Antibiofilm and antimacrofouling potential of alkylimidazolium ionic liquids

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A thesis submitted to the

Board of Studies in Life Sciences

In partial fulfillment of requirements

for the Degree of

DOCTOR OF PHILOSOPHY

of

HOMI BHABHA NATIONAL INSTITUTE

March 2021

Homi Bhabha National Institute

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List of Publications arising from the thesis

Journal

Published

1. Long alkyl-chain imidazolium ionic liquids: antibiofilm activity against phototrophic biofilms. Kiran Kumar Reddy, G., Nancharaiah, Y. V., Venugopalan, V. P., Colloids and Surfaces B: Biointerfaces, **2017**, 155: 487-496.

2. Alkylimidazolium ionic liquids as antifungal alternatives: Antifungal, antibiofilm activity against *Candida albicans* and underlying mechanism of action. Kiran Kumar Reddy, G., Nancharaiah, Y. V., Frontiers in Microbiology, **2020**, 11: 730.

3. Effect of alkylimidazolium ionic liquids on barnacle larval survival, metamorphosis and settlement. Kiran Kumar Reddy, G., Rajitha, K., Nancharaiah, Y. V., Journal of Molecular Liquids, **2020**, 302: 112497.

Communicated

1. Alkylimidazolium ionic liquids for biofilm control: Experimental studies against natural multispecies biofilms. Kiran Kumar Reddy, G., Nancharaiah, Y. V., Journal of Molecular Liquids, **2021**, (Under Review).

Chapters in books and lectures notes

Nil

Conferences

1. 1-alkyl-3-methylimidazolium ionic liquids: Novel Antibiofouling compounds, (Poster presentation). International Conference on New Horizons in Biotechnology (NHBT 2019), organised by CSIR & BRSI, Nov 20-24, 2019 at Thiruvananthapuram, Kerala, INDIA.
2. Antibiofilm activity of alkylimidazolium ionic liquids against fungal biofilms, (Oral presentation). 2nd International Conference on Contemporary Antimicrobial Research (ICCAR 2018), organised by DBT & SAR, Dec 15-17, 2018 at IIT Kharagpur, INDIA.
3. Two day training course on "Mitigation strategy for fouling in marine/shipping, civil, refinery and other industrial equipment and structures (MSF 2017)", NIGIS South zone, NACE International Gateway India Section, Apr 21-22, 2017 at Chennai, INDIA.

Others

Nil

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DEDICATION

Dedicated to my parents, for the sacrifices they went through for giving me best.

ACKNOWLEDGEMENTS

I am extremely grateful to the people whose effort and support helped me in completing this research work and PhD thesis. First and foremost, I thank my supervisor, **Prof Y. V. Nancharaiah**, whose expertise was invaluable in formulating the research questions, methodology and completion of this thesis. I am indebted to him for all his teaching, training and nurturing the naive mind of mine. He always stood by me under all circumstances, be it professional or personal. A wonderful mentor with whom I share my joy and sorrow.

I am very thankful to **Prof S. Adhikari**, the chairman of my doctoral committee, who significantly helped in understanding the chemistry and behavior of ionic liquids. His suggestions and scrutiny helped me in making the thesis better. A special mention of thanks to **Prof. Hema Rajaram**, Dean, Life Science, HBNI and also a member of doctoral committee, for the critical scrutinization of the work presented during doctoral committee meetings. I also thank other doctoral committee members, **Prof. Prasun Mukherjee** and **Prof. S. N. Jamdar** for their continuous evaluation and the valuable inputs with their microbiology and biochemistry expertise which significantly improved this thesis.

I would like to thank my colleagues Dr. P. S. Murthy, Dr. Rajesh Kumar, Dr. Hiren Joshi, Dr. Sudhir Kumar Shukla, Mr. Francis Vincent, and Mr. Atif Khan for helping me with various technical support and instrumentation. Enjoyed the technical discussions with Dr. Padmavathi in the laboratory and I thank her for the same. A special mention of appreciation to my stress busting tea group members including Dr. Abdul Nishad, Dr. Rajini Anthony and Ms. Sonal Maroo, for the joyful time spent during tea breaks. Thanks to Mr. Sarvajith and Mr. Vel Raj, research scholar friends

in the laboratory who were always ready to offer help in the laboratory. Greatly appreciate the technical support provided by research scholar and my friend, Ms. Rajitha in carrying out the macrofouling work. Am also thankful to Mr. Veeramani, Mr. Jeorge Fernandez, Mr. N. K. Pandey, Mr. Jagdish Jat and Mrs. Malathy Natarajan for their support in laboratory. Am thankful to all the members of WSCD who helped me directly and indirectly during this period.

I thank Dr. T. Subba Rao, Head, Biofouling and Thermal Ecology Section for the constant support and encouragement throughout this study. The encouragement that I got from Dr. V. P. Venugopalan, former Head of our section, needs a special mention. I greatly appreciate the support and motivation received from Dr. T. V. Krishna Mohan, Head, Water and Steam Chemistry Division during this period.

This journey would not have been possible without the support of my family, and friends. Am indebted to all my family members for encouraging me in all of my pursuits and inspiring me to follow my dreams. I am especially grateful to my parents, my sister, whose sacrifice has made me what I am in my life and career. My wife Deepika has always stood by my side during this wonderful journey. I always cherished the moments spent with my daughter, Dhruthi Reddy during the stressful times. Big thanks to all my training school batch mates who provided all the support I needed throughout. I thank my friends Hari Krishna, Arun, Mahesh and Ramesh for the joyful evening walks and long drives, during the turbulent times.

(Signature)
G. Kiran Kumar Reddy

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CHAPTER 6

Overall Findings of the Thesis and Future Directions

6.1. Overall findings of the thesis

Biofouling is the sessile growth of living organisms on the submerged surfaces. Growth of microorganisms such as bacteria, microalgae and fungi in a self-produced EPSs matrix in the form of biofilms is termed as microfouling [227]. Attached growth of bacteria and fungi on tissues and medical implants constitutes the medical biofilms. Most (~80%) of the microbial infections in humans are linked to biofilm formation [10]. Environmental biofilms are mainly constituted by bacteria and microalgae. Controlling biofilm formation on various medical and industrial surfaces is essential to prevent infection, contamination and instrument failures [11]. Antimicrobials which prevent planktonic growth are poorly effective against biofilms as biofilm bacteria are about 1000-times more tolerant to antimicrobials [229]. Invertebrate macrofoulers such as barnacles, mussels, hydroids and tubeworms attach to various surfaces of marine industry and cause biofouling [185]. Macrofouling blocks various conduits, heat exchangers and reduces water flow in industrial cooling water systems. Biofouling buildup can significantly increase the pressure drop leading to decreased flow rates and pump failures.

Challenges of current biofouling control agents/methods including poor efficiency and environmental toxicity [84] are the driving force for researching efficient and environmentally benign antibiofouling approaches. Natural and synthetic compounds are being evaluated for developing environmentally benign antifouling formulations. In this context, ionic liquids have received attention for developing new generation antifouling formulations. Ionic liquids are composed of combinations of cations and anions and allow synthesizing large number of compounds (10^{18}) with tunability of structure/properties. In addition to diverse industrial applications, these

compounds have numerous biotechnological and pharmaceutical applications. In particular, ionic liquids containing imidazolium cation have attracted considerable interest as antimicrobials. Alkylimidazolium ionic liquids have demonstrated efficient antibiofilm activity against certain pathogenic bacteria [97; 229]. However, the efficacy of imidazolium ionic liquids in controlling biofilms such as phototrophic biofilms, fungal biofilms and natural multispecies biofilms is not fully known. There were no prior studies on the efficacy of these compounds on settlement of macrofouling organisms (e.g., barnacles). Moreover, there are limited studies on mechanism of action of these compounds on biofouling organisms.

Alkylimidazolium ionic liquids that differ in alkyl side chain length were chosen for the investigations on bacteria, diatoms, fungi, biofilms and barnacle larvae. The selected ionic liquid compounds were tested on the growth and biofilm formation of a marine diatom, *Navicula* sp. An inverse relationship between alkyl side chain length and growth/ biofilm inhibition was observed. Among the 3 tested compounds, 1-hexadecyl-3-methylimidaazolium chloride ([C_{16}(MIM)][Cl]) showed the highest activity with complete biofilm inhibition at concentrations as low as 5 μM. Another compound [C_{12}(MIM)][I] also caused >90% inhibition at 10 μM. However, [C_4(MIM)][Cl] failed to inhibit biofilm formation even at 100 μM. Inhibition was clearly dependent on the carbon chain length of alkyl group. The constituents of ionic liquids (anions and 1-methylimidazole) did not inhibit the growth and biofilm formation even at elevated concentrations of 1000 μM, indicating that imidazolium cation with alkyl side chain is solely responsible for the antimicrobial action. SYTOX® Green staining and chlorophyll leakage studies revealed that, ionic liquids increase cell membrane permeability in *Navicula* sp. cells leading to cytotoxicity.

Most effective alkylimidazolium ionic liquid, [C_{16}(MIM)][Cl] was effective in preventing polymicrobial phototrophic biofilms in natural waters. At 5 μM [C_{16}(MIM)][Cl] and above, biofilm formation on test coupons in natural waters (freshwater and seawater) was completely inhibited. Phototrophic biofilms formed in control contained different types of diatoms, microalgae, dinoflagellates and cyanobacteria. Results showed that formation of multispecies phototrophic biofilms in natural waters can be retarded by ionic liquid and have prospective applications in controlling phototrophic biofilms in cooling water systems.

Alkyl side chain dependent antifungal and antibiofilm activity was observed when tested against laboratory and drug resistant clinical *C. albicans* strains. [C_{16}MIM][Cl] showed highest activity followed by [C_{12}MIM][I]. -butyl side chain containing [C_4MIM][Cl] did not exert any antifungal or antibiofilm activity against growth or biofilm formation against all the tested *C. albicans* strains. Dislodgement (dispersal) of pre-formed *C. albicans* biofilms was achieved only with [C_{16}MIM][Cl]. Although [C_{12}MIM][I] did not disrupt the pre-formed *Candida* biofilms, it was able to penetrate and kill the biofilm-resident cells of laboratory and drug resistant clinical *C. albicans*. Experimental analysis indicated the membrane damage, leakage of intracellular contents (metal cations and aromatics), and shrinking of *C. albicans* cells upon exposure to antifungal ionic liquid. *C. albicans* cells grown in the presence of antifungal ionic liquid had reduced ergosterol content, indicating cell membrane as the primary target in ionic liquid induced toxicity. *C. albicans* cells exposed to antifungal ionic liquid also exhibited severe oxidative stress, loss of mitochondrial membrane potential and loss of mitochondrial dehydrogenases activity. These results inferred that, in addition to membrane damage, tested antifungal ionic liquids also damages

other cellular components and impair cellular metabolism. Mechanisms involving multiple cellular targets are likely responsible efficient antifungal activity of ionic liquids.

For evaluating antimacrofouling activity of imidazolium ionic liquids, barnacle larvae (*Amphibalanus reticulatus*), a dominant macrofouler in the seawater cooling system of MAPS at Kalpakkam was chosen as the model test organism. All the three alkylimidazolium ionic liquids induced mortality and inhibition of cypris settlement in a dose dependent manner. Among the three compounds, $[C_{12}MIM][I]$ exhibited superior antimacrofouling activity, requiring lesser concentrations for preventing larval settlement. At micromolar concentrations, the tested ionic liquids were lethal to cypris larvae. However, settlement of cypris was strongly inhibited at sub-lethal nanomolar concentrations. The anti-settlement activity of the tested ionic liquids on barnacle larvae decreased in the following order: $[C_{12}MIM][I] > [C_{16}MIM][Cl] > [C_4MIM][Cl]$. Similar anti-larval activity patterns were observed when tested for survival and metamorphosis of different naupliar larval stages. The constituent groups of tested ionic liquid compounds such as anions (Cl^-, and I^-) and 1-methylimidazole did not induce mortality or caused settlement inhibition even at higher concentrations. Therefore, the effect of ionic liquids on larvae was linked to 1-alkyl-3-methylimidazolium cation of the ionic liquid compound. Therapeutic ratio ($T_R = LC_{50}/EC_{50}$), a parameter used to evaluate the toxicity of prospective antifouling compounds was found to be very satisfactory for the tested ionic liquids. T_R values for the all the three tested ionic liquid compounds were at >28 to 900 and much higher than the reference value (>15) qualifying them as promising non-toxic or environmentally benign antifoulants. Moreover, the EC_{50} and LC_{50} concentrations of

all the three ionic liquid compounds were found to be non-lethal to non-target organism, *Artemia salina*. This study provided data for the first time that settlement of barnacle larvae settlement can be completely inhibited in *in vitro* assays using nanomolar concentrations of ionic liquids. Moreover, the tested ionic liquids exhibited either minimal toxicity or no toxicity to *Artemina salina* at effective concentrations.

Lastly, the efficacy of antimicrobial alkylimidazolium ionic liquids was evaluated for prevention of heterotrophic biofilms in natural waters. For this, formation of multispecies biofilms was optimized in natural freshwater and seawater under different amendment/ incubation conditions. Although, there were some differences in the antibiofilm activity in freshwater and seawater, $[C_{12}MIM][I]$ and $[C_{16}MIM][Cl]$ were effective in inhibiting formation of multispecies biofilms. $[C_{16}MIM][Cl]$ also exhibited dispersal activity on the pre-formed multispecies biofilms. The potent antimicrobial ionic liquid ($[C_{16}MIM][Cl]$) inhibited biofilm formation under dynamic conditions in freshwater and seawater at 100 µM and 250 µM , respectively, when a laboratory scale re-circulating system was operated for 12 days. These results indicate potential use of $[C_{16}MIM][Cl]$ for minimizing biofilm growth in cooling water systems, dental water lines or other niche applications.

In summary, alkylimidazolium ionic liquids ($[C_{12}MIM][I]$ and $[C_{16}MIM][Cl]$) showed strong antibacterial, antialgal, antifungal, antibiofilm and anti-larval activity in various laboratory assays. Higher T_R values and non-toxicity towards *Artemia salina* indicated qualify them for use in non-toxic or environmentally benign antifouling formulations. Although potent ionic liquids deciphered strong antibiofouling activity against all the tested organisms, selection of the compound and required concentration depends on the organisms being targeted in biofouling control.

For example, [C_{16}MIM][Cl] can be preferred for biofilm control due to its dual activity in terms of inhibiting biofilm formation and dispersing pre-formed biofilms. On the other hand, [C_4MIM][I] can be considered for developing environmentally benign antifouling paints for controlling macrofouling by barnacles.

6.2. Future directions

Although a detailed analysis of biofouling control activities of alkylimidazolium ionic liquids was carried out against diverse organisms, other studies are required for employing these compounds as alternate antibiofouling agents. Data indicated efficient activity against growth and biofilm formation by phototrophs in short-term laboratory experiments. For potential applications as alternate algicides and biofilm control agents in cooling water systems, a detailed study in pilot scale cooling tower needs to be carried out. Efficacy and cost comparison analysis with commercial proprietary algicides would yield valuable information on possible application of these compounds in industrial cooling water systems.

Against diatom cells, membrane damage followed by leakage of intracellular contents was found to be the primary cause of toxicity. However, the changes in biochemistry of cells in the presence of sub-lethal concentrations in terms of oxidative stress, damage to biomolecules or an increase in expression levels of enzymes involved in cellular defence systems are not known and needs additional experimentation. These experiments would detail the overall mechanisms behind the efficient antialgal and antibiofilm activity of imidazolium ionic liquids against phototrophic organisms.

Strong antifungal, antibiofilm and detailed toxicity mechanisms for prevalent fungal pathogen (*C. albicans*) has been presented. However, the application on tissue

surfaces for treating infections depends on the cytotoxicity of these compounds. Detailed antifungal and cytotoxicity assays with skin cell lines and *in vivo* animal models would allow the use of these compounds for topical applications in preventing or treating fungal infections.

To make use of the broad-spectrum antimicrobial and antibiofilm activity against bacterial and fungal pathogens, incorporation of these compounds into surfaces of medical implants needs detailed studies. Studies pertaining to slow release of these compounds from the incorporated polymers for inhibiting settlement of pathogens would generate valuable information for their application in biomedical systems.

In contrast to biofilm control, all the tested compounds were effective in preventing barnacle larval settlement. This can be a great advantage in targeted macrofouling control, where in non-toxic $[C_4MIM][Cl]$ can be dosed to prevent macrofouling in critical components of cooling water systems. Incorporation of this environmentally benign ionic liquid on the biocide release coatings for preventing attachment of macrofoulers needs thorough experimentation. Mechanism behind settlement inhibition induced by nanomolar concentrations of ionic liquids needs detailed analysis. Gene expression and proteomic analysis would provide the useful information for understanding settlement inhibition mechanisms.

Experiments against natural multispecies biofilms from freshwater and seawater indicated the efficient antibiofilm activity under dynamic flow conditions in lab-scale recirculation systems. Activity assays using a pilot scale recirculation system connected with heat exchangers and comparison of activity with commercial biocides

would produce the valuable information on using these compounds in industrial scale recirculating cooling water systems.

SUMMARY

1-Alkyl-3-methylimidazolium ionic liquids with different alkyl groups ($[C_nMIM]^+[X]^-$, n=4, 12 and 16) were investigated for determining antibacterial, antialgal, antifungal, antibiofilm, and anti-larval activities and for understanding mechanism of action on biofouling organisms. The antibiofilm activity on *Navicula* sp. was dependent on the side chain length wherein $[C_{16}MIM][Cl$ displayed the highest antibiofilm activity. Whereas, $[C_4MIM][Cl]$ was ineffective even at millimolar concentration. $[C_{12}MIM][I]$ exhibited moderate antibiofilm activity on diatom cells. Ionic liquid treatment induced membrane damage and leakage of intracellular chlorophyll *a* from *Navicula* sp. cells. Formation of multispecies phototrophic biofilm in natural waters (freshwater and seawater) under static conditions was prevented by the potent ionic liquid, $[C_{16}MIM][Cl]$.

The antimicrobial ionic liquids such as $[C_{12}MIM][I]$ and $[C_{16}MIM][Cl]$ exhibited strong antifungal and antibiofilm activity on standard and clinical *Candida albicans* strains. In addition to biofilm prevention, $[C_{16}MIM][Cl]$ exhibited dispersal action on pre-formed *C. albicans* biofilms. Antibiofilm activity was found to be similar on laboratory and drug resistant clinical strains of *C. albicans*. Antifungal ionic liquid induced significant membrane damage and leakage of intracellular contents such as biomolecules and metal cations from *C. albicans* cells. At sub-lethal concentrations, the antifungal ionic liquid decreased cell membrane ergosterol content. Additionally, antifungal ionic liquid caused mitochondrial dysfunction, loss of mitochondrial membrane potential, and decreased mitochondrial dehydrogenase activity. Increased endogenous reactive oxygen species due to severe oxidative stress was also observed in the *C. albicans* exposed to antifungal ionic liquid. It is evident

that antifungal ionic liquid has multiple cellular targets in *C. albicans* for exerting antifungal and antibiofilm action.

All the three tested ionic liquids were found to be effective in exerting mortality and settlement inhibition in barnacle (*Amphibalanus reticulatus)* larvae. Interestingly, non-antimicrobial ionic liquid ([C_4MIM][Cl]) was found to exert both larvicidal and settlement inhibitory action on barnacle larvae. Comparatively, both [C_{12}MIM][I] and [C_{16}MIM][Cl] exerted strong anti-larval activities. At micromolar concentrations, all three ionic liquid compounds were found to be lethal to cypris larvae. At nanomolar concentrations, settlement of cypris was inhibited without affecting survival. The tested compounds were also found to impact the survival and metamorphosis of various stages of nauplius larvae. The effective concentrations for preventing barnacle larval settlement were found to be not lethal to non-target organisms such as shrimp (*Artemia salina*) larvae. This shows that ionic liquids are promising candidates for developing environmentally benign antifouling formulations.

Lastly, the antimicrobial ionic liquids were found to effectively prevent biofilm formation of multispecies heterotrophic biofilms in natural waters (e.g., freshwater, seawater). Under static and dynamic experimental conditions, biofilm formation was prevented by antimicrobial ionic liquids ([C_{12}MIM][I] and [C_{16}MIM][Cl]). The data indicates that antimicrobial ionic liquids are promising candidates for preventing polymicrobial bacterial biofilms.

CHAPTER 1

General Introduction

1.1. Introduction

Biofouling constitute the unwanted accumulation of organisms on natural or man-made surfaces [1]. Depending on the organisms involved in the biofouling, it can be termed as either microfouling majorly caused by colonization of microorganisms (biofilms) or macrofouling caused majorly by colonization of macroorganisms. Biofouling has negative implications in diverse fields including food processing, drinking water, power, oil recovery, paper manufacturing, maritime and other industries [2]. Biofilms pose health risks when pathogenic microorganisms colonize tissues or medical devices. Microfouling and macrofouling impacts various marine and terrestrial industrial processes leading to operational issues, financial losses and environmental impact. Schematic diagram showing different biofouling organisms and the related susceptible fields is shown in Fig. 1.1.

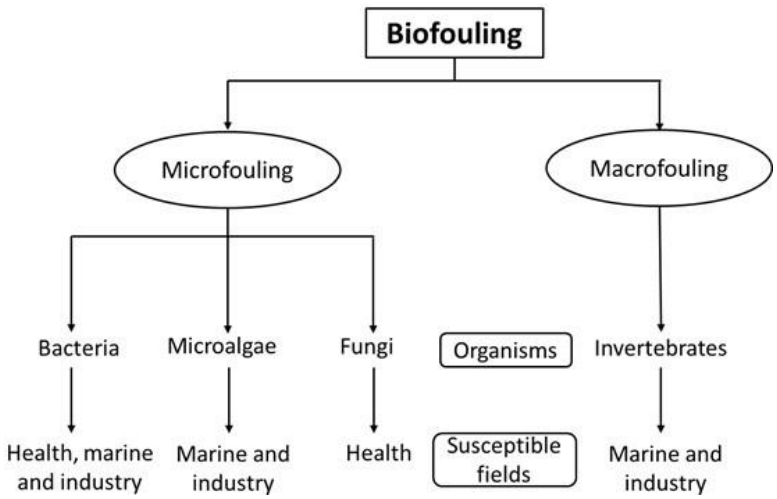


Fig. 1.1. Major biofouling organisms and their implications in various fields.

Mitigation of biofouling is important for controlling persistent infections, minimizing equipment failure, and avoiding operational problems in industrial processes [3, 4, 5]. Details of the biofouling organisms, affected surfaces/processes,

current biofouling control methods and the need for alternate biofouling control agents are detailed in this chapter.

1.2. Biofilms

Microfouling is mainly caused by biofilms. In natural and engineered settings, microorganisms prefer to thrive in attached communities (called biofilms) embedded in a self-produced extracellular polymeric matrix substances (EPSs) matrix. The biofilm growth is the most prevalent form of microbial growth and many of the microbes including bacteria, microalgae and fungi can form biofilms. The first discovery of biofilms is credited to Antonie Van Leeuwenhoek, whose first observations on dental plaque specimens under light microscope are actually related to attached microbial growth and biofilms [6]. High bacterial cell number on the immersed surfaces than the surrounding water medium supported the notion that attached growth of microbes is a preferred living style for microbes in natural environments [7]. With the advent of electron microscope, Jones and co-workers provided evidence for diverse morphologically distinct microorganisms in the biofilms formed on trickling filters of a sewage treatment plant [8]. However, detailed studies on mechanism of adhesion on biotic/abiotic surfaces, basic and applied aspects of biofilms were initiated by J.W. Costerton's group [9]. Over the years, biofilm research has gained prominence across diverse fields. The bacterial biofilms are more prevalent in the medical, marine and industrial settings. Phototrophic biofilms dominated by microalgae and cyanobacteria are of relevance in marine and industrial cooling water systems. Fungal biofilms have received more research attention in health. About 80% of microbial infections in humans are estimated to be caused by biofilms [10]. Controlling biofilm formation on various medical and industrial

surfaces such as medical devices, implants, food processing equipment and heat exchangers is essential to prevent infection, contamination, operational issues and equipment/material failures [11].

