroxyl functional group in cations and anions of imidazolium ionic liquids from this project.

As a recommendation, further study need to be done in order to determine the effect of having hydroxyl functional group in anions of imidazolium ionic liquids. Besides that, histopathological examination needs to be done on the fish taken from each aquarium to determine the status of health of the fish. This is because although the fish did not die during the experiment, there are chances that they are being infected internally by the ionic liquids. In histopathological examination, the fish will be fixed in a 10% buffered formalin solution. The entire fish body will be longitudinally sectioned, and placed directly into pre-labelled histological cassettes. Fixed samples will be processed and included in paraffin-blocks. 5 mm thick whole body sagital sections will be cut and stained with Hematoxylin-Eosin for histological evaluation.

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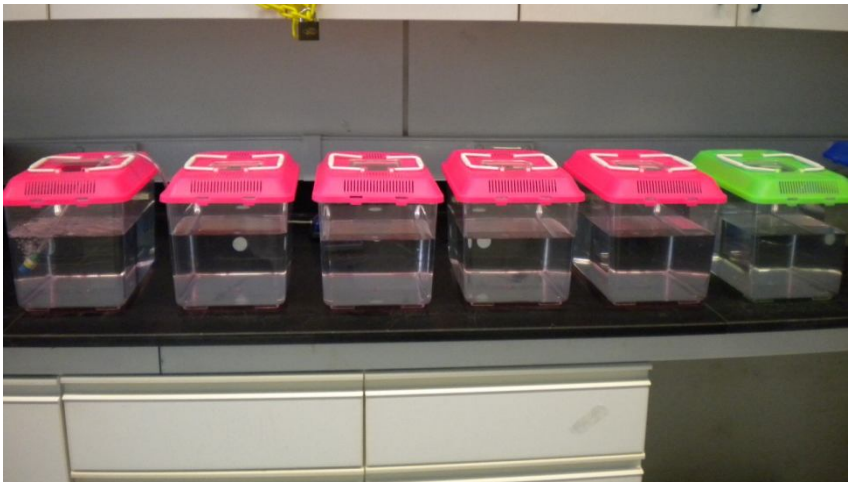
APPENDIX

APPENDIX 1: PICTURES OF EXPERIMENTAL APPARATUS



Arrangement of experimental apparatus

Eutech Instruments pH 510 Bench Meter

Mettler Toledo Portable DO Meter (Model MO128)

Guppy (*Poecilia reticulata*)