1.2.1. Biofilm life-cycle

During biofilm formation, microbes undergo a developmental process, from a free floating unicellular to a stationary multicellular state. Transition between these two lifestyles is triggered by several environmental stimuli and a variety of internal stressors such as lack of nutrients and other environmental variables [12]. The biofilm cycle involves five different stages as shown in Fig. 1.2.

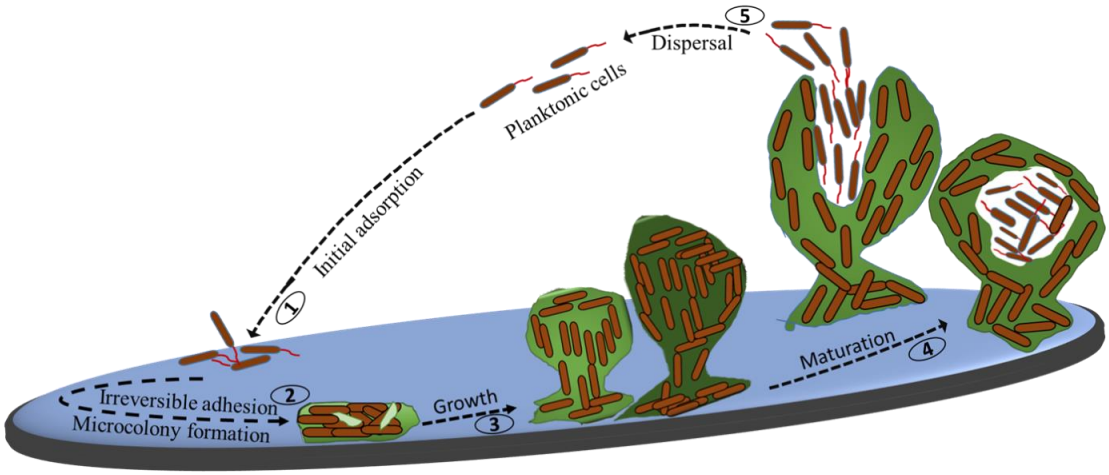


Fig. 1.2. Pictorial representation of different developmental stages in biofilm life-cycle. (Adapted from [1]).

Adhesion of cells to solid-liquid interface by planktonic microorganisms (e.g., bacteria) initiates the biofilm formation. This initial adsorption of cells on the surface is guided by physical interactions and the attachment is feeble and reversible [1, 6]. Stable binding on surface upon irreversible attachment induces the cells to replicate to form microcolonies and initiate the secretion of sticky EPSs [11]. The attached cells divide and grow further with the secretion of copious EPSs. Further growth of

biofilms in a three-dimensional structure with several water channels matures the biofilms into mushroom like structures as observed in *in vitro* flow cells [12]. Cell-cell communication within the biofilm or in response to the adverse environmental cues such as starvation or stress induces the dispersal/disassembly of biofilms to release the planktonic cells for the continuation of biofilm life cycle [13]. During the biofilm life-cycle, changes in gene expression and corresponding phenotypic changes occur at the cellular level for successful transition between planktonic to sessile lifestyle [14].

1.2.2. The biofilm composition

The biofilms comprise of the microorganisms and the EPSs matrix. In biofilms, the EPSs matrix alone accounts for about 90% by dry weight basis [13]. Biochemical composition of EPSs matrix is mainly dependent on the biofilm forming organisms, medium nutrient composition and prevailing hydrodynamic conditions. Matrix composition also varies in response to the altered environmental conditions [15, 16]. Although there are differences in the chemical composition of EPSs matrix among different biofilms, the commonly observed molecules include polysaccharides, proteins, lipids, nucleic acids and other molecules. Depending on the type of dominant microorganisms, the biofilms are classified into heterotrophic (bacterial), phototrophic or fungal biofilms.

1.2.2.1. Bacterial biofilms

Bacteria being the diverse and dominant microorganisms in different environments, most of the biotic and abiotic surfaces submerged in waters experience biofilm formation by bacteria [17]. Due to their predominance and diverse implications, bacterial biofilms are well studied and characterised over other

microbes involved in microfouling. Most of the Gram-positive and Gram-negative bacteria can form biofilms. Bacterial biofilms can also be part of multispecies biofilms along with autotrophs in phototrophic biofilms and with fungi in medical biofilms [3, 18]. Bacterial biofilms affect diverse fields such as health, water treatment/distribution systems, marine industry, and food processing industry.

Table 1.1. Bacterial biofilms in tissue-related infections.

Tissue-related infections	Biofilm bacteria	Ref.
Burns, wounds, skin ulcers	*Pseudomonas aeruginosa, Staphylococcus aureus, Acenatobacter* sp., *Staphylococcus* sp., *Micrococcus* sp., *Sphingomonas* sp.	[19, 20]
Cystic fibrosis	*P. aeruginosa, Burkholderia multivorans, Achromobacter xylosoxidans, Stenotrophomonas maltophilia, Dyella* sp., *Mycobacterium abscessus.*	[21]
Dental caries, periodontal diseases	*Lactobacillus acidophilus, Bifidobacterium* sp., *Streptococcus mutans, Streptococcus* sp., *Porphyromonas gingivalis, Prevotella intermedia, Campylobacter rectus, Campylobacter gracilis, Tannerella forsythia, Actinomyces* sp., *Scardovia wiggsiae.*	[22]
Infective endocarditis	*S. aureus, Enterococcus faecalis, Streptococcus gallolyticus.*	[23]
Duodenal, gastric ulcers and gastric cancer	*Helicobacter pylori.*	[24]
Urinary tract infections	*E. coli, Enterococcus* sp., *P. aeruginosa, Klebsiella pneumonia, Enterobacter* sp., *Proteus mirabilis, Staphylococcus* sp.	[25]
Otitis media	*Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis*.	[26]

Bacterial biofilms formed on tissues or medical devices are responsible for the persistent infections due to their high tolerance to antimicrobials and evasion of host-immune responses [27, 28]. Biofilms are responsible for delayed healing of cuts, burns or wounds [20]. Different tissue and device related biofilm infections along with major bacterial species present in the biofilm are given in Table 1.1 and 1.2 respectively.

Table 1.2. Bacterial biofilms in device-related infections.

Device-related infections	Biofilm bacteria	Ref.
Urinary catheters	*E. coli, P. mirabilis, S. aureus, E. faecalis, P. aeruginosa, Providencia sp., K. pneumoniae.*	[29]
Endotracheal tubes	*E. faecalis, S. aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa, Enterobacter sp.*	[30]
Central venous catheters	Coagulase-negative *Staphylococci, S. aureus,* enteric Gram-negative bacilli.	[31]
Cardiac pacemaker	*Staphylococcus epidermidis, Propionibacterium acne.*	[32, 33]
Orthopedic prosthesis	*S. epidermidis, S. aureus, Streptococcus* sp., *Enterococcus* sp.	[34]
Cochlear implants	*S. pneumonia, S. aureus, P. aeruginosa, E. coli.*	[35]
Voice prosthesis	*S. aureus, Pseudomonas sp., Enterobacter sp., Klebsiella sp., Proteus sp.*	[18]
Contact lens	*P. aeruginosa, Serratia marcescens, S. aureus, Fusarium sp., Achromobacter sp., Stenotrophomonas sp., Delftia* sp.	[36]

Apart from causing persistent infections of tissues and medical devices, bacterial biofilms are responsible for causing operational problems in various

industries. Bacterial biofilms on various surfaces of food processing and dairy industry are responsible for contamination and spoilage. Bacteria from biofilms developed in water distribution systems causes contamination of drinking water. Reverse osmosis membranes of water filtration systems and dairy industry experience severe biofouling and often require replacement of expensive membranes leading to economic losses [37, 38, 39]. Biofilms on paper mill components, heat exchangers and different components of cooling water systems reduces the process quality, reduction in heat transfer efficiency, material damage from microbially influenced corrosion and infections to operating personnel [40, 41, 42]. In the marine environment, biofilms formed in seawater cooling water systems, maritime transport, oil and gas industry, aquaculture equipment, RO membranes of desalination plants reduces the efficiency of operation, causes damage to components and incur economic losses. The microbial composition and implications of biofilms formed in different industries are given in Table 1.3.

Table 1.3. Bacterial composition and implications of biofilms formed in industrial settings.

Industry	Biofilm bacteria	Implications	Ref.
Oil and gas industry	Acid producers, sulphate reducers, iron oxidizers, thermophilic archaea, methanogens, nitrate reducers.	Microbially influenced corrosion	[41]
Marine ship hulls	*Myroides odoratimimus, P. mirabilis, Exiguobacterium* sp., *Jeotgalibacillus alimentarius., Bacillus sp., Halomonas aquamarina, Halotalea alkalilenta, Micrococcus luteus, Arthrobacter mysorens.*	Increased drag and surface corrosion	[43]
Membranes in	*Ruegeria* sp., *Donghicola* sp., *Leucothrix mucor, Alteromonas* sp.,	Flux reduction of membranes	[38]

desalination plants	*Shewanella* sp., *Vibrio* sp., *Cellulophaga* sp.	requiring replacement.	
Paper industry	*Tepidimonas* sp., *Chryseobacterium sp., Deinococcus geothermalis, Bacillus sp., Clostridium* sp.	Reduced paper quality, production losses.	[40]
Recirculating cooling water systems	*Legionella pneumophila, Rubellimicrobium* sp., *Rhodobacter* sp., *Porphyrobacter* sp., *Sphingomonas* sp., *Paracoccus* sp., *Hydrogenophaga* sp., *Delftia* sp., *Rubrivivax* sp., *Erwinia* sp., *Acinetobacter* sp., *Microcella putealis, Flexibacter flexilis, Flavobacterium* sp., *Haliscomenobacter* sp., *Gemmatimonas* sp.	Operation inefficiencies, component damage, high operational and maintenance costs, infections to operating personnel.	[3, 44]
Food processing industry (food packing, dairy, fish, poultry, meat)	*Listeria* sp., *Lactobacillus* sp., *Enterobacter* sp., *Micrococcus* sp., *Streptococcus* sp., *Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp., *Salmonella* sp., *Aeromonas* sp., *Campylobacter* sp., *Paracoccus* sp., *Serratia* sp., *Rhodococcus* sp., *E. coli*., *Acinetobacter calcoaceticus.*	Food borne diseases, contamination and spoilage	[4, 45]
Drinking water distribution systems	*Legionella* sp., *Pseudomonas* sp., *Aeromonas* sp., *Campylobacter* sp., *E. coli, Helicobacter* sp., *Salmonella* sp., *Vibrio* sp., *Shigella* sp., *Mycobacterium* sp., *Enterobacter* sp., *Enterococcus* sp.	Contamination posing health risks, corrosion of pipes, unpleasant taste and odors.	[42]
Reverse osmosis membranes	*Pseudomonas* sp., *Corynebacterium* sp., *Bacillus* sp., *Acinetobacter* sp., *Cytophaga* sp., *Moraxella* sp., *Micrococcus* sp., *Aeromonas* sp., *Mycobacterium* sp., *Flavobacterium* sp., *Arthrobacte* sp., *Serratia* sp., *Lactobacillus* sp.	Flux reduction forcing expensive replacements.	[37]

1.2.2.2. Phototrophic biofilms

Phototrophic biofilms are formed by colonization of microalgae and cyanobacteria on material surfaces submerged in cooling waters (freshwater and seawater) and exposed to direct sunlight. Microalgal communities dominated by diatoms and cyanobacteria are commonly reported in the biofilms formed in marine aquaculture, maritime transport, desalination plants and cooling water systems [46, 47, 48, 49].

Table 1.4. Examples of phototrophic biofilms, community structure and implications in industrial settings.

Industry	Community composition	Implications	Ref.
Marine aquaculture	*Cladophora* spp., *Codium fragile* spp. Fragile, *Undaria pinnatifida.*, *Antithamnion sp.*, *Ectocarpus spp.*, *Enteromorpha* spp., *Gracilaria* sp., *Ulva* spp.	Poor water quality, fluctuations in oxygen availability, shell erosion.	[47]
Industrial cooling water systems	Cyanobacteria (*Calothrix* sp., *Chroococcus* spp., *Cyanothece* sp., *Nostoc* sp., *Oscillatoria* sp., *Pseudanabaena* sp., *Synechocistis* sp., *Microcoleus* sp., *Gleocapsa* spp., *Phormidium* spp.) Diatoms (*Navicula* spp., *Nitzschia* spp., *Pinnularia* sp., *Surirella* sp., *Amphora* sp., *Cymbella* sp.) Green algae (*Pseudococcomyxa simplex, Scenedesmus* sp., *Stigeoclonium* sp., *Ulothrix* sp., *Vischeria* sp., *Cladophora* spp., *Ulothrix* spp.).	Performance loss and damage to equipment.	[3, 48, 49, 50]
Ship hulls	*Amphora delicatissima, Entomoneis pseudoduplex,*	Increased drag and	[46]

	Cyclophora tenuis, A. delicatissima, Achnanthes manifera, Amphora bigibba, C. tenuis, Cylindrotheca sp., *Nitzschia* sp., *Achnanthes* sp., *Amphiprora* sp., *Navicula* sp., *Stauroneis* sp.	surface corrosion.	
Desalination membranes	*Nitzschia* sp., *Minutocellus* sp., *Halamphora* sp., *Psammodictyon* sp.	Loss of flux and high maintenance costs.	[51]

The phototrophic microbes are often part of other microfouling communities (commonly with bacteria) [3] or with macrofoulers (in marine environments) [46]. Composition of phototrophic biofilms and their industrial implications are given in Table 1.4.

1.2.2.3. Fungal biofilms

Biofilm formation by yeast and fungal pathogens on tissues and medical implants is of importance due to their prevalence and implications in disease. However, biofilms constituted by yeast and filamentous fungi are also reported from food processing plants and drinking water distribution systems, respectively [52, 53]. Formation of fungal biofilms was reported on epithelial, endothelial surfaces and also on medical implants [54]. The fungal pathogens include yeasts (*Candida* spp., *Blastoschizomyces capitatus, Saccharomyces* spp., *Malassezia pachydermatis, Trichosporon asahii, Cryptococcus neoformans)* and filamentous fungi (*Aspergillus* spp., *Fusarium* spp.). Pathogenic yeasts such as *Candida albicans*, *Paracoccidioides brasiliensis*, and *Histoplasma capsulatum* are dimorphic in nature, wherein they switch from yeast to filamentous hyphae during colonization of surfaces, virulence

development, and in response to nutritional, and environmental changes [55, 56]. Enhanced resistance to antifungals and evasion of host defense systems are responsible for the chronic and persistent infections by fungal biofilms [57]. In food processing industry and water distribution systems, formation of fungal biofilms is associated with contamination and infection of human beings [52, 53]. Fungi often co-exist with bacteria in polymicrobial biofilms formed on tissues and medical implants. For example, biofilms containing *C. albicans and S. aureus* as well as *A. fumigatus* and *P. aeruginosa* are frequently reported in various infections [56]. The details of fungal biofilms formed various tissues, implants and industrial systems are summarised in Table 1.5.

Table 1.5. Fungal biofilms in health and industry.

Biofilm source	Identified yeast/ fungi	Ref.
Wounds, diabetic foot ulcers	*C. parapsilosis, C. tropicalis, C. albicans, Cladosporium herbarum, Aspergillus* spp., *Penicillium* spp., *Alternaria* spp., *Pleospora* spp., and *Fusarium* spp.	[58]
Nail infection, discoloration and thickening	*Trichophyton rubrum, T. mentagrophytes, Epidermophyton floccosum, Acremonium sp., Alternaria sp., Aspergillus sp., Cladosporium carrionii, Fusarium sp., Geotrichum candidum, Lasiodiplodia theobromae, Onychocola sp., Scopulariopsis sp., Scytalidium sp., Candida sp.*	[59]
Chronic rhinosinusitis	*A. fumigatus, Bipolaris papendorfii.*	[60]
Oral thrush, dental caries, periodontal disease	*C. albicans, C. dubliniensis, C. tropicalis, C. krusei, C. glabrata.*	[61, 62]
Vulvovaginal candidiasis	*C. albicans, C. glabrata, C. dubliniensis, C. parapsilosis, C. tropicalis, C. lusitaniae, C.*	[63]

	guilliermondii.	
Cardiac devices, Prosthetic heart value, Joint replacements, urinary catheters	*C. albicans, C. galbrata, C. parapsilosis, C. tropicalis, A. fumigatus.*	[64, 65]
Central venous catheter (CVC)-related infections	*Candida spp., Blastoschizomyces capitatus.*	[66]
Breast implants	*Trichosporon beigelii.*	[67]
Food industry	*Debaryomyces hansenii, S. unisporus, S. cerevisiae, C. vini, C. krusei, Pichia membranifaciens, Hansenula polymorpha, Dekkera bruxellensis, Saccharomycodes ludwigii, Schizosaccharomyces pombe, Wickerhamomyces anomalus, Dekkera anomala, Pichia anomala, Saccharomyces* spp., *Zygosaccharomyces* spp., *Candida* spp., *Rhodotorula* spp.	[52]
Reverse osmosis membranes	*Sporopachydermia lactativora, Magnusiomyces spicifer, Saprochaete clavate, C. pseudoglobosa, Sporopachydermia lactativora.*	[68]
Drinking water distribution systems	*Acremonium* sp., *Alternaria* sp., *Aspergillus sp., Candida sp., Cladosporium sp., Cryptococcus sp., Mucor racemosus, Nectria* sp., *Paecilomyces* sp., *Papulaspora* sp., *Penicillium* sp., *Phoma* sp., *Rhizoctonia* sp., *Rhodotorula* sp., *Sporotrichum* sp., *Sporothrix* sp.	[53]

1.3. Macrofouling and barnacle biofouling

Macrofouling represents undesirable attachment and growth of macroscale organisms on the submerged surfaces. Various surfaces submerged in the seawater are invariably and frequently colonized by various macrofouling organisms. Although more than 4000 macrofouling species are listed, most commonly reported organisms

include barnacles, mussels, hydroids, bryozoans, tube worms, and macroalgae [69, 70, 71, 72]. In natural conditions, microfouling often precedes macrofouling. The surfaces submerged in seawater are conditioned and rapidly colonized by microfoulers such as bacteria and microalgae resulting formation of biofilms. This is followed by the attachment of fouling larvae which would eventually grow as a large macrofouler [69]. The sequence of events leading to the formation of climax macrofouling community is shown in Fig. 1.3.

Fig. 1.3. Steps in macrofouling development on submerged surfaces (Adapted from [69]).

Macrofouling community composition mainly depends on the native-fouling organisms present in the seawater/freshwater. For example, macrofouling community in European ecoregions include kelps (macroalgae, *Laminaria* spp., *Saccharina latissima* and *Saccorhiza polyschides*), bryozoans (*Bugula* sp., *Celleporella hyaline*), mussels (*Mytilus edulis, M. galloprovincialis*), acorn barnacles (*Perforatus perforates*, *Chirona hameri*) and calcareous tubeworms (*Serpula vermicularis*) [70]. Macrofouling community in the Indian East Coast is dominated by barnacles (*Balanus* sp, *Chthamalus stellatus*), followed by mussels (*Perna viridis* and *Modiolus* sp.), oysters (Crassostrea madrasensis), tubicolous polychaetes (Serpulids sp.) and goose barnacles (*Lepas anatifera*) [71, 72].

Apart from native fouling species, other factors which influence the marine biofouling include seawater temperature, depth and light availability, water currents and distance to shore [70]. Material chemistry, colour, topography and wettability of substratum can also influence marine biofouling [70]. Depending on the man-made surfaces on which they colonize, macrofouling has various economic and ecological implications. Macrofouling impacts different industries such as maritime transport, marine aquaculture, coastal cooling water systems, and marine buoys. Biofouling on ship hulls increases the drag and reduces fuel efficiency. Ship hull fouling is also responsible for the introduction of non-native, invasive species into the new marine environments which can have ecological implications by disturbing native biodiversity [73]. Macrofoulers on marine aquaculture equipment causes damage to components and reduces the target culture yield [74]. Fouling on various components of marine cooling water systems have severe economic impacts by causing material damage, by reducing heat transfer efficiency and flow blockages [72, 75]. Macrofouling communities and their implications are summarised in Table 1.6.

Table 1.6. Macrofouling communities on various surfaces and their industrial implications.

Industry	Macrofoulers identified	Implications	Ref.
Marine aquaculture	Ascidians (*Didemnum* sp., *Styela* sp.), terbellaria (*Stylochus* sp.), Polychaeta (*Polydora* sp., *Hydroides elegans*), Porifera (*Callyspongia fibrosa, Cliona* sp.), Bivalve molluscs (*Mytilus* sp., *Pinctada* sp., *Pinna* sp., *Pteria* sp.), Hydrozoa (*Amphisbetia bispinosa*, *Ectopleura* sp., *Tubularia* sp.),	Increased weight and drag on equipment; physical damages; restriction of water exchange causing drop in dissolved oxygen and accumulation of waste.	[47, 74]

	Arthropoda (*Balanus* sp.)		
Maritime shipping	*Aiptasia pallida*, *Haliplanella lineata*, *Hydroides* sp., *Brachidontes* sp., *Mytella* sp., *Mytilopsis leucophaeta*, *Littorina* sp., *Amphibalanus* Sp., *Megabalanus* sp., *Conchoderma* sp., *Lepas* sp., *Caprella* sp., *Pachygrapsus* sp.	Increased drag, high fuel consumption, corrosion, introduction of invasive species.	[73, 76]
Coastal cooling water systems	Barnacles (*Balanus reticulatus*), Mussels (*Perna viridis, Modiolus straitulus*), bryozoans, hydroids, tunicates and serpulid worms.	Material damage, flow blockage, increased pressure drop, reduction in heat exchanger efficiency.	[72, 75]
Sensor mounted moored data buoys	Gooseneck barnacle (*Lepas anatifera*)	Reduction of quality of data, increased frictional resistance, sensor drifts.	[77]

1.4. Strategies for biofouling control

Sessile growth of organisms on biotic and abiotic surfaces is associated with serious health, economic and ecological implications [28, 17]. Selection of appropriate control treatment is necessary for mitigating biofilm and macrofouling problems. Although treatment choice depends on major fouling organisms, and industry type, broad-spectrum treatment controlling both biofilm and macrofouling is commonly used in industrial settings. However, irrespective of the treatment, biofilm and macrofouling control is necessary for avoiding persistent infections, contamination and to prevent process/ equipment failures [1]. Development of resistance to existing biocides, ineffectiveness and stringent environmental regulations on the use of

antifouling agents are some of the issues in biofouling mitigation. Therefore, development of novel and alternate antibiofouling strategies are being explored.

1.4.1. Biofilm control strategies

Treatment of pathogenic bacterial and fungal biofilms on tissues or medical implants is challenging due to general antimicrobial resistance including multi-drug resistance, and biofilm resistance [78, 79]. Examples of biofilm infections on tissues include cystic fibrosis and chronic wounds. More than 60% cases in chronic wounds involve formation of biofilms. This can cause complicated medical conditions in diabetic, obese and aged population. The current treatment of chronic wounds relies on the use of topical and systemic antibiotics. Use of topical antiseptics in wound dressing helps in controlling acute infections [78]. About 80% of cystic fibrosis patients, the infection is dominated by *P. aeruginosa* biofilm. Current clinical management relies on chronic antibiotic therapy involving an extended course of nebulized and systemic antibiotics [79].

Lack of vascularisation in medical implants protects the microbes from host immune system. Additionally, a small bacterial load being sufficient to colonize the abiotic surface, medical implants routinely gets affected by biofilms and cause persistent infections [10]. Current treatment of device-related infections includes administration of high doses of antibiotics based on the severity of disease. In the case of recurrent and persistent infections, surgical replacement of implants is the only viable treatment. Incorporation of antibiotics (e.g., gentamicin, tobramycin, and vancomycin) on surfaces of medical devices for localized release and attachment inhibition are being investigated (e.g., antibiotic-loaded hydroxyapatite coatings) [10, 79]. However, many of these strategies can only delay the onset of biofilm formation

but may not completely inhibit biofilm development and disease progression. Catheter-related blood stream infections caused by bacterial and fungal biofilms is controlled by antibiotic lock therapy in high risk patients by loading high concentrations of antibiotics in catheter lumen [79, 80]. Biofilm formation in industrial cooling water systems is controlled by employing oxidizing bioicdes, non-oxidizing biocides or combination of both. Chlorine, hypochlorite, bromine, ozone, and hydrogen peroxide are the commonly used oxidizing biocides. Non-oxidizing biocides such as glutaraldehyde, thiocyanates, isothiazolones, and quaternary ammonium salts are also used for controlling biofouling in power plant cooling water systems. Surfactants, detergents, dispersants, and commercial enzyme formulations are used for controlling established biofilms [3].

In lieu of challenges in controlling biofilms with existing methods, alternate antibiofilm strategies are being investigated [3, 81]. The market for antibiofilm agents is estimated to reach about 2.4 billion$ by 2025, indicating great potential for novel antibiofilm strategies (https://www.reportlinker.com/p05894592/Biofilms-Treatment-Market-by-Products-Wound-Type-End-User-Global-Forecast-to.html?utm_source=PRN). Some of the alternate biofilm control strategies include the development of novel antibiofilm agents, quorum sensing inhibitors, surface modification or coatings for medical devices and industrial surfaces, and physical therapeutics such as photo and sonodynamic therapy have received research attention.

1.4.2. Macrofouling control strategies

Macrofouling is controlled using physical methods (by manual cleaning, screens, heat treatment, circulating sponge rubber balls, ultrasounds), chemical methods (oxidizing biocides such as chlorine, ozone, hydrogen peroxide and non-

oxidizing biocides such as amines, isothiazolones, and aldehydes) or combination of both [82, 83]. Biocide containing antifouling paints are used in protecting ships, cooling water system components and other marine equipment. These paints often use copper along with booster biocides such as Irgarol 1051, Sea-Nine 211, Diuron, Chlorothalonil, zinc pyrithione (ZnPT) and copper pyrithione (CuPT) [84]. These paints minimize biofouling by releasing biocides in a controlled manner from the paint matrix. The foul release antifouling coatings are based on the surface chemistry of the coating which allows weak attachment of fouling organism and attached organisms gets detached under hydrodynamic flow. Foul release antifouling coatings are prepared by the use of hydrophobic surfaces such as silicone- (e.g., Poly(dimethylsiloxane)), fluorine- (e.g., Poly(tetrafluoroethylene)) or combination of fluorine-silicon- based materials [85, 86].

Most of the currently applied methods for biofouling control are often poorly active or inactive against certain fouling species or produce toxic metabolites. Oxidizing biocides such as chlorine negatively alters the water quality and produce carcinogenic by-products. Methods such as ozonation are expensive and relatively short half-life from rapidly interacting with organics hampers the efficacy [3]. The antifouling paints disturb the marine ecology due to the non-specific biocidal activity [84]. Foul release coatings often suffer from poor mechanical stability, low activity under static or low hydrodynamic forces [86]. The alternate approaches include development of novel chemicals with natural or synthetic origin along with high efficacy and low non-target toxicity. In this direction, agents such as antimicrobial peptides, glycoproteins, polysaccharides, and enzymes which degrade/disrupt the adhesives/EPSs/intercellular communication involved in settlement are being tested

[87, 88]. Other green approaches include use of natural bioactive compounds from sponge-associated bacteria, bacterial biofilms, microalgae, and cyanobacteria for bulk water treatment or inclusion in antifouling paints for environmentally benign biofouling control [75, 89]. Alternate strategies for marine fouling control are preparation of antifouling coatings based on biomimetic approach following naturally occurring antifouling microtopographies or slippery surfaces [86]. The attachment inhibition is achieved by retaining the hydration layer on the surface through the use of poly(ethylene glycol) based materials [85]. Zwitterionic or PEG-based copolymers which are amphiphilic in nature and can effectively inhibit macrofouling [86]. Other alternate physical approaches for biofouling control include ultraviolet radiation, electrolysis, ultrasound, magnetic fields, and radioactive coatings [88].

Ionic liquids have attracted attention as new generation antimicrobials because of huge structural diversity (theoretically, 10^{18} compounds can be synthesized) and tunability of structure which enable attaining desired biological activity including antimicrobial activity [90, 91]. The properties, antimicrobial and antibiofilm activities of ionic liquids are detailed below.

1.5. Ionic liquids

Ionic liquids are the molten salts completely composed of ions with a melting point below 100 °C and many exists as liquid at room temperature [92]. The size difference between the bulky cation and relatively smaller anion prevents the crystal lattice formation leading to low melting points in ionic liquids [93]. The interest in ionic liquids gradually increased in the past two decades owing to their diversity in composition, tunability in properties and corresponding applications [94]. Important properties of ionic liquids include negligible vapor pressure, high chemical and

thermal stability, high conductivity, wide electrochemical window, liquid form over a wide range of temperature, and excellent solvation properties [95]. The properties of ionic liquids can be fine-tuned with the modification in constituent ions which aids their application in diverse fields of chemistry, hence called “designer solvents”. As this characteristic can be modulated to suit the reaction conditions, these are also called “task specific ionic liquids” [92]. An ionic liquid consists of a cation, an organic ring with alkyl groups and an inorganic/ organic counter anion. Commonly used ionic liquids and their structures are given in Fig. 1.4.

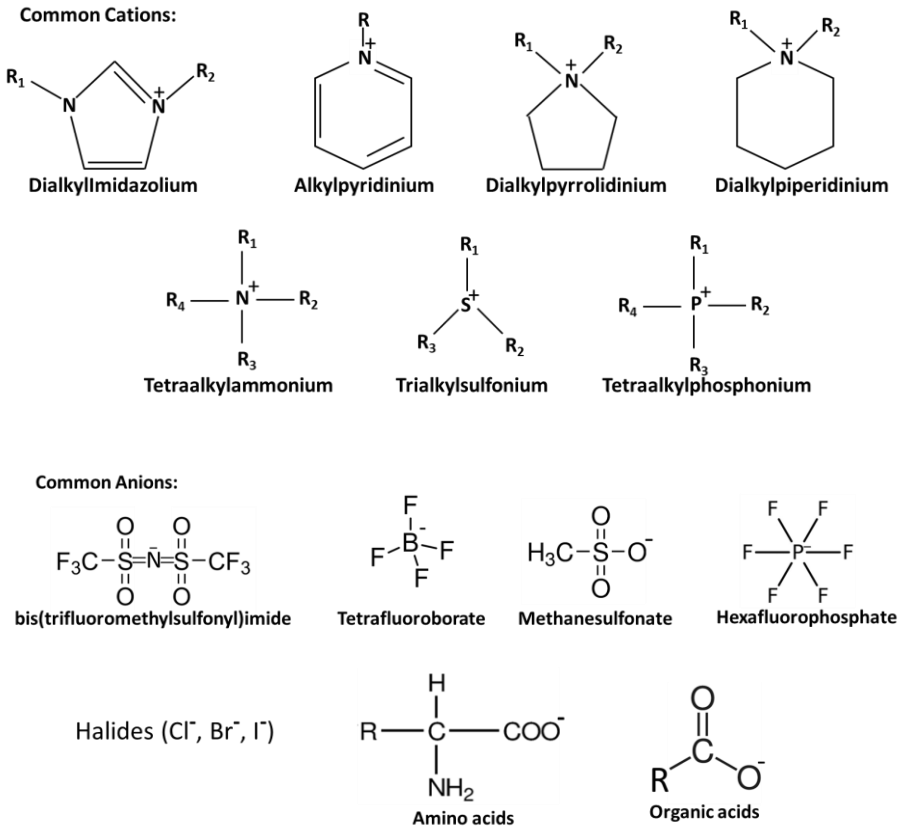


Fig. 1.4. Chemical structure of common cations and anions used in synthesizing ionic liquids.

Apart from diverse industrial applications, some of these ionic liquids have been explored as antimicrobials, antiseptics and antifouling agents [90, 96, 97]. At the beginning of this thesis study, there were only few studies on the evaluation of antibacterial, antifungal and antibiofilm activities of ionic liquids. Some of the important studies along with the recent reports are discussed below.

1.5.1. Antimicrobial activity of ionic liquids

Ionic liquids were studied as antimicrobial agents against various pathogenic and non-pathogenic microbes including bacteria and fungi. Ionic liquids with different cation and anion combinations were synthesized and tested against microbes. Among the different classes, alkylimidazolium ionic liquids with different alkyl side chain lengths, varied organic/ inorganic anions, and functionalization with different organic moieties were commonly tested against diverse groups of microbes [98, 99, 100, 101, 102]. Important studies reporting the antimicrobial activities of ionic liquids are summarised in Table 1.7. These results collectively indicated the potential antibacterial and antifungal activity of ionic liquids. Alkyl side chain length in cation ring significantly contributes to the antimicrobial activity, with large chains being highly active [103]. Membranes were found to be the primary targets in deciphering the antimicrobial activity. Hydrophobic interactions between the long alkyl side chains and cell membranes can cause the cell damage and induce the mortality [97].

Table 1.7. Antimicrobial activity of ionic liquids against single culture bacteria and fungi.

Ionic liquids	Microbes tested	Observation	Ref.
3-alkoxymethyl-1-methylimidazolium salts (chain length: C_3 to C_{12}, C_{14}, C_{16});	*Micrococcus luteus*, *Straphylococcus* sp., *E. hirae*, *E. coli*, *P. vulgaris*, *K.*	Increased activity with side chain length with –dodecyl being highly active against bacteria	[96]

Anions: Cl^-, $[BF_4]^-$, $[PF_6]^-$.	*pneumoniae, P. aeruginosa, C. albicans, R. rubra.*	and –hexadecyl against fungi.	
1-alkyl-3-methyl imidazolium, 1-alkyl-3-methylpyridinium bromide. (Chain length: C_4, C_6, C_8)	*E. coli, S. aureus, B. subtilis, P. fluorescens, S. cerevisiae.*	Antimicrobial activity: Imidazolium > pyridinium. Increases with side chain length. Gram-positive bacteria was more sensitive.	[104]
1-alkyl-quinolinium bromide (Chain length: C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18}).	*Staphylococcus* sp., *E. coli*, *K. aerogenes*, *B. cereus*, *P.s mirabilis*, *P. aeruginosa*, *C. tropicalis.*	Optimum activity by -tetradecyl and -hexadecyl side chain containing ionic liquids.	[103]
1-dodecyl-3-methylimiazolium iodide.	*S. aureus and P. aeruginosa.*	Sensitivity: Gram-positive > Gram-negative. Activity was comparable to CTAB.	[97]
1-butyl-3-methylimidazolium cation, Anions: Alkylfumarates with C_1, C_4, C_8, C_{12} side chains.	*B. Subtilis, E. Coli, S. cerevisiae.*	Activity increased with anion side chain length for *B. Subtilis*. Monobutylfumarate for *E. Coli* and monooctylfumarate for fungi were more active.	[99]
1-hexadecyl-3-methylimidazolium cation with Cl^-, $[CH_3O_3S]^-$, $[NTF_2]^-$ anions.	*Fusarium graminearum.*	Activity: Cl^- > $[CH_3O_3S]^-$ > $[NTF2]^-$.	[105]
1-Acetyl-3-hexylbenzotriazolium cation with benzoate or sorbate anion	*E. coli* and *Rhodococcus erythropolis.*	Efficient antibacterial activity compared to their precursors benzotriazole, sodium benzoate and potassium sorbate.	[106]
1-alkyloxycarbonyloxyethyl-3-methylimidazolium chlorides with C_{10}, C_{12},	*Kocuria rhizophila, S. epidermidis, S. aureus, B. subtilis, E. coli, K. pneumonia, P. aeruginosa, C. albicans.*	Side chain functionalization with carbonate ester moiety enhanced self-aggregation, adsorption	[100]

C_{14} side chains.		at the air-water interface, antimicrobial activity.	
N-Cinnamylimidazolium chlorides with C_1, C_6, C_8, C_{10} side chains.	*S. epidermidis, S. aureus, S. pyogenes, E. coli, P. aeruginosa, A. baumannii.*	Compound with –decyl side chain showed potential antimicrobial activity against infection causing gram-positive bacteria.	[101]
1-(2-hydroxyethyl)-3-methylimidazolinium chloride and 1-ethyl-3-methylimidazolinium chloride.	Bacterial population present in freshwater was enriched in open air for 24 h and inoculated for the experiment.	Antibacterial activity of ionic liquids in dose dependent manner, inhibited cell attachment and corrosion of steel surface. Activity: hydroxyethyl group > ethyl group.	[102]

1.5.2. Antibiofilm activity of ionic liquids

After the initial reports on antimicrobial activity of different classes of ionic liquids, Carson and co-workers demonstrated the *in vitro* antibiofilm activity of alkylimidazolium ionic liquids against laboratory, clinical and drug-resistant strains of bacteria and fungi [107]. The alkylimidazolium ionic liquids with –octyl, -decyl, -dodecyl and -tetradecyl side chains were able to eradicate the biofilms, albeit at higher concentrations (up to 16X MIC, depending on strains). Increase in alkyl side chain length increased the biofilm eradication activity. Eradication of biofilm formation by Gram-positive bacteria required lower concentrations of ionic liquids over Gram-negative bacteria or fungi.

Table 1.8. Activity of ionic liquids against single culture bacterial and fungal biofilms.

Ionic liquids	Biofilms tested	Observation	Ref.

1-alkyl-3-methylimidazolium chloride with $C_{8,}$ C_{10}, C_{12}, C_{14} side chains.	*Staphylococcus* sp., *E.coli*, *P. aeruginosa*, *K. aerogenes, B. cenocepacia, P. mirabilis, C. tropicalis*	Side chain length dependent activity with C_{14} being highly active.	[107]
1-alkylquinolinium bromide with C_8 to C_{18} side chains.	*Staphylococcus* sp., *E coli*, *P. aeruginosa*, *K. aerogenes, B. cenocepacia, P. mirabilis, C. tropicalis*	Mean biofilm cell killing: C_{14} > C_{12} > $C_{16.}$	[103]
1-dodecyl-3-methylimidazolium Iodide	*S. aureus, P. aeruginosa*	Antibiofilm activity: Gram-positive > Gram-negative bacteria.	[97]
1-hexadecyl-3-methylimidazolium with $[MeOSO_3]^-$, Cl^- anions	Multidrug-resistant six *C. tropicalis* isolates	Activity: Cl^- > $[MeOSO_3]^-$. Efficient than commercial antifungals.	[108]
Imidazolium and pyridinium ionic liquids with $C_{4,}$ C_6, C_8 side chains and I^-, PF_6^-, Cl^-, $[BF_4]^-$, $[NTf_2]^-$, tosylate, nitrate anions.	*S. aureus, K. pneumoniae, S. typhimurium, P. aeruginosa, E. coli, Bacillus tequilensis, B. subtilis.*	N-hexylpyridinium nitrate showed the highest anti-adhesion activity.	[109]
Alkylimidazolium, alkylpyrrolidinium, alkylpiperidinium bromides with –dodecyl side chains.	*S. aureus* and *P. aeruginosa.*	All the compounds exhibited antibiofilm activity. *S. aureus* biofilms were more sensitive.	[110]

Similar results were observed when the antibiofilm activity of 1-alkylquinolinium bromides with varied alkyl side chain length were evaluated against these bacterial and fungal isolates [103]. Increase in side chain length known to alter the surface activity and critical micelle concentration (CMC), thereby influencing the biological activity [100]. These results indicated the tuneability of ionic liquid cation in addition to side chain length alters the antibiofilm activity towards different

microorganisms. This tuneability offers flexibility for development of ionic liquids for various biocidal applications as antiseptics, disinfectants and antifouling agents. Ionic liquids with different functional groups and alkyl side chains exhibited activity against bacterial, fungal biofilms and important studies are summarised in Table 1.8.

1.6. Objectives and thesis outline

Biofilms and biofouling have diverse implications in health, industry and environment as explained in previous sections. Due to the ecotoxicity and environmental impacts of the existing antibiofouling methods, search for alternate and environmentally benign agents with high biofouling control activity and low ecotoxicity are being developed. Ionic liquid compounds were shown to possess antimicrobial and antibiofilm activity against bacteria and fungi. Among ionic liquids, imidazolium ionic liquids with different alkyl side chains and anions are well studied for antimicrobial and antibiofilm applications (Table 1.7 and 1.8). Most of the antibiofilm studies using these compounds are restricted to bacteria and fungi in monoculture biofilms. However, naturally occurring biofilms are polymicrobial (multispecies) comprising of diverse groups of organisms. An efficient antibiofilm agent should be broad-specific with high activity towards diverse groups of organisms. Studies are not available on antibiofilm activity of ionic liquids against phototrophic biofilms, which are part of natural biofilm communities and frequently developed in parts of cooling water systems exposed to sunlight. Toxicity mechanisms of these compounds were not clearly elucidated for these compounds. Additionally, there were no reports on efficacy of ionic liquids on macrofouling which is a dominant and severe problem in marine aquatic systems.

For the quest of understanding broad-spectrum antibiofouling potential of alkylimidazolium ionic liquids and the mechanism of action, following objectives were investigated during this thesis work.

(1). Antimicrobial and antibiofilm activity of alkylimidazolium ionic liquids using model bacteria, microalgae and fungal strains.

(2). Antibiofilm activity of ionic liquids against natural, multispecies heterotrophic/ phototrophic biofilms in static and dynamic assays.

(3). Antimacrofouling activity of alkylimidazolium ionic liquids using barnacle larvae and non-target toxicity.

(4). Understanding the mode of action of ionic liquids on biofouling organisms.

The thesis was divided into 6 chapters on the basis of the research work carried out to fulfil the thesis objectives. Chapter 1 provides a general introduction to the research work described in this thesis. It provides the background to this study with detailed up to date review of the related literature on biofilms, its implications, the need for alternative antibiofouling strategies and ionic liquids as new generation antibiofouling agents. Next four chapters (Chapters 2 to 5) constitute the explicit details of materials used, experimental methodology, results and discussion separately addressing each one of the thesis objectives. Chapter 2 provides experimental results on the antibiofilm activity of alkylimidazolium ionic liquids against model biofilm forming diatom, *Navicula* sp., and natural phototrophic biofilms. The mechanism of action of ionic liquid on diatom cells was characterised. In Chapter 3, the efficacy of ionic liquid compounds on model *C. albicans* biofilm formation and drug-resistant clinical *C. albicans* strains was provided. Detail mode of action of antifungal ionic

liquid on *C. albicans* in terms of multiple cellular targets was elucidated in this work. Chapter 4 details the antimacrofouling potential of alkylimidazolium ionic liquids on larvae of *Amphibalanus reticulatus*. Survival and settlement of barnacle larvae in presence of ionic liquids was characterised. Effect of mortality and metamorphosis in various stages of larvae were characterised. Non-target toxicity was studied using shrimp larvae. Chapter 5 states the results related to antibiofilm activity of natural, multispecies bacterial biofilms from freshwater and seawater origin in static and dynamic assays. Results of antibiofilm activity in laboratory scale recirculating system feeding natural freshwater and seawater were also presented. The final chapter (Chapter 6) is the summary of major findings, concluding remarks and the future perspectives emanating from this study. References cited in the study are listed in the references section. Appendices and copies of published journal articles are included towards the end of thesis.

CHAPTER 2

Efficacy of alkylimidazolium ionic liquids on prevention of phototrophic biofilms

2.1. Introduction

Phototrophic biofilms are observed on surfaces in terrestrial, marine and freshwater environments. Phototrophic biofilms are responsible for the aesthetically unacceptable discoloration of surfaces like rooftops and concrete buildings. In industrial cooling systems that employ cooling towers, surfaces are directly exposed to sunlight and encounter the problem of algal biofouling [111]. Copious algal growth in spray ponds and cooling towers interferes with the cooling process by blockage of the screens and pipes by detached algal biomass. Microalgae are significant contributors of biofilm growth in marine environments [112]. The biofilms formed on ship hulls are often dominated by diatoms [113]. Diatoms such as *Amphora*, *Cocconeis*, *Navicula* and *Nitzschia* sp. are commonly observed in the biofilms formed in coastal waters [111, 113, 114, 115]. Consequently, diatoms are used as the model organisms for studies on phototrophic or algal biofilms [116, 117, 118].

Chlorination is routinely used for minimizing biofouling in industrial cooling water systems [114, 119, 120]. Biofouling on surfaces (e.g., ship hulls, off shore structures, meshes of intake wells) submerged in the marine environment is controlled by using toxic antifouling paints containing copper compounds (e.g., cuprous oxide, cuprous thiocyanate), or organotin compounds (e.g., tributyltin oxide, tributyltin fluoride) [121]. Due to extreme toxicity to biota [122], use of triorganotin and its compounds in antifouling paints is restricted. Copper-based formulations are coming under increased scrutiny. Copper (Cu) ions are very effective in preventing the growth of phototrophic organisms. But, use of Cu is not preferred in industrial cooling systems due to galvanic corrosion of structural materials (e.g., steels). The efficacy of chlorination is decreased because of alkaline pH of water due to photosynthesis during

day time. Foul-release coatings based on hydrophobic silicone-based coatings are ineffective for preventing biofilm growth dominated by diatoms [113]. Hence, alternative methods are being investigated for biofilm prevention. During the last decade, ionic liquid compounds have attracted attention as potential antimicrobial agents [99]. However, there are no reports on the use of ionic liquids for prevention of phototrophic biofilms.

To develop new generation antifouling agents, the efficacy of alkylimidazolium ionic liquids was investigated for preventing phototrophic biofilms. The efficacy of 1-alkyl-3-methylimidazolium ionic liquids with three different alkyl side chains was determined on monospecies (*Navicula* sp.) and multispecies phototrophic biofilms. Structure based activity relationships were analyzed on planktonic growth and biofilm formation.

2.2. Materials and methods

2.2.1. Ionic liquids and CTAB

Alkylimidazolium ionic liquids such as 1-butyl-3-methylimidazolium chloride ([C_4MIM][Cl]), and 1-dodecyl-3-methylimidazolium iodide ([C_{12}MIM][I]) were purchased from Sigma-Aldrich (USA). 1-hexadecyl-3-methylimidazolium chloride ([C_{16}MIM][Cl]) was purchased from Acros (USA). CTAB [$(C_{16}H_{33})N(CH_3)_3$]Br was also procured from Sigma-Aldrich (USA). A 100 mM stock solution of each compound was prepared in ultrapure water and stored at room temperature until further use. Chemical structure of ionic liquids and CTAB are given in Fig. 2.1.

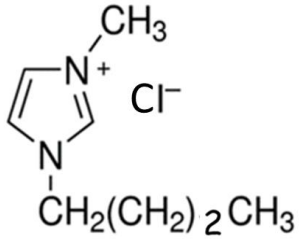
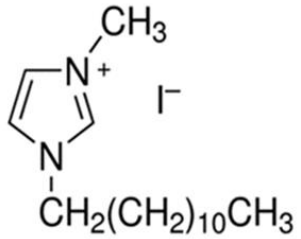
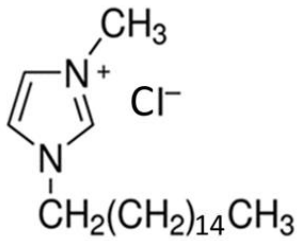
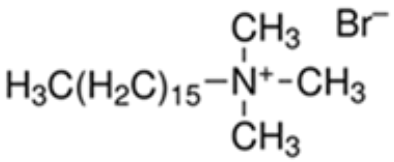
1-butyl-3-methylimidazolium chloride [C_4MIM][Cl]	
1-dodecyl-3-methylimidazolium iodide [C_{12}MIM][I]	
1-hexadecyl-3-methylimidazolium chloride [C_{16}MIM][Cl]	
Cetyltrimethylammonium bromide [CTAB]	

Fig. 2.1. Chemical structure of ionic liquids and CTAB used in the study.

2.2.2. Algal strain

Axenic *Navicula* sp. culture was maintained with regular sub-culturing in sterile Guillard's *f*/2 medium [123] prepared in filtered seawater. The culture was grown in a temperature-controlled room (24±1 °C) illuminated with white fluorescent lamp (Philips 40W) at 60 $\mu Em^{-2}\ s^{-1}$ irradiance in 12:12 h light: day cycles.

2.2.3. Natural phototrophic biofilms

Natural multispecies biofilms were developed by exposing fibre reinforced plastic (FRP) panels to seawater or freshwater in the laboratory. The FRP coupons used as the substratum for biofilm development contained glass fibres reinforced in an

epoxy polymeric matrix. The FRP materials are commonly used in manufacturing tanks, pipes and insulation rods. Seawater and freshwater samples were collected from the intake well of a seawater cooled madras atomic power station [124], and reservoir of freshwater cooled fast breeder test reactor, respectively [118]. The incubation time for development of natural phototrophic biofilms were 12 d and 20 d respectively, for freshwater and seawater.

2.2.4. *Navicula* sp. growth and minimum inhibitory concentrations (MIC)

Minimum inhibitory concentrations (MIC) of different alkylimidazolium ionic liquids and CTAB were estimated by spiking each of them in 100 ml sterile *f/2* media in 250 mL sterile Erlenmeyer flasks. Different concentrations (0 to 100 µM) of each of the compound were prepared using a 2-fold dilution method. Actively growing log phase *Navicula* sp. cells were harvested, concentrated in sterile *f/2* media and inoculated into each of the culture flasks at the final cell density of 2×10^4 cells/mL. Each experiment was performed in duplicate. Under mixing conditions, all the flasks were incubated for 6 days under the culture conditions described above. A 10 mL sample was collected from each flask every 2 days for measuring chlorophyll *a* content. Chlorophyll *a* was extracted with 90% acetone (Merck, India) by overnight incubation at 4 °C. The extracted chlorophyll *a* was estimated spectrophotometrically by trichromatic method (647, 664, 750 nm with turbidity correction at 630 nm and fitting the obtained values in standard formula) [125]. MIC values represented the lowest concentration of the compound at which the growth of *Navicula* sp. was completely retarded.

2.2.5. *Navicula* sp. biofilm

Navicula sp. biofilms were grown in sterile, polystyrene 24 well microtiter plates (Nest biotech, China) with 1 mL working volume per well. Different concentrations of ionic liquids and CTAB were prepared by 2-fold dilution method. The wells were inoculated with *Navicula* sp. at a final cell density of ~2×10^5 cells/mL. The plates were incubated under the culture conditions mentioned above. After 5 d of incubation, the medium was poured off and the amount of *Navicula* sp. biofilm was estimated as per reported method [116]. Chlorophyll *a* was extracted by adding 1 mL dimethyl sulfoxide (DMSO) to each well and incubating for 30 min in the dark. Each well was mixed thoroughly and 200 µL from each well was transferred to a fresh well in a 96-well microtiter plate (Nest biotech, China). Chlorophyll *a* was measured by quantifying florescence at an excitation of 360 nm and emission of 670 nm using multimode microplate reader (Synergy H1, Biotek®, USA). The fluorescence values were presented as relative fluorescence units (RFU). A set of three wells was used for quantifying the effect of each concentration.

2.2.6. *Navicula* sp. adhesion

Adhesion of *Navicula* sp. cells was determined in a 24 well microtiter plate at the end of 6 h incubation. Supernatant suspension of *Navicula* sp. culture growing in a static culture flask was poured off and the cells attached at the bottom (biofilm) of the flask were removed using a sterile brush, dispersed by vortexing, concentrated by centrifugation and used for inoculation of wells at cell density of ~10^6 cells/mL. An equal amount of inoculum was added to each well having different concentrations of [C_{16}MIM]Cl ranging from 0 to 10 µM. The microtiter plate was incubated for 6 h under light illumination as mentioned previously. After 6 h, non-adherent and loosely

attached cells were poured off and attached cells were estimated by chlorophyll *a* extraction and quantification using fluorescence [126].

2.2.7. Natural multispecies phototrophic biofilms

Natural seawater (1 L) was augmented with *f*/2 media components and 0 to 5 µM [C_{16}MIM]Cl in glass beakers. A FRP coupon (15×8.6×0.2 cm) was placed in each beaker and the beakers were incubated directly under natural sunlight. After 20 d, the biofilm developed on each panel was scraped using nylon brush, pelleted and estimated for total wet weight and chlorophyll *a*. Results are represented as the amount of wet biomass deposited per unit area (mg cm^{-2}) and mg chlorophyll *a* per unit area (mg cm^{-2}). Experiment was performed in duplicates.

For freshwater phototrophic biofilms, freshwater was augmented with BG11 media [126] components and 0 to 5 µM [C_{16}MIM][Cl] in glass beakers. A FRP coupon (15×8.6×0.2 cm) was placed in each baker and incubated under the conditions as mentioned previously for experiments of *Navicula* sp. After 12 d, biofilm was scraped and processed for measuring wet weight and chlorophyll *a*.

2.2.8. SYTOX® Green staining and microscopy

Diatom cells were stained using the viability stain, SYTOX® Green (Molecular Probes Inc., USA), according to a reported protocol [127]. Briefly, 2 mL of actively growing *Navicula* sp. cells (~10^6 cells/mL) were pelleted, washed and re-pelleted in Eppendorf tube. The [C_{16}MIM]Cl solutions were added to cell pellets, mixed well and incubated at 30 °C, 150 RPM for 1 h. After the incubation, the cells were centrifuged, washed and stained with SYTOX® Green (5 mM stock in DMSO, diluted 250x prior to staining). After 5 min of incubation in the dark, the cells were observed under epifluorescence microscope equipped with a mercury arc lamp for excitation and

FITC cut-off filter for emission (Carl Zeiss Axio Scope A1, USA). Fluorescence images were acquired using a Leica DC500 CCD camera (Leica Microsystems, Germany) and presented without any image processing. Red and green/lemon yellow cells were counted in the fluorescence images for determining live and dead ratios. A minimum of 200 cells were counted from multiple fields and the percentage of live cells (with green fluorescence) were estimated. This experiment was repeated twice.

2.2.9. Chlorophyll *a* leakage from *Navicula* sp. cells

Membrane damage upon exposure of *Navicula* sp. cells to ionic liquid was estimated by the release of chlorophyll *a* into the suspension. A 2 mL of actively growing cells with a cell density of 2×10^6 cells/mL were centrifuged. The washed cell pellet was incubated with different concentrations of $[C_{16}MIM]Cl$ in the dark at 4 °C. At the end of 1 and 2 h incubation, each tube was centrifuged and 0.2 mL of supernatant was transferred to 96 well microtiter plate. Along with respective $[C_{16}MIM]Cl$ concentration controls, chlorophyll *a* released into the supernatant was quantified as Relative Fluorescence Units (RFU). Any increase in RFU measurements in the cell-free supernatant indicates the release of intracellular chlorophyll *a* into the surrounding aqueous medium.

2.2.10. Statistical analysis

Unpaired t-test was performed to compare test data points with respective controls. The level of significance was incorporated in Figures.

2.3. Results

2.3.1. Effect of ionic liquids on *Navicula* sp.

Growth pattern of *Navicula* sp. in the presence of three alkylimidazolium ionic liquids and CTAB is shown in Fig. 2.2 and 2.3. Growth was not inhibited in the

presence of 1 µM of [C_4MIM][Cl], while it was retarded in the presence of 5 to 100 µM of [C_4MIM][Cl]. Growth inhibition was mainly evident in terms of an extended lag phase. The growth of *Navicula* sp. was observed even at concentrations as high as 50 and 100 µM, but with a prolonged lag phases (Fig. 2.2A).

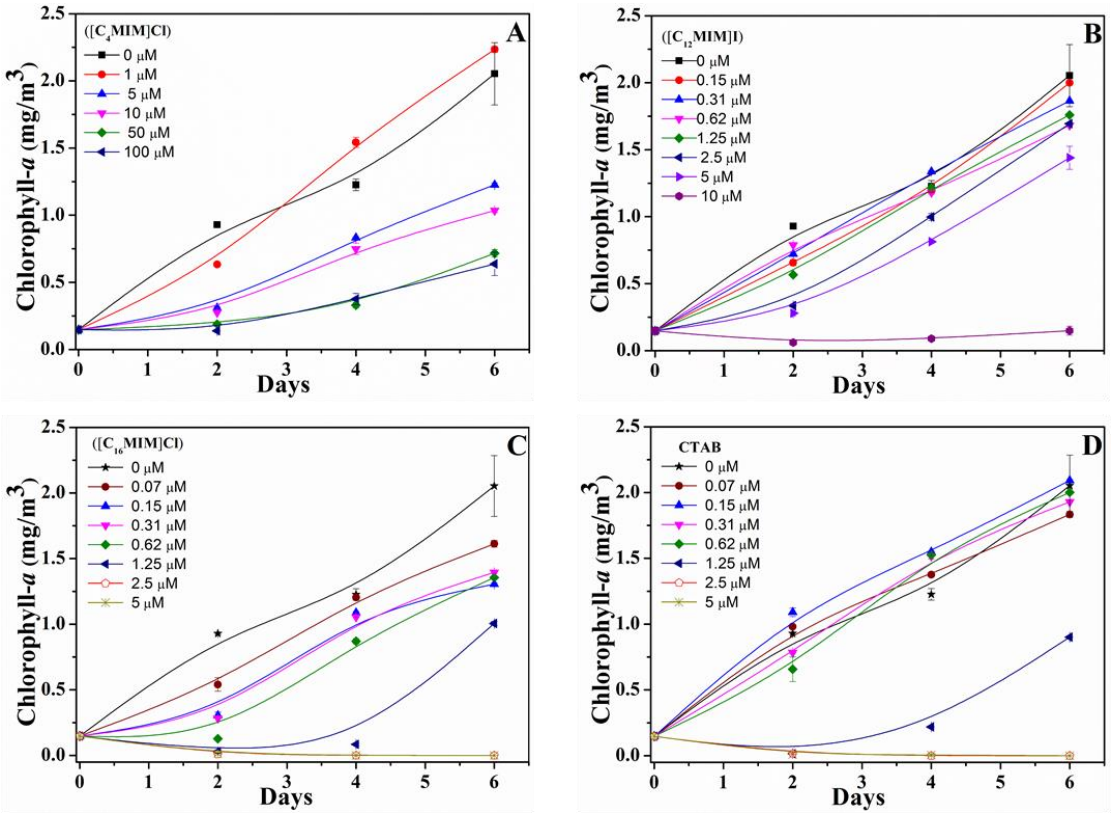


Fig. 2.2. Planktonic growth of *Navicula* sp. cells in the presence of different alkylimidazolium ionic liquids and CTAB. Different concentrations of [C_4MIM][Cl] (A), [C_{12}MIM][I] (B), [C_{16}MIM][Cl] (C) and CTAB (D) were spiked in sterile *f*/2 media. Equal amount of inoculum (*Navicula* sp. cells) was added and growth was estimated in terms of chlorophyll *a* using acetone extraction method.

In contrast, the growth was completely inhibited in the presence of 10 and 2.5 µM of [C_{12}MIM][I] and [C_{16}MIM][Cl] (Fig. 2.2B, C). The MIC values for [C_4MIM][Cl] and [C_{12}MIM][I] were > 100 µM and 10 µM, respectively. While, the MIC value for [C_{16}MIM][Cl] was determined to be 2.5 µM. Among the three ionic liquids tested, [C_{16}MIM][Cl] strongly inhibited the growth of *Navicula* sp. Extended

lag phase was clearly evident at 1.25 µM of [C_{16}MIM][Cl]. The cationic biocide, CTAB also exhibited growth inhibition (Fig. 2.2D) on *Navicula* sp. in a manner similar to that of [C_{16}MIM][Cl]. Complete inhibition in *Navicula* sp. growth was caused by both CTAB and [C_{16}MIM][Cl] at 2.5 µM. The results showed that the growth inhibition activity of ionic liquids increased with an increase in the alkyl side chain length. Compound with longer alkyl side chain showed potent activity at lower concentrations as compared to ionic liquid with shorter alkyl chain.

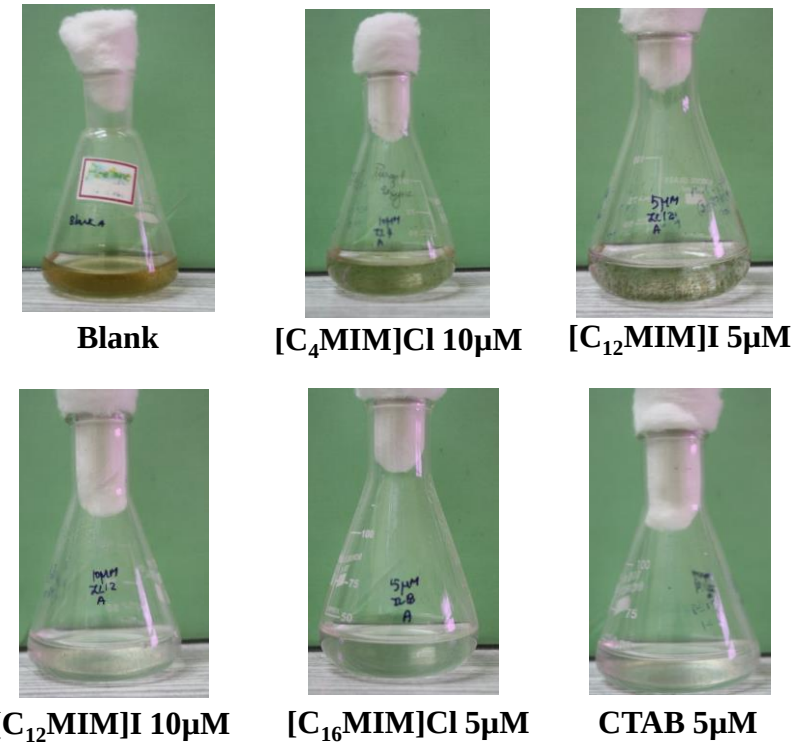


Fig. 2.3. Representative images of planktonic growth of *Navicula* sp. cells in the presence of different alkylimidazolium ionic liquids.

The concentrations required for the inhibition of biofilm formation in the presence of different alkylimidazolium ionic liquids and CTAB were comparable to those needed for inhibition of planktonic growth (Fig. 2.4). Biofilm formation was not inhibited in the presence of up to 10 µM of [C_4MIM][Cl]. Partial inhibition in biofilm

formation was observed only at 50 and 100 µM of [C_4MIM][Cl] (Fig. 2.4A), while complete inhibition in biofilm formation was observed in the presence of [C_{12}MIM][I] at 10 µM (Fig. 2.4B). In the case of [C_{16}MIM][Cl] and CTAB, complete biofilm inhibition was achieved at 5 µM (Fig. 2.4C and D). It was clearly evident from the data that [C_{16}MIM][Cl] exhibited strong inhibition on planktonic and biofilm growth of *Navicula* sp. Therefore, [C_{16}MIM][Cl] was chosen for further experiments on adhesion of *Navicula* sp. cells, natural multispecies biofilm formation under phototrophic conditions and to determine the mechanism of action.

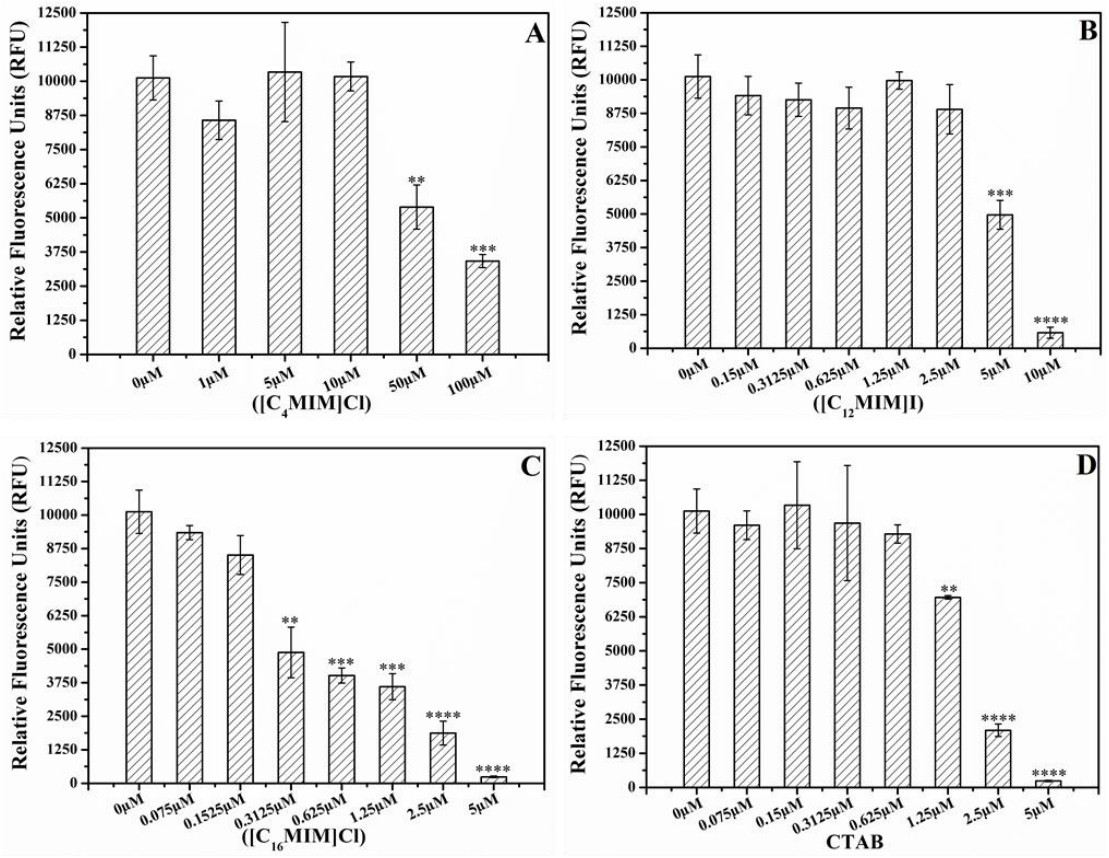


Fig. 2.4. Biofilm formation by *Navicula* sp. in the presence of different concentrations of alkylimidazolium ionic liquids and CTAB. Biofilm formation in the presence of different concentrations of [C_4MIM][Cl] (A), [C_{12}MIM][I] (B), [C_{16}MIM][Cl] (C) and CTAB (D) was estimated in 24-well microtiter plates. Biofilm was represented as the total chlorophyll *a* in terms of relative fluorescence units (RFU). Unpaired *t*-test was performed to compare test data points with respective controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

1-methylimidazole group and the halides (Cl^- and I^-) are the other constituents of alkylimidazolium ionic liquids beside side chain length. In order to link the activity of ionic liquids to the alkyl chain length, *Navicula* sp. growth and biofilm formation were determined in the presence of different concentrations of 1-methylimidazole, chloride (as NaCl) and iodide (as KI). Growth and biofilm formation were not inhibited in the presence of 1-methylimidazole and halides (Fig. 2.5).

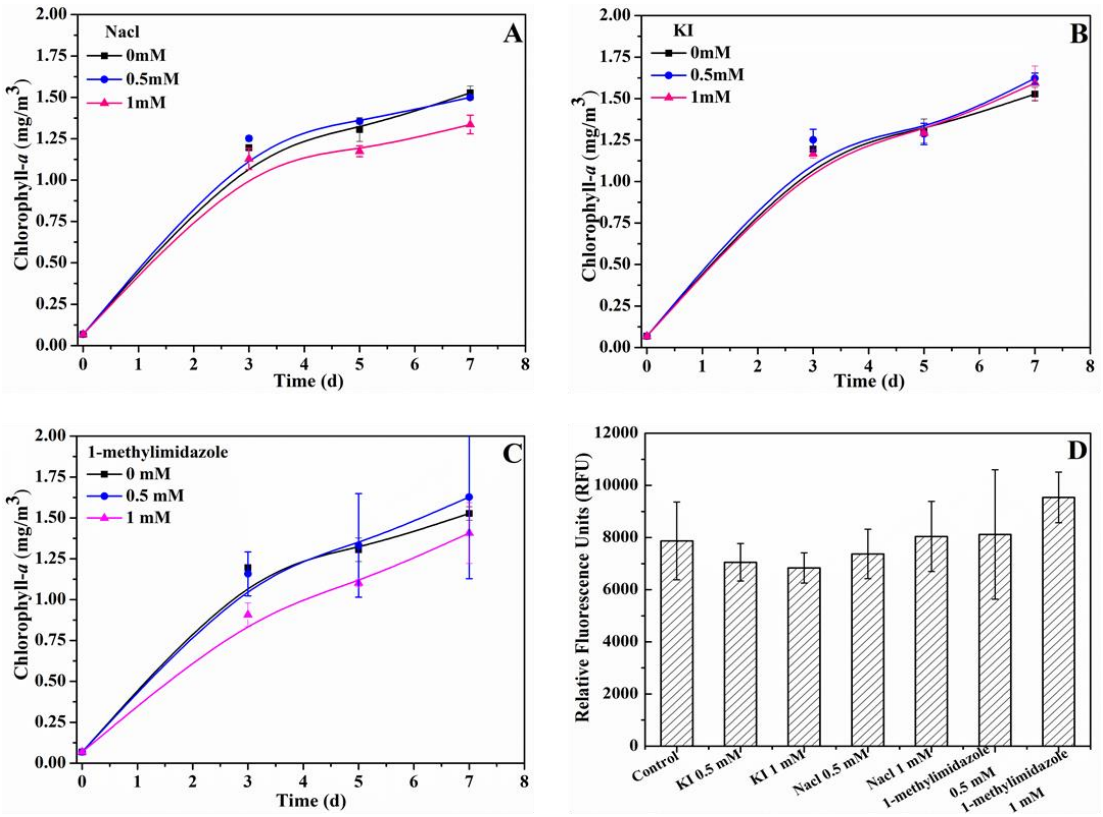


Fig. 2.5. *Navicula* sp. growth and biofilm formation in presence of 1-methylimidazole and anions. Planktonic growth in flasks at different concentrations of sodium chloride (A), potassium iodide (B) and 1-methylimidazole(C). Biofilm formation in microtiter plates (D).

In order to determine the effect on initial stages of biofilm formation, adhesion of *Navicula* sp. cells was determined in the presence of different concentrations of $[C_{16}MIM][Cl]$. Strong inhibition in the adhesion of diatom cells was evident at 10 µM of $[C_{16}MIM][Cl]$ (Fig. 2.6).

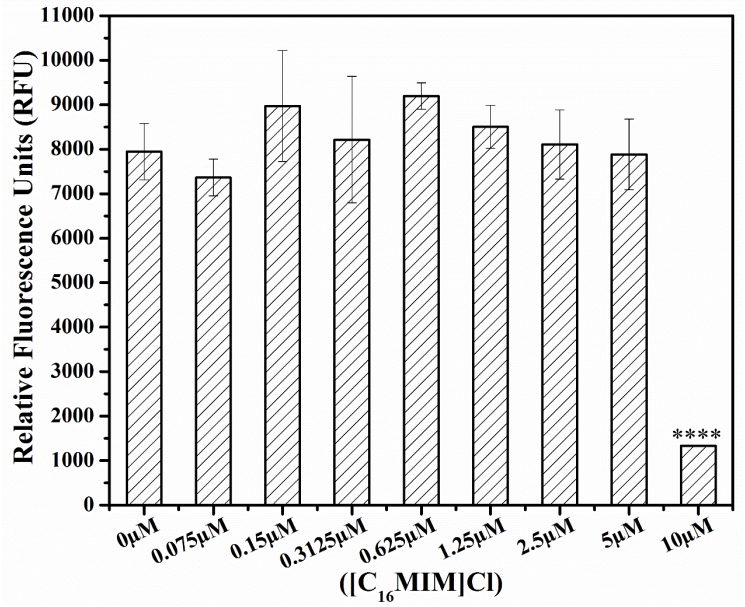


Fig. 2.6. Adhesion of *Navicula* sp. cells in the presence of different concentrations of ionic liquid ([C_{16}MIM]Cl). Biofilms of *Navicula* sp. were scraped and dispersed by vortexing. Concentrated cells were used for adhesion assay in 24-well microtiter plates. After 6 h of incubation, adhered cells were quantified and represented as chlorophyll *a* relative fluorescence units (RFU). ****: $p<0.0001$.

2.3.2. Mechanism of action of ionic liquid on diatom cells

Mechanism of toxicity of [C_{16}MIM][Cl] on *Navicula* sp. cells was probed by assessing the cell membrane damage and leakage of intracellular components. SYTOX® green is a membrane impermeant dye, which cannot enter the cells with intact cell membrane. The stain enters the cytoplasm of membrane-compromised cells, binds to DNA, and exhibits green fluorescence upon excitation. The intact untreated *Navicula* sp. cells after staining with SYTOX® Green appeared red due to intrinsic chlorophyll *a* autofluorescence. But, the membrane compromised cells after staining with SYTOX® Green appeared green or lemon yellow upon excitation. The ratio of green cells to red cells increased with an increase in ionic liquid concentration, suggesting membrane permeabilization as the possible mechanism of cell damage (Fig. 2.7). Percentage of live cells were found to be 95, 93, 49, 8 and 0%, at 0, 3.125, 6.25,

12.5 and 25 mM [C_{16}MIM][Cl], respectively. At 12.5 and 25 µM of ionic liquid, most of the diatom cells were stained with SYTOX® Green and appeared green indicating extensive cell membrane permeabilization by the ionic liquid treatment.

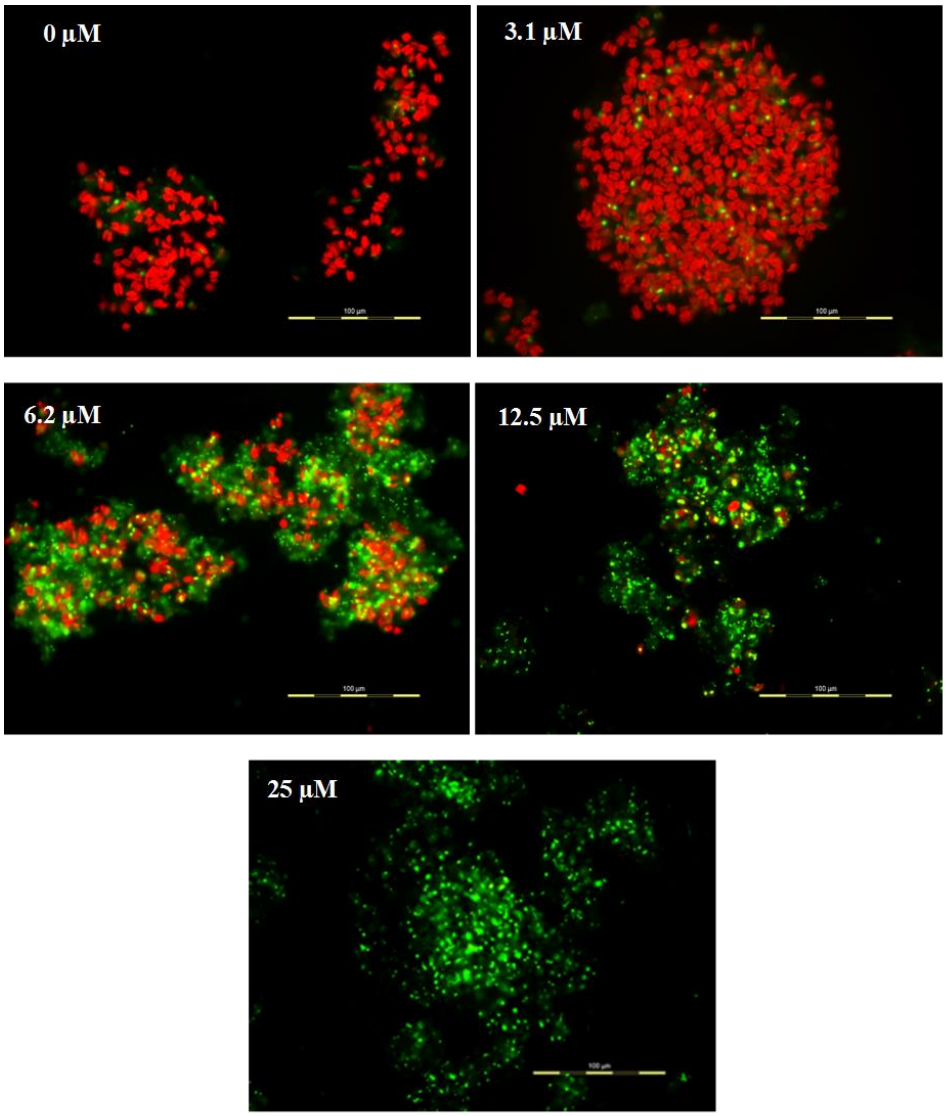


Fig. 2.7. Cell viability in *Navicula* sp. cells treated with the ionic liquid [C_{16}MIM]Cl. *Navicula* sp. cells were incubated with different concentrations of [C_{16}MIM]Cl for 1 h contact time, stained with SYTOX® Green and imaged using epifluorescence microscope. Red indicates viable while green indicates membrane compromised cells. Red = chlorophyll autofluorescence, Green or lemon yellow = SYTOX® Green fluorescence.

The live/dead data was corroborated by the leakage of chlorophyll *a* into the surrounding aqueous phase during exposure to ionic liquid. In fact, increased chlorophyll *a* fluorescence was noted in the supernatant with an increase in ionic liquid concentration and contact time (Fig. 2.8). In controls with untreated cells and medium blank with ionic liquid did not show chlorophyll *a* fluorescence in the supernatant.

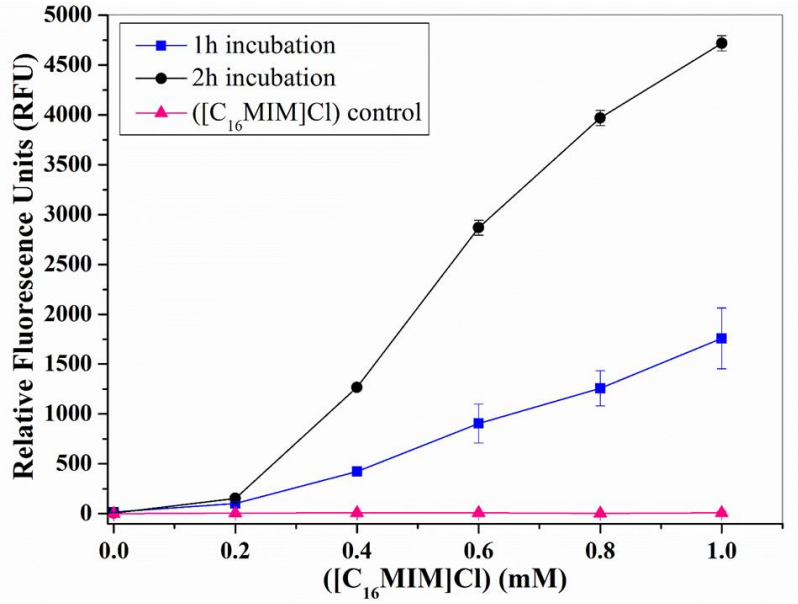


Fig. 2.8. Chlorophyll *a* leakage from *Navicula* sp. cells upon acute exposure to [C_{16}MIM][Cl]. *Navicula* sp. cells were incubated with different concentrations of [C_{16}MIM][Cl] for different contact times (1 h and 2 h). After the incubation, chlorophyll leaked into the supernatant was measured and represented as relative fluorescence units (RFU).

2.3.3. Effect of ionic liquid on natural multispecies phototrophic biofilms

Formation of phototrophic biofilms in the presence of different concentrations of [C_{16}MIM][Cl] in freshwater (Fig. 2.9) and in seawater (Fig. 2.10) are shown. Visual observation and phototrophic biofilm estimation by biomass amount, chlorophyll *a* fluorescence indicated that, phototrophic biofilm formation on FRP coupons was inhibited in a dose-dependent manner. In fact, complete inhibition of phototrophic biofilm was caused in the presence of 5 µM of [C_{16}MIM][Cl]. Analysis of

phototrophic biofilm communities revealed diverse phototrophic organisms including microalgae, diatoms, dinoflagellates and cyanobacteria. The phototrophic organisms observed in from the biofilms formed in freshwater and seawater are summerised in Table 2.1 and Table 2.2, respectively.

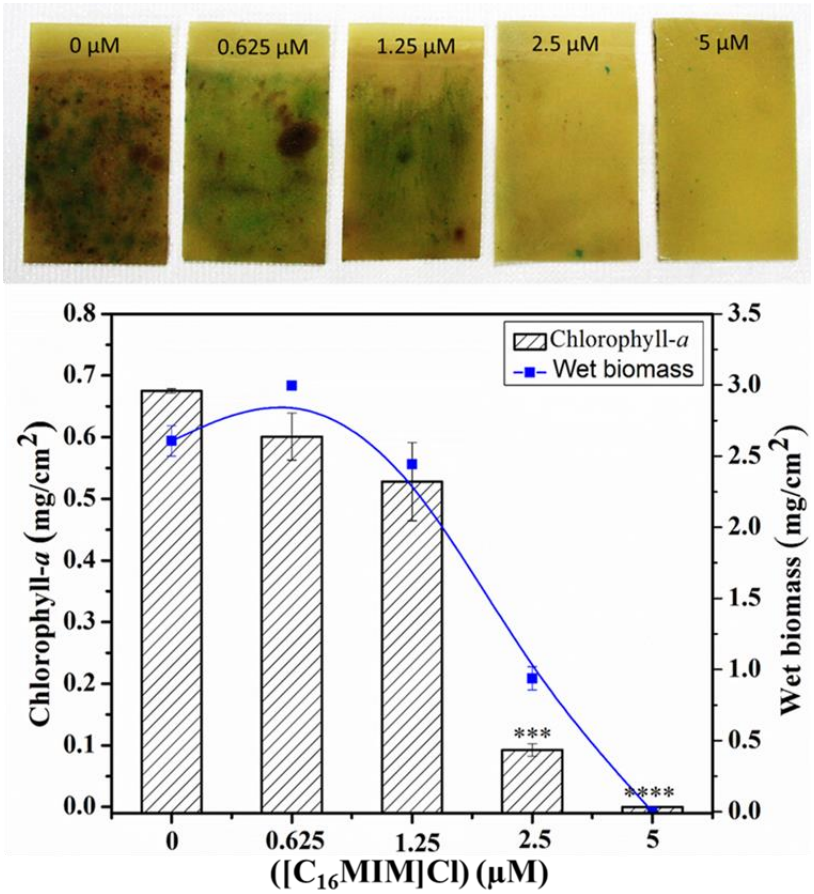


Fig. 2.9. Phototrophic biofilm formed in freshwater in the presence of varied concentrations of [C_{16}MIM][Cl]. Digital images (A) and quantification (B) of natural phototrophic biofilms formed on fibre reinforced plastic coupons immersed in freshwater in the presence of different concentrations of ionic liquid, [C_{16}MIM][Cl]. At the end of 12 days of incubation, the biofilm was scraped and used for quantification of chlorophyll *a* and wet biomass deposition. ***: $p<0.001$, ****: $p<0.0001$.

Phototrophic biofilms formed in freshwater contained microalgae (*Pediastrum* sp. *Cosmarium* sp., *Rhodomonas* sp., *Scenedesmus* sp.), cyanobacteria

(*Cylindrospermopsis* sp., *Anabaena* sp., *Oscillatoria* sp.) and diatoms (*Nitzschia* sp., *Amphipleura* sp., *Synedra* sp.) (Table 2.1).

Table 2.1. Photoautotrophs of phototrophic biofilms formed in natural freshwater.

Organism	Characteristics	Ref.
Pediastrum sp.	These are the **green algae** (Chlorophyceae) and occur as non-motile surface sediment assemblages in freshwater lakes and ponds.	[128]
Cosmarium sp.	These are bi-lobed **green algae** of the family Desmidiaceae. Part of the phototrophic biofilms formed on monuments.	[129]
Rhodomonas sp.	Flagellate, small, unicellular **red algae** belongs to Cryptophyceae.	[130]
Scenedesmus sp.	Colonial, **green algae** (Chlorophyceae) are reported in forming phototrophic biofilms.	[131]
Nitzschia sp.	It's a pennate **diatom** and is reported to be a dominant player in freshwater phototrophic biofilms across various successions.	[118]
Amphipleura sp.	These are the tube dwelling **diatoms** present in the freshwater.	[132]
Synedra sp.	Fresh water **diatoms** usually attach to the substratum by secreting polysaccharide and amino acid rich basal pads.	[133]
Cylindrospermopsis sp.	It is a freshwater **cyanobacterium** often reported in phototrophic biofilms on monuments.	[134]
Anabaena sp.	Nitrogen fixing filamentous **cyanobacteria** often found in phototrophic cyanobacterial biofilms.	[135]
Oscillatoria sp.	Freshwater filamentous **cyanobacteria** reported to be present in phototrophic biofilms developed on monuments.	[136]

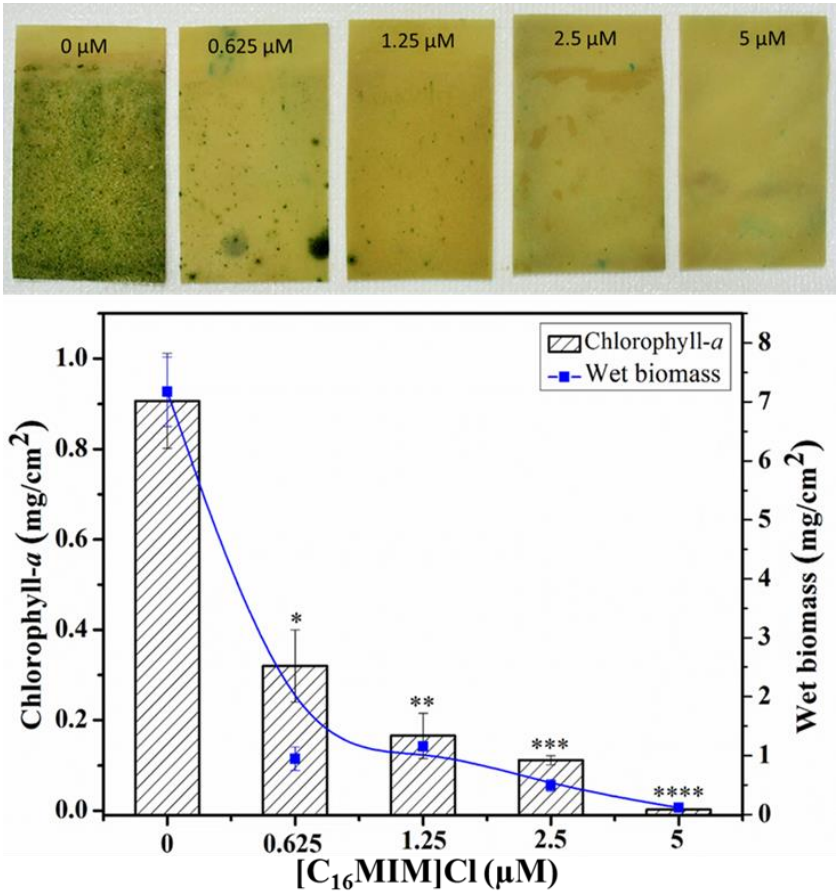


Fig. 2.10. Phototrophic biofilm growth in natural seawater in the presence of varied concentrations of [C_{16}MIM][Cl]. Digital images (A) and quantification (B) of natural phototrophic biofilms formed on fibre reinforced plastic coupons immersed in seawater in the presence of different concentrations of ionic liquid, [C_{16}MIM][Cl]. At the end of 20 days of incubation, the biofilm was scraped and used for quantification of chlorophyll *a* and wet biomass deposition. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

The phototrophic biofilms formed in seawater contained diatoms (*Thalassiothrix* sp., *Amphora* sp., and *Navicula* sp.) as the dominant members. Other phototrophs included cyanobacteria such as *Oscillatoria* sp., *Gloeotrichia* sp., *Spirulina* sp., *Rivularia* sp., and a dinoflagellate, *Prorocentrum* sp. (Table 2.2).

Table 2.2. Photoautotrophs of phototrophic biofilms formed in natural seawater.

Organisms	Characteristics	Ref.
Diatoms: *Amphora* sp., *Navicula* sp., *Thalassiothrix* sp.	These are the common biofilm forming diatoms on the surfaces submerged in Indian coastal waters and on ship hulls.	[112, 121, 137]
Cyanobacteria: *Gloeotrichia* sp., *Spirulina* sp., *Rivularia* sp., *Oscillatoria* sp., *Prorocentrum* sp., *Chroococcus* sp.	These cyanobacteria are reported to be the constituents of natural phototrophic biofilms developed on submerged marine environments.	[138]

2.4. Discussion

Sessile growth of microorganisms in self-immobilized polymeric matrix is more prevalent and has negative implications in health and industrial environments [97, 116]. Phototrophic biofilms dominated by diatoms, microalgae and cyanobacteria are part of the biofilms formed on ship hulls, submerged surfaces and frequently encountered in sunlight exposed surfaces of cooling water equipment [111, 113]. Phototrophic organisms being part of natural biofilms and predominant in cooling water systems, activity of alkylimidazolium ionic liquids against these biofilms would generate data on the broad-spectrum antibiofilm activity of the tested compounds. Hence, selected alkylimidazolium ionic liquids with different alkyl side chain length were studied against model biofilm forming diatom, *Navicula* sp. and natural phototrophic biofilms.

Navicula sp. growth was not inhibited in presence of short (–butyl) side chain containing [C_4MIM][Cl]. Similar results were observed, wherein short side chain containing imidazolium ionic liquids were not effective against bacterial and fungal

growth [98]. The growth inhibition of *Navicula* sp. observed in the presence of ionic liquids was corroborating with the previous work [139], wherein the activity of ionic liquids on microalgal cultures was dependent on the length of alkyl side chain. Growth inhibition was observed in presence of alkylimidazolium ionic liquids with –dodecyl and –hexadecyl side chains. Moreover, comparable activity and MIC values for [C_{16}MIM][Cl] and CTAB, suggested that -hexadecyl group linked to 1-methylimidazolium was responsible for the observed growth inhibition.

Antibiofilm activity of alkylimidazolium ionic liquids against *Navicula* sp. biofilms followed the similar trend as growth inhibition. [C_4MIM][Cl] did not inhibit biofilm formation completely even at 100 µM concentration. With increase in alkyl side chain length to –dodecyl in [C_{12}MIM][I], complete inhibition in biofilm formation was achieved. This compound was previously reported to be effective in inhibiting the bacterial biofilms [97, 107]. The highly active ionic liquid [C_{16}MIM][Cl] was very potent in inhibiting the biofilm formation and the activity was similar to CTAB, a known cationic biocide in deciphering the antibiofilm activity. The results were in agreement with the previous studies, where in antibiofilm activity was dependent on alkyl side chain length [103, 107].

In sessile mode of growth, adhesion is the foremost step and important event in biofilm formation [140], during which microorganisms adhere to the target surfaces and start colonizing with extensive EPSs production. Several factors which can influence the adhesion of bacteria and microalgae have been studied [140, 141]. The concentration required to prevent adhesion was relatively higher as compared to 5 µM needed for biofilm prevention (Fig. 2.6). The difference was mainly because of the higher initial cell densities and short incubation periods used in the adhesion

experiment. This observation also shows that the concentrations of ionic liquids required for prevention of adhesion and biofilms was dependent on the inoculum cell density and the contact time. Since most natural waters are oligotrophic and the total number of microorganisms available is much less as compared to the cell densities used in the laboratory assays, the compounds used in this study can be expected to be more effective even at lower concentrations.

The toxicity towards *Navicula* sp. cells indicated membrane damage from SYTOX® green staining with increase in ionic liquid concentrations. Release of intracellular contents such as Chlorophyll *a* into the supernatant was dependent on the ionic liquid concentration and contact time. The data presented in the Fig. 2.7 and 2.8 convincingly show that $[C_{16}MIM][Cl]$ causes significant change to membrane permeability in diatom cells and thereby contributes to release of intracellular contents including chlorophyll *a*. $[C_{16}(MIM)]Cl$ contains a hydrophobic -hexadecyl hydrocarbon chain and a polar hydrophilic imidazolium ring. The overall positive charge of imidazolium ring of ionic liquid will help in the interactions with negatively charged cells, while the hydrocarbon chain of $[C_{16}(MIM)]Cl$ can interact with lipid bilayer through hydrophobic interactions. These molecular interactions between the -hexadecyl side chain of ionic liquid and hydrophobic tails of lipid bilayer can lead to membrane permeabilization and leakage of intracellular components.

Cooling water systems which are directly exposed to the sunlight invariably encounter problems of excessive algal growth on surfaces. Manual scraping of cooling tower deck to remove algal biofilm is a laborious process and operators use chemical biocides to control it [142]. Chlorination and application of algicides [143, 144] are routinely used to control algal biofilm in cooling water systems. However,

chlorination has a disadvantage that it can cause delignification of the cooling tower wood [145]. Quaternary ammonium salts like CTAB are potent biocides for use in re-circulating cooling water systems, but, their interaction with corrosion inhibitors such as phosphonates can make them less efficient [146]. Besides manual cleaning, other non-biocidal approaches include the use of ultrasonic sound to control biofilm [144]. Ultrasonic sound is effectively used for the control of cyanobacterial blooms. Disadvantage of this technique is that it is effective only to algae carrying gas vacuoles [147]. High energy consumption and release of toxins are other disadvantages of this technique.

The properties of ionic liquids are tunable by varying the cation, anion and alkyl side chain. For example, biocompatible ionic liquids are designed for biomass pre-treatment to avoid toxicity on downstream fermentation processes [148]. Similarly, antimicrobial ionic liquids are being designed for applications in pharmaceutical agents and antimicrobial formulations [91]. Recently, Garcia and co-workers reported antimicrobial activity of amide-functionalized methylimidazolium or pyridinium ionic liquids against bacterial and fungal strains [100]. Several studies have reported the antimicrobial properties of ionic liquids, although there are limited studies on biofilm control [100]. As such, the toxicity of an ionic liquid depends on cation, anion and alkyl side chain, thus modification of these groups allows designing either lesser or more toxic compounds. In general, alkylimidazolium ionicliquids with shorter alkyl side chains were found to be nontoxic [149], as compared to their counterparts with longer alkyl side chains.

Ionic liquids are attractive for controlling biofilms because they disrupt cell membrane and may not allow development of resistance mechanisms. In the case of

their application as biocides in re-circulating systems, the other advantages include their stability even at high operating temperatures. The efficacy of biocides like chlorine and ozone is linked to temperature and they tend to escape from the water at relatively high temperatures encountered in cooling systems. Negligible vapor pressures of ionic liquids offer them to be stable at wide range of operating temperatures. This study provided evidence for prevention of natural multispecies phototrophic biofilms from freshwater and seawater by an alkylimidazolium ionic liquid with hexadecyl group. Exposure of diatom cells to this ionic liquid led to membrane disruption and release of intracellular contents such as chlorophyll *a*.

2.5. Conclusions

- All three ionic liquids used in this study inhibited the growth of fouling diatom *Navicula* sp. The growth was delayed at higher concentrations of ionic liquid with butyl side chain, whereas, complete inhibition in the growth of diatom cells was caused by relatively lower concentrations of ionic liquids either with -dodecyl or -hexadecyl side chain.
- Alkylimidazolium ionic liquid with -hexadecyl side chain [C_{16}MIM][Cl] showed remarkable inhibition on growth, adhesion and biofilm formation in *Navicula* sp. at concentrations low as 5 µM [C_{16}MIM][Cl].
- SYTOX® Green staining showed evidence for membrane permeabilization in *Navicula* sp. cells exposed to [C_{16}MIM][Cl]. Release of chlorophyll *a* into the surrounding aqueous medium was observed during acute exposure of cells to ionic liquid. Moreover, the mechanism of action of ionic liquid on diatom cells appeared to be similar to that of CTAB, a potent cationic biocide. Experimental data

confirmed that the toxicity of [C_{16}MIM][Cl] on phototrophic microbes was exerted through membrane damage.

- In order to extend the algal antibiofilm activity to natural conditions, the effect of ionic liquid on natural phototrophic biofilm formation was evaluated in natural waters (freshwater and seawater). Ionic liquid prevented natural phototrophic biofilm growth, showing their potential in impeding the growth of multispecies biofilms. The results presented in this study are first of its kind on ionic liquids for inhibiting biofilm formation by phototrophic organisms which are important members of biofilms in streams and re-circulating cooling water systems.

CHAPTER 3

Alkylimidazolium ionic liquids for prevention of fungal biofilms and underlying mechanism of action

3.1. Introduction

Fungal pathogens are a major health issue causing over 1.6 million deaths annually [150]. Several species of *Candida* are responsible for the fungal infections, collectively called as candidiasis. *Candida* species are commensal organisms in healthy individuals but, in immunocompramised or diseased patients, they become opportunistic and cause infections ranging from superficial (oral or vaginal) to life threatening systemic infections. About 50 to 70% of systemic fungal infections are caused by *Candida* spp. [151]. *Candida albicans* is the most frequently observed organism in candidiasis. Persistent *Candida* infections are increasingly being reported in medically implanted devices leading to high mortalities [151, 152, 153]. Biofilm growth by *Candida* spp. further complicates the treatment as the cells reside in biofilms are about 2000 times more resistant to fluconazole and amphotericin B than their planktonic counterparts [108].

Fluconazole is most commonly used for treating candidiasis [154], but, well documented resistance of *Candida* spp. to fluconazole makes this drug a less attractive antifungal agent in the current treatment scenario. Besides drug efflux mechanisms, alterations in target sites/gene expression, current challenges in treatment include biofilm formation which can directly or indirectly enhances the drug resistance [155]. Current treatment strategies are ineffective for prevention of biofilms warranting prospective new antifungal agents [153, 156, 157]. As an effective alternative, ionic liquids have received attention as antifungal agents [105, 158, 159, 160, 161, 162]. Due to effective antifungal activity, Ionic liquids are currently seen as promising asset for fighting fungal infections [163]. However, the effect of ionic liquids on preformed fungal biofilms (biofilm eradication potential) is largely unknown. Evaluation of

antifungal ionic liquids on preformed biofilms is of clinical relevance as the biofilm formation often precedes treatment. There are also limited studies on the mechanism of action of ionic liquids on fungal pathogens [159].

Due to limited understanding of mechanisms, studies aimed at identifying potential targets and underlying mode of action of antifungal ionic liquids are desired for their prospective use in treating infections. This study was aimed to determine the antifungal, antibiofilm and biofilm eradication activities of three imidazolium ionic liquids against *C. albicans* strains and to understand the mode of action of potent antifungal imidazolium ionic liquid.

3.2. Materials and methods

3.2.1. Organisms, media and growth conditions

This research work was conducted using *C. albicans* ATCC 10231 (Microbiologics, USA), a reference strain commonly used for evaluating antifungal agents. Experiments were also conducted with two clinical strains of *C. albicans* (CA i16 (GenBank No. MG757722.1) and CA i21 (GenBank No. MG757724.1)) which were isolated from sputum samples of patients. The clinical strains were obtained from University of Madras, India. These cultures were routinely maintained on potato dextrose agar (PDA) (HiMedia, India). For liquid cultures, a single colony was picked from PDA, transferred to potato dextrose broth (PDB) and incubated for 24 h at 30 ºC and 120 RPM in a temperature controlled orbital shaker. Cells harvested from PDB were used for the growth and biofilm experiments.

Filter sterilised RPMI 1640 medium (L-Glutamine, phenol red, 2 g l^{-1} glucose and 0.165 M MOPS buffer, pH 7.0) (HiMedia AT-180, INDIA) was used for biofilm

experiments. Cultures were grown in PDB for 24 h, pelleted by centrifugation, re-suspended in RPMI 1640 and adjusted to desired cell density for performing biofilm experiments. For determining the mechanism of action, cells were re-suspended in phosphate buffered saline (PBS).

3.2.2. Imidazolium ionic liquids and antifungal drugs

Chemical structures of three ionic liquids used in this study are given in Chapter 2 (Fig. 2.1). Stock solutions (100 mM) of ionic liquids were prepared in sterile, ultrapure water and stored at room temperature until further use. Fluconazole and amphotericin B were purchased from Sigma-Aldrich (USA). Stock solutions of fluconazole (32 mM) and amphotericin B (13 mM) were prepared, respectively, in ethanol and dimethyl sulfoxide (DMSO). These stock solutions were stored at 4 °C until use.

3.2.3. MIC and MFC of ionic liquids against *C. albicans* ATCC 10231

MIC of ionic liquids was determined by microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [164]. Actively growing log phase culture in PDB was pelleted by centrifugation (8000 rpm for 5 min). Cell pellet was re-suspended in RPMI 1640 and cell density was adjusted. MIC and MFC values were determined in RPMI 1640 medium containing initial cell densities of 10^3 or 10^6 cfu ml^{-1}. Initial cell density of 10^3 cfu ml^{-1} is recommended for determining MIC according to CLSI antifungal susceptibility testing [164]. Initial cell density of 10^6 cfm ml^{-1} often employed in biofilm inhibition studies was also used for determining MIC and MFC values [165]. A 2-fold series dilution of ionic liquids and antifungal drugs (fluconazole and amphotericin B) were prepared in RPMI 1640 containing *C. albicans* cells. Working concentrations in the range of 0 to 1000 µM for

[C_4MIM][Cl], 0 to 100 µM for [C_{12}MIM][I] and 0 to 50 µM for [C_{16}MIM][Cl] were prepared in RPMI 1640 and tested. Concentrations of fluconazole and amphotericin B were tested, respectively, in the range of 0 to 3265 µM and 0 to 13 µM. Subsequently, 200 µl of these dilutions were transferred to the wells of 96-well microtiter plate. Five replicates were set up for each concentration. Control wells received cells in RPMI without the compound. After 24 h of incubation in an orbital shaker at 37 ºC and 120 RPM, growth was determined by measuring absorbance at 600 nm using a microplate reader (Biotek®, USA). The lowest concentration that prevented *C. albicans* growth (measured as absorbance at 600 nm) was represented as MIC. For MFC estimation, aliquots of suspension from selected microtiter wells were plated on PDA plates and incubated at 37 ºC for 48 h. MFC was the lowest concentration at which no colonies of *C. albicans* appeared on PDA plates.

3.2.4. Antibiofilm activity of ionic liquids

For estimating incubation time for maximum biofilm formation, RPMI 1640 containing 10^6 cfu ml^{-1} was aliquoted (200 µl) in 96-well sterile, flat bottom, polystyrene microtiter plates (Tarsons, India). Pierce and co-workers recommended using 10^6 cfu ml^{-1} as inoculum cell density for developing *C. albicans* biofilms in 96-well plates [165]. Hence, 10^6 cfu ml^{-1} cells were used for all biofilm experiments. Multi-well plates containing cells in RPMI 1640 were incubated at 120 RPM and 37 ºC for 6, 12, 18, 24 or 48 h. At the end of incubation, biofilm mass and metabolic activity were estimated by crystal violet (CV) and 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT), respectively. Based on time-course experiment on biofilm formation, 24 h incubation time was chosen for quantifying the effect of ionic liquids and antifungal drugs on biofilm formation.

Antibiofilm activity was determined by incubating planktonic and adherent cells separately in the presence of ionic liquids and antifungal drugs. *C. albicans* cell suspensions (200 µl, 10^6 CFU/ml in RPMI 1640) were transferred to each well of a 96-well microtiter plate. Ionic liquids and antifungal drugs were added and serially diluted using 2-fold dilution. Final concentrations in the range of 1000 to 125 µM for [C_4MIM][Cl], 50 to 2.3 µM for [C_{12}MIM][I] and 25 to 1.1 µM for [C_{16}MIM][Cl] were tested. Amphotericin B was tested in the range of 13 to 0.168 µM. Fluconazole concentrations were tested up to 3265 µM. Effect of anions (Cl^- and I^-) and 1-methylimidazole on growth and biofilm formation of *C. albicans* was determined by incubating with excess concentrations (500-1000 µM) of NaCl, KI and 1-methylimidazole. For each concentration, five replicate wells were used. The plates were incubated at 37 ºC in an orbital shaker at 120 rpm to allow biofilm formation. For determining antibiofilm activity using adherent cells, the cell suspensions were transferred to each well of a microtiter plate and incubated for 3 h at 37 ºC in an orbital shaker at 120 rpm to allow adhesion. Subsequent to adhesion, the non-attached cells were carefully removed from the wells. Then 200 µl of RPMI medium containing different concentration of ionic liquids and antifungal drugs was added to each well. The plates were incubated for 24 h as described above to allow biofilm formation.

The biofilm was quantified using CV [97] and XTT reduction assays [166]. For CV assay, biofilms were stained with 0.1% CV (HiMedia, India) for 10 minutes and excess stain was removed by washing the wells the demineralised water [97]. Plates were air-dried for overnight and CV bound to the biofilm was eluted with 33% glacial acetic acid. Eluted CV was measured by reading absorbance at 570 nm. Eluted

CV was diluted with 33% glacial acetic acid whenever the absorbance exceeded 2. For XTT reduction assay, working solutions of XTT (0.5 mg ml^{-1}) were prepared in sterile PBS, stored as 1.8 ml aliquots at -18 ºC. As an electron coupler, a stock solution of 0.32 mg ml^{-1} phenazine methosulfate (PMS) was prepared in PBS and stored at -18 ºC in 0.2 ml aliquots. Prior to the assay, 1.8 ml of XTT was mixed with 0.2 ml PMS and added immediately to each well of the microtiter plate. The plates were incubated at 37 ºC for 2 h to develop orange coloured formazan which was estimated at 492 nm [167].

For visualisation of biofilms, sterile glass slides were inserted in 50 ml falcon tubes containing 25 ml of RPMI 1640 with 10^6 cfu ml^{-1} cells and different concentrations of ionic liquids. Tubes were incubated at 37 ºC, 120 RPM for 24 h. Slides were washed with PBS to remove loosely bound cells, stained with BacLight® live/dead stain (Invitrogen, USA) for 15 min and observed under inverted fluorescence microscope (Carl Zeiss, Germany).

3.2.5. Eradication of preformed biofilms by ionic liquids

For biofilm eradication experiments, 24 h old biofilms were cultivated in RPMI 1640 as described above. After 24 h, spent media was discarded from wells and washed with PBS buffer to remove loosely bound *C. albicans* cells. Different concentrations of ionic liquids were prepared by 2-fold dilution in PBS and 200 µl aliquots were transferred to wells. Five wells were used for each concentration. Plates were incubated again for 24 h at 37 ºC and 120 RPM. After the challenge period, contents in wells were discarded and washed with PBS to remove detached or loosely bound cells. The biofilm remained after exposure to test compounds was quantified

with CV assay. Viability and metabolic activity of cells in the challenged biofilms was determined using BacLight® staining and XTT reduction, respectively. For this, working solution of Syto 9 and propidium iodide (PI) mixture was prepared as per manufacturer's recommendations. Challenged biofilms were stained with 200 µl of stain in the dark for 15 min. Syto 9 and PI fluorescence was estimated by multimode reader (Biotek®, USA) using 488 nm excitation. Syto 9 and PI signals were collected at 520 nm and 620 nm, respectively. The results were represented as the ratio of Syto 9 to PI fluorescence (Live/Dead ratio). XTT reduction assay was performed as mentioned previously.

3.2.6. Effect of ionic liquids on clinical *C. albicans* isolates

Biofilm forming clinical isolates (CA i16 and CA i21) were screened for evaluating the efficacy of fluconazole, amphotericin B and ionic liquids. These isolates were cultured overnight in PDB and adjusted to a cell number of 10^6 cfu ml^{-1} in RPMI 1640. Biofilm formation by CA i16 and CA i21 was determined at different time intervals as mentioned above. Then antibiofilm and biofilm eradication experiments were performed in the presence of ionic liquids. Similar experimental procedures, incubation time and estimation assays were used, as mentioned previously.

3.2.7. Mechanism of action of antifungal ionic liquids

Based on MIC, MFC, antibiofilm and biofilm eradication against *C. albicans* and clinical isolates, the potential ionic liquid $[C_{16}MIM][Cl]$ was selected for understanding possible mode of action. From the previously estimated MIC values, a 10-fold MIC concentration of $[C_{16}MIM][Cl]$ and amphotericin B were prepared in sterile PBS and incubated with 10^6 cfu ml^{-1} for 3 h at 120 RPM. At the end of

incubation, cells exposed to the ionic liquid were harvested by centrifugation, washed with PBS and used for various assays (morphological changes, cell membrane permeabilisation, leakage of intracellular material, reactive oxygen species and mitochondrial dysfunction) described below. For determining effect on ergosterol content, *C. albicans* 10231 was grown in PDB with sub-MIC concentrations of antifungal ionic liquid.

3.2.8. Morphological changes upon ionic liquid exposure

C. albicans cells exposed to [C_{16}MIM][Cl] were harvested and observed under bright field microscope. The images were analyzed using *ImageJ* 1.37V software for determining the size of cells in terms of overall length. For each treatment, a minimum of 220 cells were analyzed and average cell length was determined.

3.2.9. Effect on membrane permeabilisation

Alteration in cell membrane permeability was determined by investigating the propidium iodide (PI) uptake by *C. albicans* cells. *C. albicans* cells exposed to ionic liquid were harvested by centrifugation, washed with PBS and stained with 20 µM PI (Invitrogen, USA) for 15 min. Experiment was performed in triplicates. PBS was used as the control. Inverted fluorescence microscope (Carl Zeiss, Germany) was used for visualizing cells exhibiting PI fluorescence. PI uptake by the *C. albicans* cells was measured quantitatively using 485 nm excitation and 630 nm emission settings with multimode reader (Biotek®, USA).

3.2.10. Leakage of intracellular material

Leakage of intracellular contents was indirectly measured by the increase in concentrations of metal cations such as potassium and calcium and increase in 260 nm

absorbance in cell free supernatants. *C. albicans* cells (10^6 cfu ml^{-1}) in ultra-high pure (UHP) water was exposed to 0.1, 0.25, 0.5, 0.75 and 1 mM of [C_{16}MIM][Cl]. After 3 h incubation at 37 °C and 120 RPM, cell suspensions were centrifuged to collect the supernatant. Absorbance of the supernatant was measured at 260 nm using UV-Visible spectrophotometer (Shimadzu). Metal cations were measured in the supernatants by inductively coupled plasma-atomic emission spectrometer (ICP-AES) (Horiba Jovin Yvon, France). Appropriate controls (UHP water, UHP water with 0.1 to 1 mM [C_{16}MIM][Cl]) were used for analyzing absorbance at 260 nm and quantifying metal cations.

3.2.11. Effect on ergosterol content

For this, *C. albicans* cells were cultured in 50 mL Erlenmeyer flasks containing 20 ml PDB with sub-MIC concentrations (MIC/2, MIC/4 and MIC/8) of [C_{16}MIM][Cl] and fluconazole (5 and 50 µM). Flasks were incubated for 24 h at 120 RPM and 37 °C. Then, cells were harvested, washed with PBS and used for extracting total sterols through saponification [168]. Briefly, 3 mL of 25% alcoholic KOH solution (25 g KOH dissolved in 36 ml UHP water with a 100 mL final make up with 100% ethanol) was added to each of the cell pellet in falcon tubes. Each of these suspensions were mixed by vortexing for a minute and incubated for 1 h in 80 °C water bath. After incubation, tubes were cooled to room temperature and sterols were extracted by adding a mixture of water (1 mL) and n-hexane (3 mL). These suspensions were vigorously mixed by vortexing for 3 min and left for phase separation. The hexane layer containing sterols was diluted with absolute ethanol and scanned between 200 to 300 nm using UV-Vis spectrophotometer. Ergosterol content

was determined and normalized to wet biomass content using established formulae [168].

3.2.12. Effect on intracellular reactive oxygen species (ROS)

C. albicans cells exposed to [C_{16}MIM][Cl] were analyzed for intracellular ROS using 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, USA) [169]. Cell number, treatment with [C_{16}MIM][Cl], incubation and washing procedures were similar to that of membrane permeabilisation experiment. Cells exposed to ionic liquid were incubated with 20 µM DCFH-DA at 37 ºC under dark for 30 min. Experiment was performed in four replicates with appropriate controls. The cells were then observed for 2′,7′-dichlorofluorescein (DCF) fluorescence using an inverted fluorescence microscope (Carl Zeiss, Germany). The fluorescence of DCF was quantified with 485 nm excitation and 538 nm emission settings using multimode reader (Biotek®, USA).

3.2.13. Effect on mitochondrial membrane potential and mitochondrial activity

Mitochondrial membrane potential ($\Delta\psi_m$) in *C. albicans* after exposure to [C_{16}MIM][Cl] was measured using Rhodamine 123 (Rh123) (Sigma, USA) as per Lopes and co-workers with minor modifications [170]. Cells were exposed to [C_{16}MIM][Cl] as detailed in the membrane permeabilisation experiment. Experiment was conducted in four replicates. The cells exposed to ionic liquid were stained with 20 µM Rh123 for 30 min in the dark. After incubation, excess stain was removed and washed with PBS. Cells were re-suspended in PBS and observed for Rh123 fluorescence under inverted fluorescence microscope (Carl Zeiss, Germany) through

FITC filter. Fluorescence of Rh123 was also quantified with 485 nm excitation and 530 emission settings using multimode reader (Biotek®, USA).

MTT assay was used for determining mitochondrial activity of *C. albicans* cells [170]. Cells exposed to [C_{16}MIM][Cl] were harvested, re-suspended in PBS containing 0.5 mg ml^{-1} MTT. Cells were incubated at 120 RPM and 37 °C for 2 h. End of the incubation, cell suspensions were centrifuged and washed with PBS. Purple formazan product developed from the MTT reduction by mitochondrial dehydrogenases was solubilised in 200 µL DMSO by vigorous vortexing. Suspensions were centrifuged and supernatants were collected for estimating formazan absorbance at 510 nm using UV-Visible spectrophotometer (Shimadzu, Japan). Experiment was performed in duplicates with necessary controls.

3.2.14. Statistical analysis

Data was processed from replicates and presented as mean ± standard deviation (SD). Student's t-test was used for determining the statistical significance. Differences between the control and treatment samples were considered to be significant at *P* values < 0.05, <0.01, and <0.001.

3.3. Results

3.3.1. MIC and MFC of ionic liquids and antifungal drugs

The antifungal activity of three ionic liquids and antifungal drugs was expressed as MIC and MFC against *C. albicans* 10231 (Table 3.1). Growth of *C. albicans* 10231 was not inhibited in the presence of [C_4MIM][Cl] even at the highest concentration (1000 µM) tested using initial cell densities of 10^3 and 10^6 cfu ml^{-1}. Thus, growth of *C. albicans* 10231 in the presence of [C_4MIM][Cl] was similar to that of control.

Table 3.1. The antifungal activities of imidazolium ionic liquids and antifungal drugs against *C. albicans* 10231.

Fungal strain	Compound (µM)									
	$[C_4MIM][Cl]$		$[C_{12}MIM][I]$		$[C_{16}MIM][Cl]$		Fluconazole		Amp B	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
C. albicans 10231	> 1000	ND	25	75	4.68	6.25	>3265	ND	1.62	1.62
CA i16	>1000	ND	25	50	4.68	6.25	>3265	ND	0.34	0.68
CA i21	>1000	ND	37.5	75	9.38	12.5	>3265	ND	0.67	1.34

ND: Not determined.

However, the growth of *C. albicans* 10231 was severely inhibited in the presence of imidazolium ionic liquids containing –dodecyl or –hexadecyl alkyl groups. $[C_{12}MIM][I]$ completely inhibited the growth of 10^3 and 10^6 cfu ml^{-1} cell densities, respectively, at 6.25 and 25 µM. MFC values at these cell densities was determined to be 37.5 and 75 µM, respectively. Among the tested compounds, $[C_{16}MIM][Cl]$ showed maximum potency with MIC value of 2.34 and 4.68 µM, for 10^3 and 10^6 cfu ml^{-1}, respectively. The MFC concentrations for $[C_{16}MIM][Cl]$ were determined to be 4.68 and 6.25 µM, for 10^3 and 10^6 cfu ml^{-1}, respectively. The MIC values for fluconazole and amphotericin B were determined to >3265 µM and 1.62 µM against 10^6 cfu ml^{-1} *C. albicans* 10231, respectively. The MFC values showed that $[C_{16}MIM][Cl]$ was more effective in killing fungal cells than other two ionic liquids and fluconazole. The MFC of $[C_{16}MIM][Cl]$ at 6.25 µM was slightly greater as compared to 1.62 µM for amphotericin B.

3.3.2. Antibiofilm activity of ionic liquids

Time course assay of biofilm development by *C. albicans* 10231 revealed highest biofilm mass and metabolic activity at 24 h (Fig. 3.1). Thus, 24 h incubation

time was used for biofilm inhibition experiments. Biofilm formed in presence of different concentrations of alkylimidazolium ionic liquids was quantified by CV and XTT as shown in Fig. 3.2.

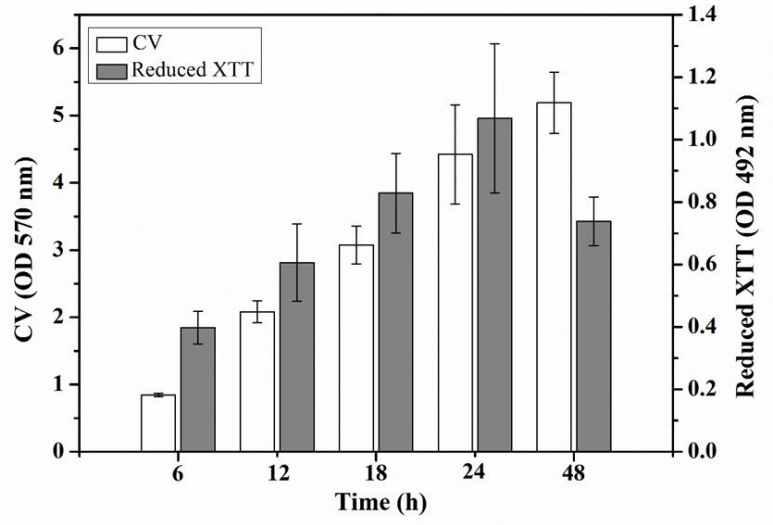


Fig. 3.1. Biofilm development by *C. albicans* ATCC 10231 at different time intervals. Biofilm biomass and metabolic activity was quantified using crystal violet (CV) and XTT, respectively.

Biofilm inhibition was not evident in the presence of [C_4MIM][Cl] (Fig. 3.2A, D). Biofilm formation by *C. albicans* 10231 was severely inhibited in the presence of imidazolium ionic liquids containing –dodecyl and –hexadecyl groups. For example, 50% and 100% inhibition in biofilm formation was achieved, respectively, using 4.6 µM and 25 µM [C_{12}MIM][I] (Fig. 3.2B). [C_{16}MIM][Cl] was found to be more potent with no biofilm formation beyond 4.68 µM (Fig. 3.2C). Biofilm formation with adhesion step revealed complete inhibition in biofilm formation beyond 25 µM and 6.25 µM for [C_{12}MIM][I] and [C_{16}MIM][Cl], respectively (Fig. 3.3). Complete inhibition in biofilm formation was achieved using 1.62 µM amphotericin B. However, biofilm formation was not prevented even using 3265 µM of fluconazole. Inhibition of *C. albicans* 10231 biofilm formation in the presence of imidazolium ionic liquids with –

dodecyl or –hexadecyl groups was clearly evident in the florescence microscopic images (Fig. 3.2D).

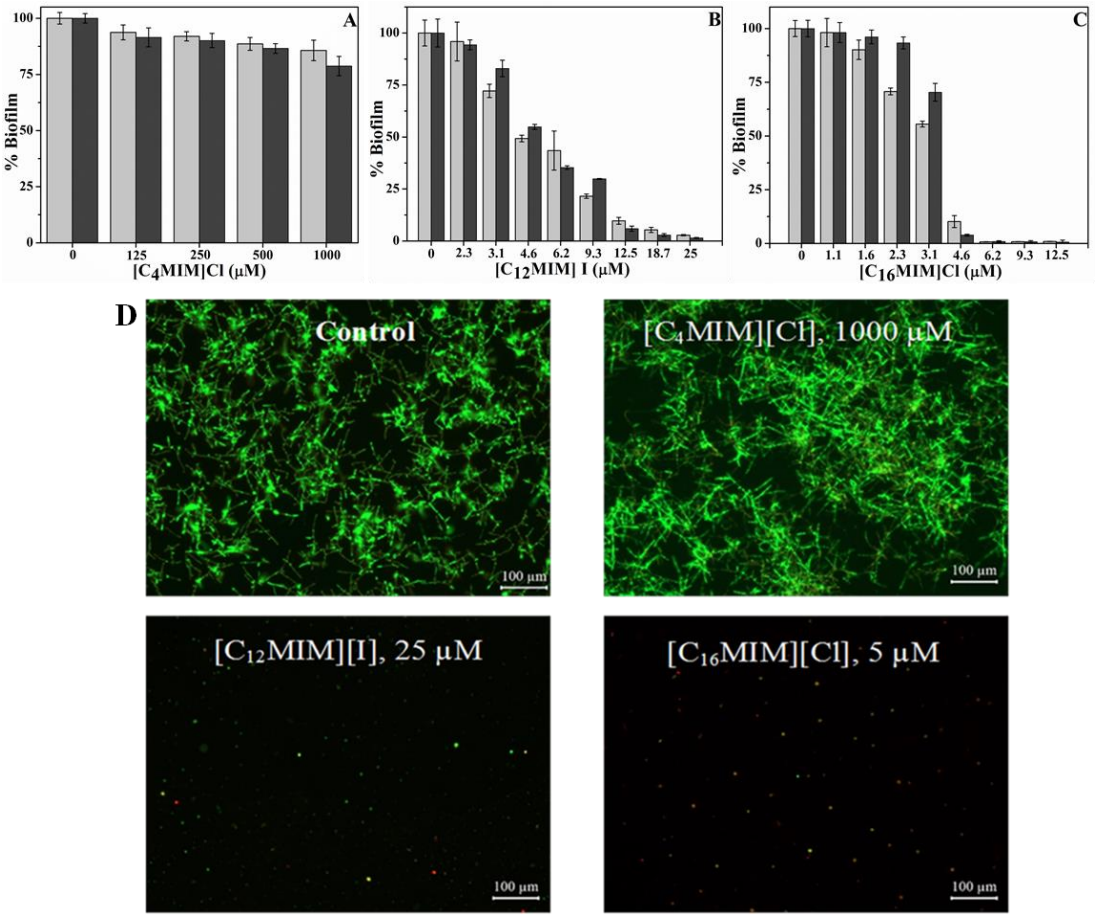


Fig. 3.2. Biofilm formation by *C. albicans* 10231 at different concentrations of ionic liquids. Different initial concentrations of [C_4MIM][Cl] (A), [C_{12}MIM][I] (B), and [C_{16}MIM][Cl] (C) were evaluated for antibiofilm activity. Biofilm quantified by CV (light grey) and XTT (dark grey) was presented. Fluorescence microscopic images of *C. albicans* biofilms in presence of different concentrations of ionic liquids (D).

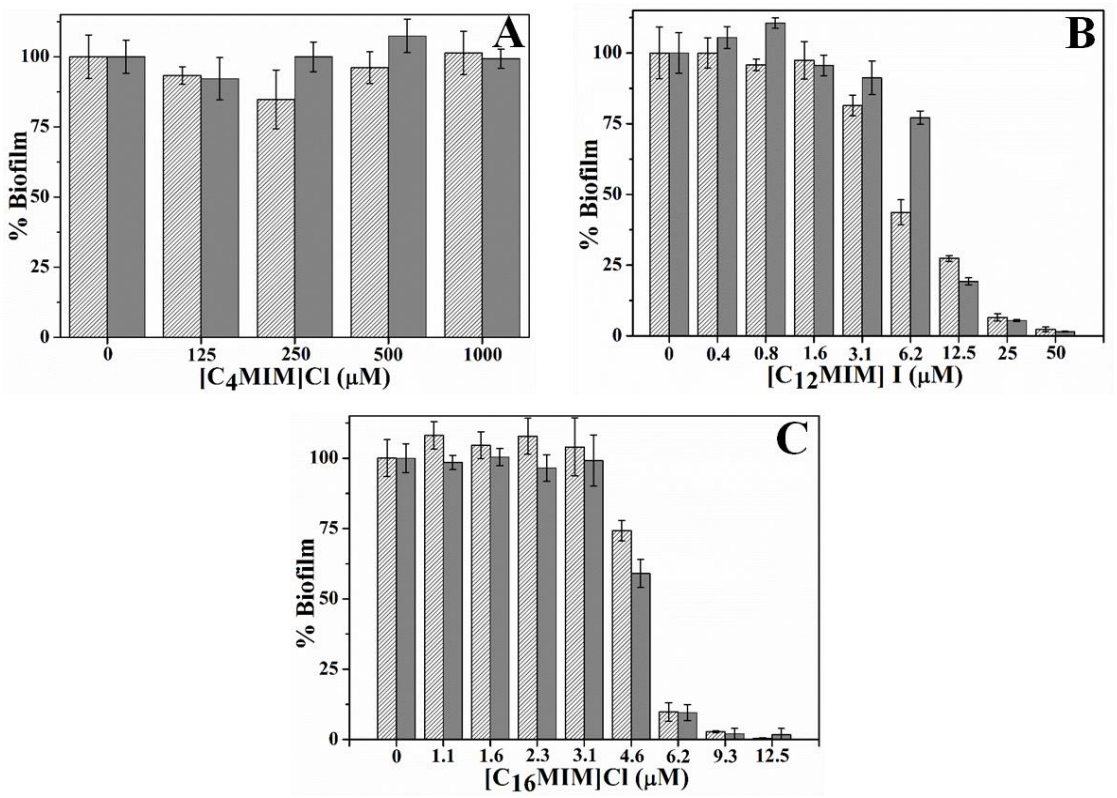


Fig. 3.3. Biofilm formation in presence of ionic liquids after initial adhesion. After a 3 h adhesion step by *C. albicans* 10231, biofilm formation at different concentrations of $[C_4MIM][Cl]$ (A), $[C_{12}MIM][I]$ (B), and $[C_{16}MIM][Cl]$ (C) was presented.

The anion constituents (Cl^- and I^-) and 1-methylimidazole ring did not show any significant effect on the growth and biofilm formation of *C. albicans* 10231 (Fig. 3.4).

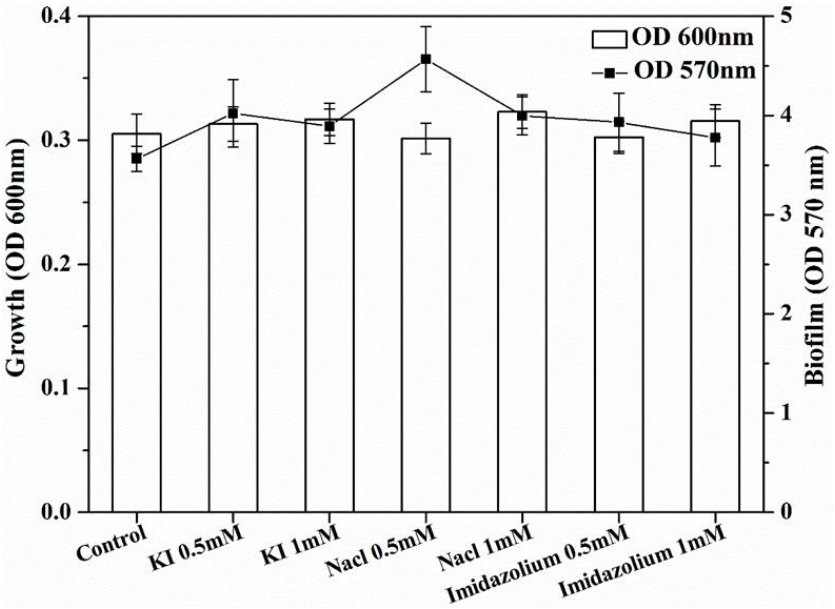


Fig. 3.4. Growth and biofilm formation of *C. albicans* 10231 in presence of 1-methylimidazole and constituent anions. Planktonic growth (OD600) and biofilm formation (Crystal violet, OD570) in presence of enhanced concentrations (500 and 1000 µM) of ionic liquid anions (Cl^- and I^-) and 1-methylimidazole.

3.3.3. Biofilm eradication potential of ionic liquids

The biofilm eradication potential of alkylimidazolium ionic liquids was determined in terms of killing of biofilm cells and dislodgement of preformed biofilm. The effect of alkylimidazolium ionic liquids on preformed *C. albicans* 10231 was shown in Fig. 3.5 and 3.6. $[C_4MIM][Cl]$ and $[C_{12}MIM][I]$ did not cause dispersal of 24 h old *C. albicans* 10231 biofilms irrespective of their concentrations (Fig. 3.5A, B). A concentration dependent biofilm dispersal was observed in the case of $[C_{16}MIM][Cl]$. However, biofilm dispersal required much higher concentrations than those required for inhibiting biofilm formation. For example, removal of >90% of biofilm was required 250 µM $[C_{16}MIM][Cl]$ (Fig. 3.5C).

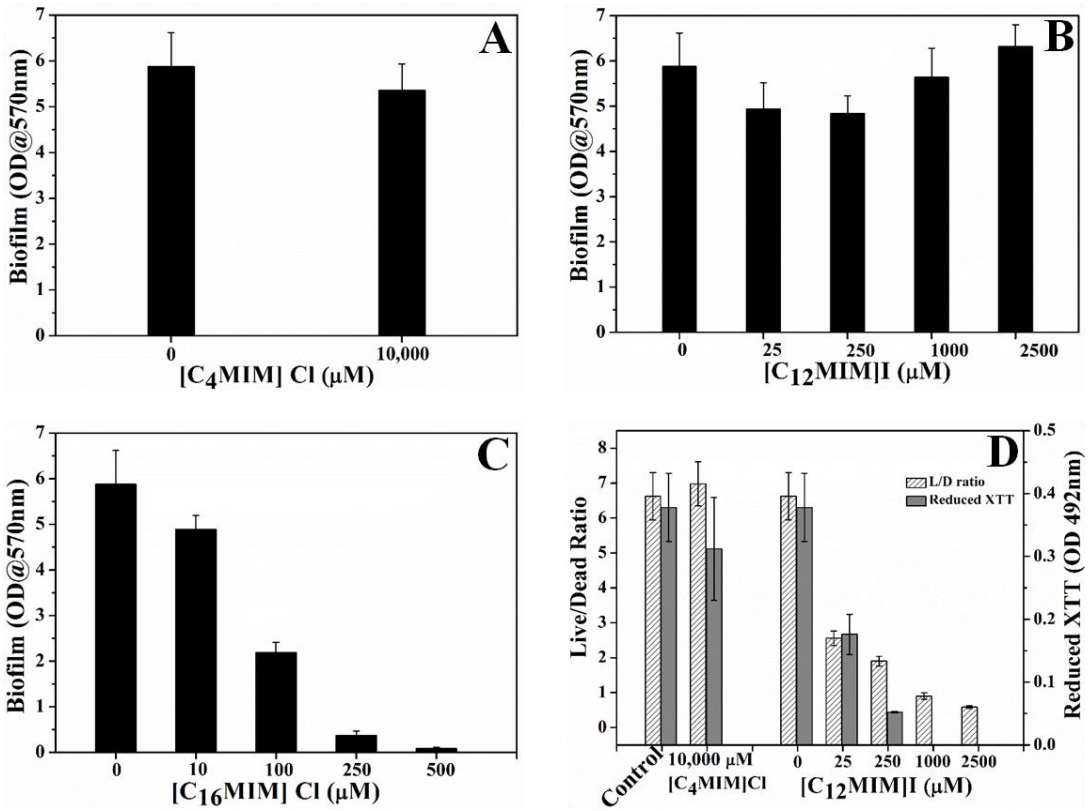


Fig. 3.5. Effect of imidazolium ionic liquids on preformed biofilms. *C. albicans* 10231 biofilms were exposed to imidazolium ionic liquids for 24 h and then residual biofilm was quantified using CV method (A, B, C). Cell viability in *C. albicans* biofilms upon exposure to ionic liquids that did not exert biofilm dispersal (D).

Since $[C_4MIM][Cl]$ and $[C_{12}MIM][I]$ were not causing any biofilm dispersal, their effect was determined in terms of viability and metabolic activity of biofilm cells (Fig. 3.5D). There was no change in the Live/Dead (L/D) status or metabolic status of *C. albicans* 10231 biofilms exposed to 1000 μM $[C_4MIM][Cl]$ for 24 h. However, the L/D status and XTT reduction potential of *C. albicans* 10231 biofilms was drastically decreased upon exposure to 25 to 2500 μM of $[C_{12}MIM][I]$.

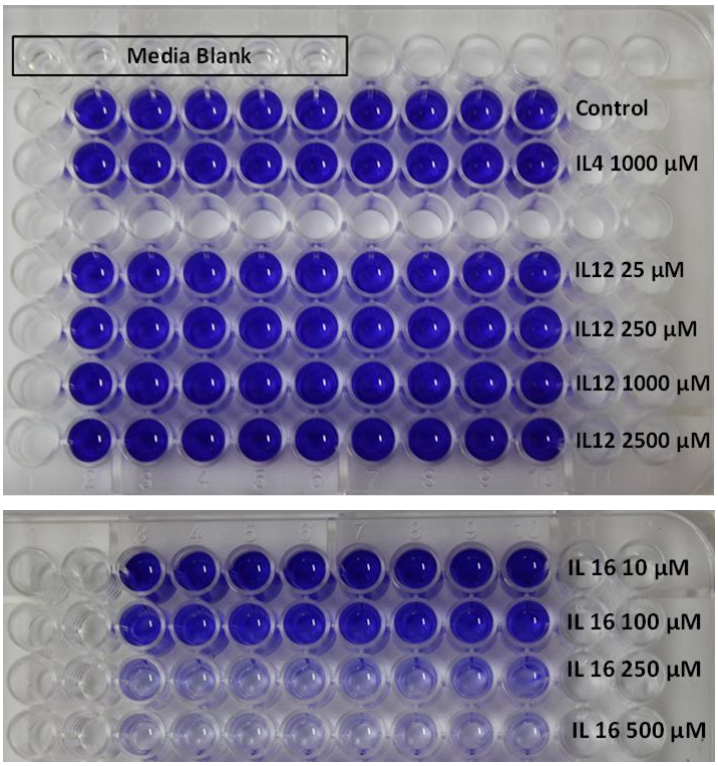


Fig. 3.6. Eradication of pre-formed biofilms in multiwell plates. 96 well plates showing the eradication of preformed *C. albicans* ATCC 10231 biofilms after challenging with different concentrations of ionic liquids.

3.3.4. Ionic liquids against fluconazole resistant clinical *C. albicans* strains

Clinical strains (CA i16 and CA i21) were selected based on their copious biofilm formation potential and high resistance towards fluconazole. CLSI has fixed MIC breakpoint of ≥64 µg ml^{-1} for fluconazole resistant strains. The clinical strains evaluated in this study were highly resistant to fluconazole and inhibition in growth was not observed up to 1000 µg ml^{-1} fluconazole. MIC and MFC values for ionic liquids and antifungal drugs against clinical *C. albicans* strains were presented in Table 3.1. Biofilm formation potential and metabolic activity of both the clinical strains at different time intervals was shown in Fig. 3.7. Biofilm formation pattern was found to be very similar

between the two clinical strains. Incubation time of 24 h was considered optimum based on highest metabolic activity.

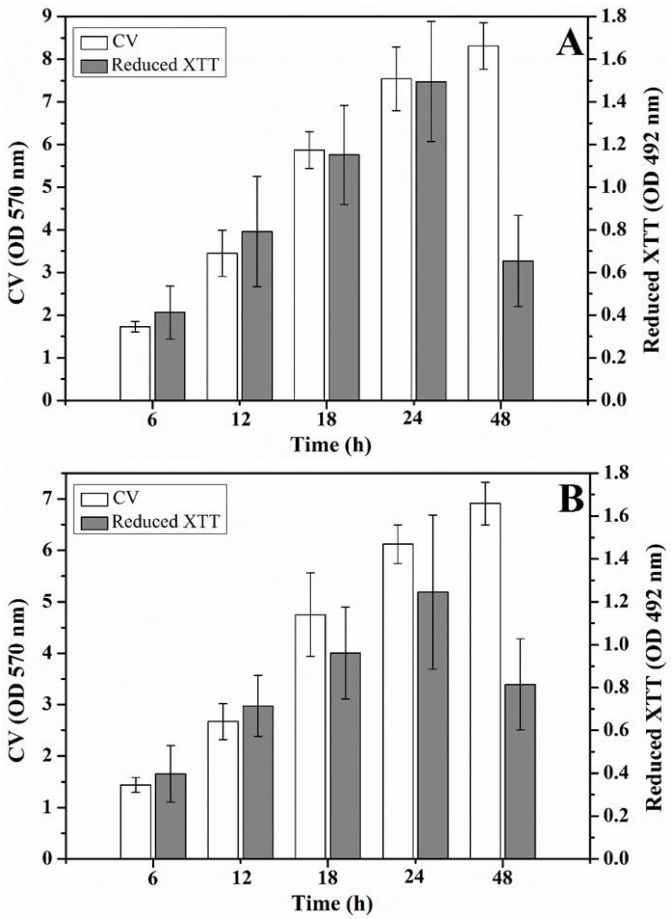


Fig. 3.7. Optimum incubation time for maximum biofilm formation by clinical *C. albicans* strains. Biofilm development by *C. albicans* clinical isolates, CA i16 (A) and CA i21 (B) at different time intervals. Biofilm biomass and metabolic activity was quantified using crystal violet (CV) and XTT, respectively.

The tested imidazolium ionic liquids with –dodecyl and –hexadecyl groups were effective in preventing biofilm formation by both the clinical strains. However, there was a marginal difference in the activity towards these two different isolates. Complete inhibition in biofilm formation by clinical isolates required 25 µM and 6.25 µM, respectively, for [C_{12}MIM][Cl] and

[C_{16}MIM][I] (Fig. 3.8). Imidazolium ionic liquid with –hexadecyl group was equally effective in dispersing preformed biofilms of clinical strains. Removal of preformed biofilms of clinical strains was about 75% and 100% at 100 and 250 µM, respectively (Fig. 3.8D).

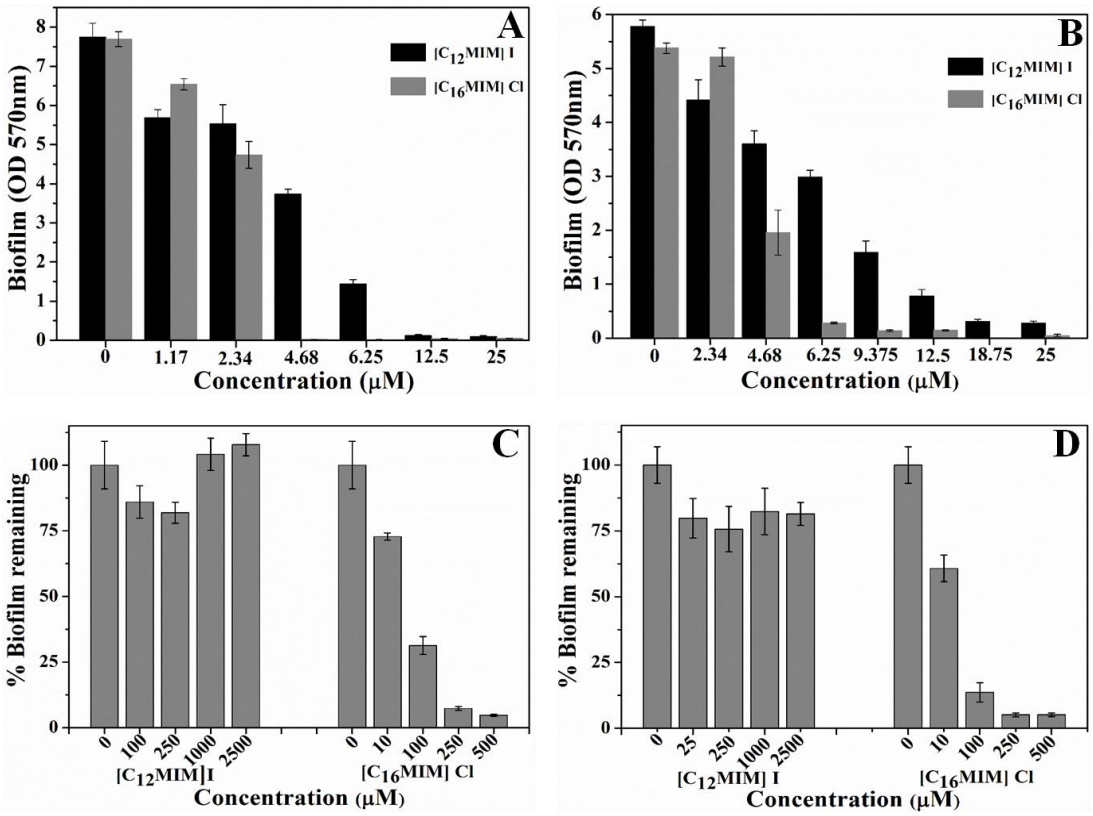


Fig. 3.8. Effect of ionic liquids against biofilms of clinical *C. albicans* isolates, CAi16 (A, C) and CAi21 (B, D). Antibiofilm (A, B) and biofilm eradication potential (C, D) of ionic liquids on fluconazole resistant clinical *C. albicans* isolates.

3.3.5. [C_{16}MIM][Cl] causes shrinking of *C. albicans* cells

Microscopic observations revealed that potent ionic liquids (containing -dodecyl, -hexadecyl side chain) cause changes in cell size (data not shown). A systematic study with [C_{16}MIM][Cl] exposure to *C. albicans* 10231 cells revealed a significant decrease in the cell size (Fig. 3.9). In control population,

the cell length was 5.5±1.1 µm. However, the cell length decreased to 4.1±0.8 µm upon exposure to [C_{16}MIM][Cl]. In presence of amphotericin B, the average length of cells was further reduced to 3.4±0.8 µm (Fig. 3.9D). The results indicate that, [C_{16}MIM][Cl] decreased the cell volume leading to shrinkage of *C. albicans* 10231 cells.

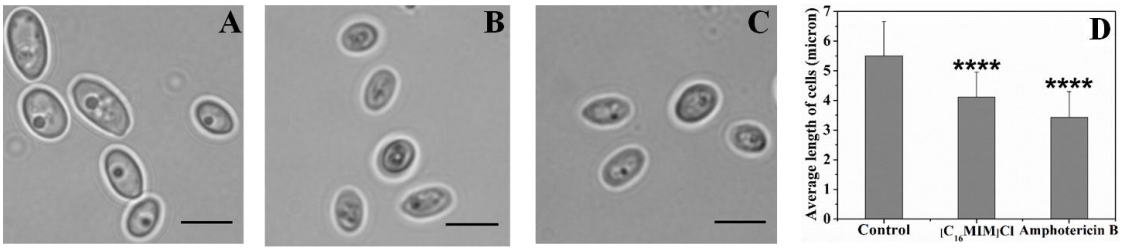


Fig. 3.9. Effect of imidazolium ionic liquids on cell size during short term exposure. Bright field microscopic images of *C. albicans* cells in control (A), [C_{16}MIM][Cl] (B) and amphotericin B (C) treatments. Scale bar = 5 µm. Graph (D) represents the average length of cells in control and treatment. Cells exposed to 10X MIC concentration for 3 h; ****P*<0.001, n=220.

3.3.6. [C_{16}MIM][Cl] induces membrane permeabilisation

Impact of [C_{16}MIM][Cl] on plasma membrane of *C. albicans* cells was monitored by PI uptake. Due to high molecular weight, PI can only enter the cells with permeabilized cell membrane. Control cells were not stained by PI (Fig. 3.10B). But, ionic liquid and amphotericin B treated cells were stained by the PI (Fig. 3.10D, F). Quantitative fluorescence measurement revealed significant uptake of PI by the cells exposed to [C_{16}MIM][Cl] than amphotericin B (Fig. 3.10G).

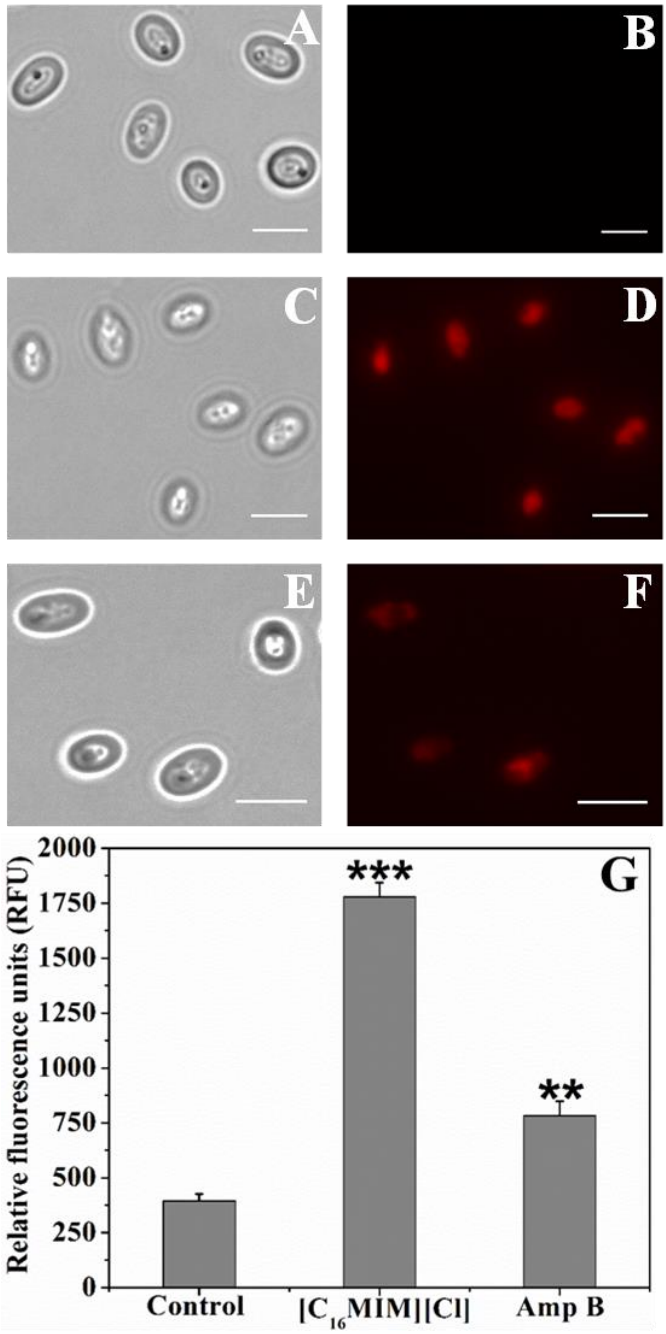


Fig. 3.10. Propidium iodide (PI) uptake by *C. albicans* cells exposed to [C_{16}MIM][Cl]. Bright field and fluorescence microscopy images of control (A, B), ionic liquid treated (C, D) and amphotericin B treated (E, F) cells. (G) PI fluorescence by control, ionic liquid and amphotericin B treated *C. albicans* cells (cells exposed to 10X MIC concentration for 3 h; ****P*<0.001, ***P*<0.01, n=3).

3.3.7. [C_{16}MIM][Cl] causes leakage of intracellular material

[C_{16}MIM][Cl] induced leakage of intracellular contents was measured from the increase in 260 nm absorbance (nucleic acids) and release of important

metal cations (i.e., potassium and calcium). A clear increase in the absorbance at 260 nm was observed in the supernatant upon exposure of cells to potent ionic liquid (Fig. 3.11A). ICP-AES data showed efflux of potassium and calcium when *C. albicans* 10231 cells were exposed to [C_{16}MIM][Cl] (Fig. 3.11B). Increased absorbance of supernatant and release of metal cations was a clear indication for leakage of intracellular contents during ionic liquid treatment.

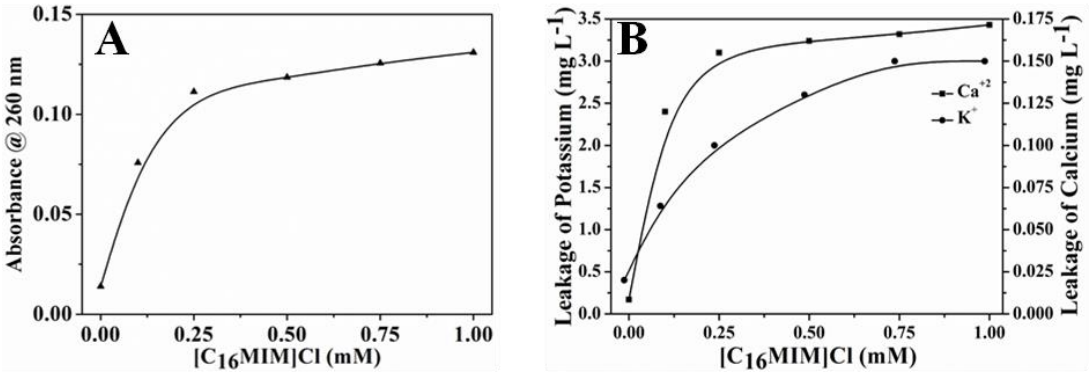


Fig. 3.11. Leakage of intracellular components from *C. albicans* cells upon exposure to [C_{16}MIM][Cl]. (A) Absorbance of cell-free supernatant at 260 nm, (B) concentration of potassium and calcium in cell-free supernatant.

3.3.8. [C_{16}MIM][Cl] decreases ergosterol content

The effect of [C_{16}MIM][Cl] on cell membrane ergosterol content was determined at sub MIC concentrations (MIC/2 to MIC/8). Absorption spectra of extracted sterols were shown in Fig. 3.12A. A decrease in the absorbance of the sterols from 250 to 300 nm was evident when *C. albicans* 10231 were grown in the presence of [C_{16}MIM][Cl]. The total sterol content, calculated from absorbance at 282 nm decreased in the cells cultured in the presence of sub MIC concentrations of [C_{16}MIM][Cl]. Normalisation to cell weight after ergosterol estimation and comparison with sterol content in control cells indicate the

negative effect of ionic liquid on ergosterol content and the effect is concentration dependent (Fig. 3.12B).

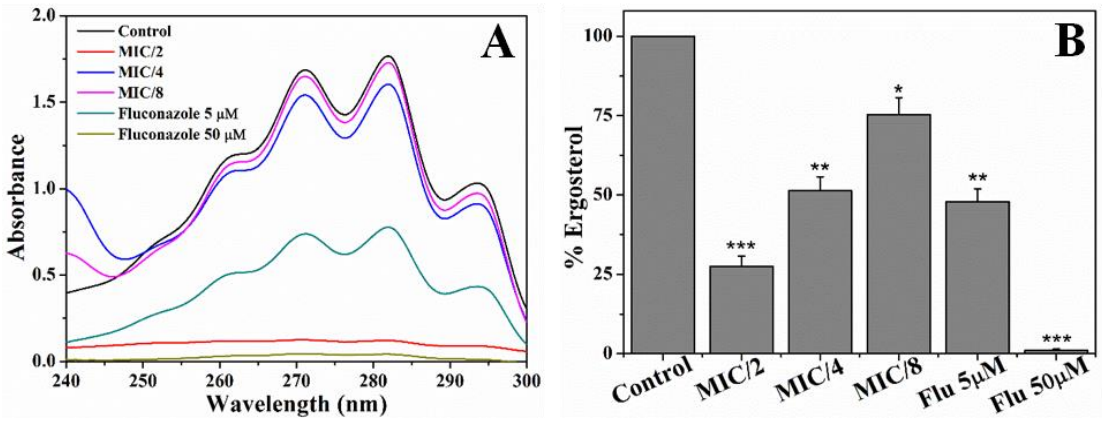


Fig. 3.12. Effect of [C_{16}MIM][Cl] on ergosterol content. (A) Absorption spectra of extracted sterols from *C. albicans* cells cultured in the presence of different concentrations of [C_{16}MIM][Cl] and fluconazole. (B) Ergosterol content in *C. albicans* cells grown in the presence of [C_{16}MIM][Cl] and fluconazole (**P*<0.05, ***P*<0.01, ****P*<0.001, n=2).

3.3.9. [C_{16}MIM][Cl] induces ROS production

C. albicans 10231 cells were exposed to [C_{16}MIM][Cl] and amphotericin B and stained with DCFH-DA for determining ROS generation. ROS include superoxide anions, hydroxyl radicals, hydrogen peroxide, and singlet oxygen, which can oxidize DCFH-DA and generate DCF. Accumulation of DCF inside the cells is a direct estimation for ROS generation. Qualitative and quantitative measurement of ROS generation in *C. albicans* 10231 cells was shown in Fig. 3.13. Fluorescence microscopic images revealed intense fluorescence from the *C. albicans* 10231 cells treated with [C_{16}MIM][Cl] (Fig. 3.13D). Cells treated with amphotericin B were also stained (Fig. 3.13F), although to a lesser extent than ionic liquid treatment The fluorimetric data indicated significantly higher ROS in the *C. albicans* 10231

cells exposed to ionic liquid (Fig.3.13G). The ROS induced by amphotericin B were found to be significantly lower than that of ionic liquid treatment.

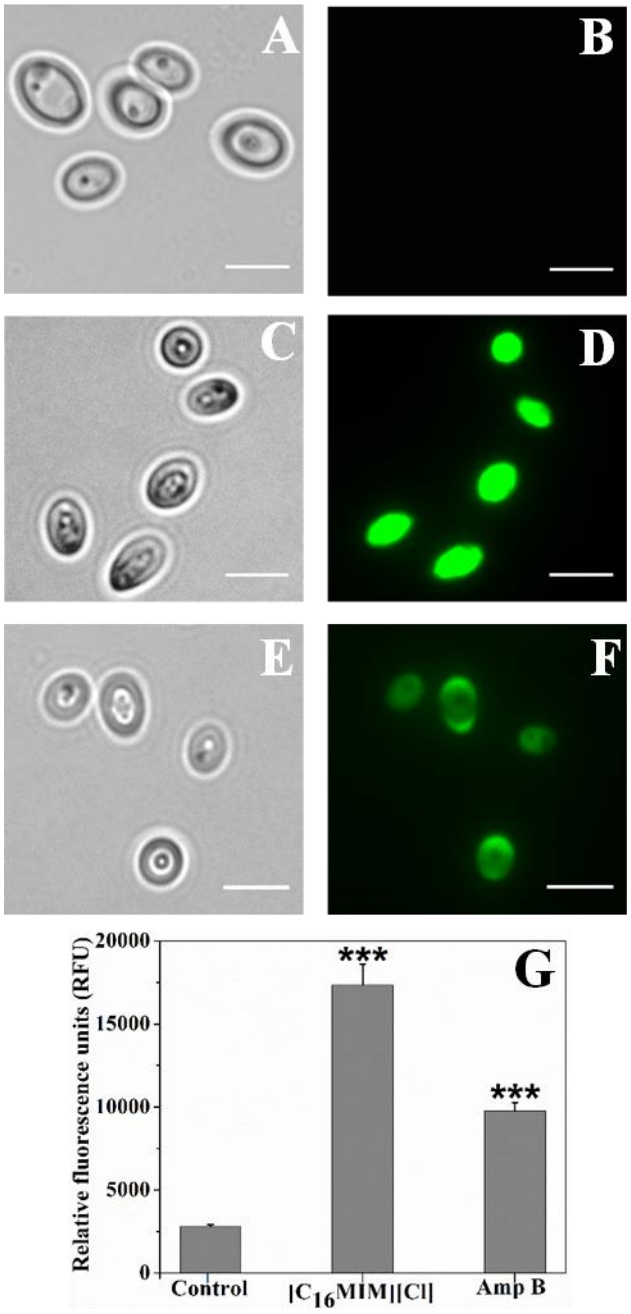


Fig. 3.13. ROS in *C. albicans* cells before and after exposure to [C_{16}MIM][Cl] and amphotericin B. Bright field and fluorescence images of control (A, B), ionic liquid treated (C, D) and amphotericin B treated (E, F) cells. Scale bar = 5 µm. Quantitative estimation (G) of ROS in control, ionic liquid and amphotericin B treated cells. (Cells exposed to 10X MIC concentration for 3 h; ***P<0.001, n=4).

3.3.10. [C_{16}MIM][Cl] decreases the mitochondrial membrane potential and inhibits mitochondrial activity

$\Delta\psi_m$ is essential for mitochondrial energy metabolism for ATP synthesis. Alterations in membrane potential can damage mitochondrial function and could affect the cell survival. Effect of [C_{16}MIM][Cl] on $\Delta\psi_m$ was investigated by Rhodamine 123 (Rho123), a dye sequestered by active mitochondria. Hyperpolarisation leads to increased accumulation of Rho123. But, depolarisation results decreased accumulation of Rho123. Intense Rho123 staining was observed in control cells indicating the active $\Delta\psi_m$ (Fig. 3.14A). Ionic liquid exposed *C. albicans* cells indicated faint staining with Rho123, indicating loss of membrane polarisation (Fig. 3.14A). Quantitative measurement of Rho123 fluorescence showed a significant decrease in ionic liquid exposed cells over control cells (Fig. 3.14B). The results suggest that, ionic liquids cause loss of $\Delta\psi_m$ in *C. albicans* 10231 cells.

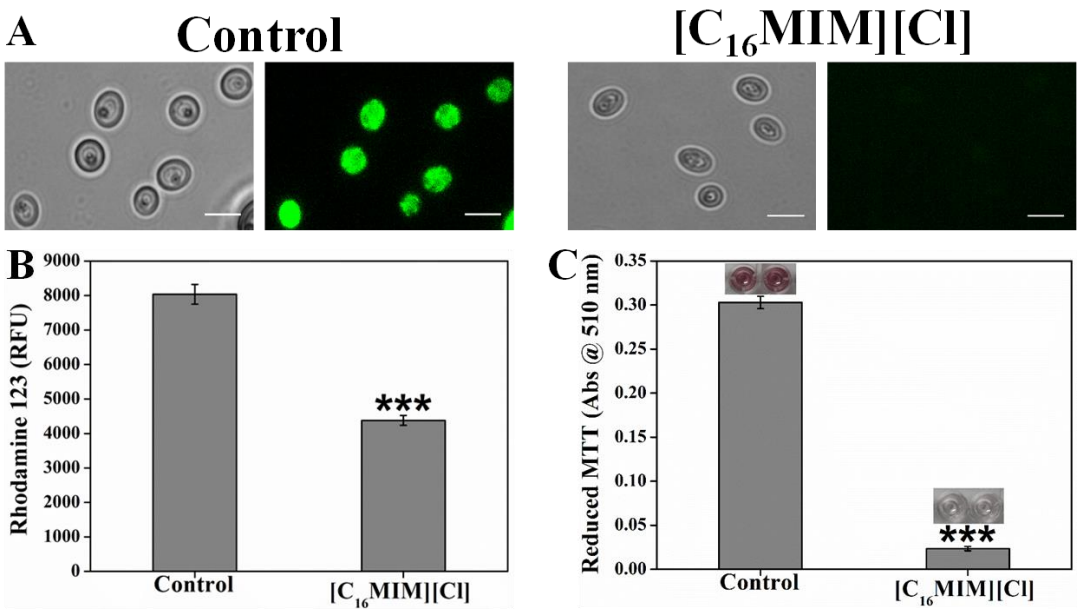


Fig. 3.14. Effect of [C_{16}MIM][Cl] on *C. albicans* mitochondrial membrane potential and mitochondrial activity. Cells exposed with [C_{16}MIM][Cl] were incubated with Rhodamine 123 (A, B) and MTT (C). Rhodamine 123 fluorescence from control and treated cells was observed under fluorescence microscope (A) and fluorescence was also quantified in multimode reader (B). MTT reduction by control and treated cells is shown in fig. (C) along with microtiter wells showing reduced MTT in control. Cells exposed to 10X MIC concentration for 3 h; $^{**}P<0.001$, n=4 for Rhodamine 123 assay, n=2 for MTT assay.

In addition to $\Delta\psi_m$, mitochondria function was also evaluated by estimating the activity of mitochondrial dehydrogenases. Metabolically active mitochondria can reduce colorless MTT to purple formazan which can be solubilised and measured calorimetrically. Failure of such reduction is an indication for the abnormal mitochondrial activity. MTT reduction to purple formazan was observed in control cells, indicating the normal activity of dehydrogenases. In case of ionic liquid exposed cells, formazan formation was severely impaired due to mitochondrial dysfunction (Fig. 3.14C). The formazan development in control *C. albicans* 10231 cells inoculated in microtiter wells can be seen in the inset of Fig. 3.14C.

3.4. Discussion

Major challenges in developing new antifungal drugs or therapeutic strategies against *C. albicans* are (i) its opportunistic pathogenicity causing both superficial and systemic fungal infections, (ii) its complex and polymorphic biofilm structure, and (iii) emergence of drug resistant *C. albicans* strains. These challenges warrant development of novel antifungal agents for effective treatment therapies. Ionic liquids have attracted attention for medical applications because of huge structural diversity (theoretically, 10^{18} compounds are possible) and tunability of structure which enable attaining desired biological activity and antimicrobial activities [90, 91].

Among others, imidazolium ionic liquids are well studied for antimicrobial activities [97] and shown to possess antifungal activity. For antibiofilm agents, it is desirable to have antimicrobial and surfactant activities [171]. Imidazolium ionic liquids particularly with long-alkyl chains can exhibit antifungal and surfactant activities. In this study, imidazolium ionic liquids with three different alkyl groups were investigated for determining (i) biofilm prevention and biofilm eradication activities on *C. albicans* strains, and (ii) possible mode of action of promising ionic liquid on *C. albicans* cells.

Although antifungal and antibiofilm activities of imidazolium ionic liquids have been studied previously [105, 108, 160, 162], their effects on preformed *C. albicans* biofilms and their molecular toxicity mechanisms are largely unknown. This study provided a clear insight into the biofilm eradication potential of alkylimidazolium ionic liquids and mechanism of action of prospective antifungal ionic liquid. Multi-marker approach was adopted for identifying targets and to discern the mode of action of promising antifungal ionic liquid.

3.4.1. Antifungal, antibiofilm and biofilm eradication activity of ionic liquids

The antifungal, antibiofilm and biofilm eradication potential of three ionic liquids was determined *in vitro* against *C. albicans* strains. Alkylimidazolium ionic liquid with -butyl group was not inhibitory to *C. albicans* cells and considered as non-antifungal ionic liquid. Ionic liquids with -dodecyl and -hexadecyl groups were effective in preventing the growth of *C. albicans* and two other clinical strains, hence referred to as antifungal ionic liquids. This study re-confirmed that the antifungal activity of alkylimidazolium ionic liquids was dependent on the carbon chain length of

alkyl group. MIC and MFC values of ionic liquids were dependent on initial cell densities, that is, low concentrations were sufficient to inhibit the growth of 10^3 cfu ml^{-1} than 10^6 cfu ml^{-1}. MIC values of potent antifungal ionic liquids were much lower than fluconazole against *C. albicans* 10231. However, the antifungal activity of two of the tested ionic liquids was comparable or slightly higher than amphotericin B. Biofilm formation by *C. albicans* 10231 and clinical strains was effectively and completely inhibited in the presence of antifungal ionic liquids. The results of this study in terms of antifungal and antibiofilm activity of alkylimidazolium ionic liquids are in agreement with previous work on *C. albicans* [159, 160]. But, the effect of these antifungal ionic liquids on preformed biofilms is largely unknown. The effect of antifungal drugs on adherent cells or biofilms is of clinical relevance because (i) biofilm formation often precedes treatment and (ii) biofilms are much more resistant to antifungals. In this context, the biofilm eradication potential of antifungal ionic liquids was determined for the first time in terms of viability loss and dispersal of preformed *C. albicans* biofilms. Antifungal ionic liquid with -hexadecyl group was able to effectively disperse 24 h old *C. albicans* biofilm (Fig. 3.5C). Interestingly, antifungal ionic liquid with –dodecyl group was not effective in dispersing the biofilm even at higher concentrations (Fig. 3.5B and Fig. 3.8C, D). But, this antifungal ionic liquid remarkably decreased the viability and metabolic activity of *C. albicans* cells in the preformed biofilm (Fig. 3.5D). The biofilm eradication potential results suggest that antifungal ionic liquids are suitable for treating *C. albicans* infections.

3.4.2. Mechanism of action of antifungal ionic liquids

The microscopic observation revealed that the cells exposed to $[C_{16}MIM][Cl]$ were smaller than the untreated control cells (Fig. 3.9). A

significant reduction in cell volume was noticed in *C. albicans* 10231 cells exposed to antifungal ionic liquid for few hours. Shrinkage of *C. albicans* cells was also observed upon exposure to antifungal agents such as apigenin, silver nanoparticles [172, 173]. The cell shrinkage was often associated with cell membrane permeabilisation and leakage of intracellular contents [172]. Ionic liquids can increase the permeabilization of cell membrane in bacterial and microalgal cells [97, 174]. An increased absorbance (at 260 nm) and concentration of metal ions (K^+ and Ca^{2+}) in the aqueous medium surrounding *C. albicans* 10231 during exposure to antifungal ionic liquid suggested cell membrane permeabilisation or damage (Fig. 3.11). Membrane permeabilisation was indeed confirmed in ionic liquid treated *C. albicans* 10231 cells by the preferential uptake of PI but not by control cells (Fig. 3.10). The intracellular concentrations of alkali metal ions (i.e., K^+, Na^+) are important for maintaining cell volume, pH and cell membrane potential in yeasts [175]. Therefore, leakage of metal cations (K^+ and Ca^{2+}) and other intracellular material (UV absorbing substances) are responsible for the observed reduction in cell volume.

Ergosterol is a major sterol in the fungal cell membrane and adequate levels are essential for maintaining membrane integrity and other membrane functions [176]. Hence, ergosterol and its biosynthetic pathways are important targets in the development of antifungal agents [177]. A significant decrease in the ergosterol content of *C. albicans* 10231 cells cultured in the presence of antifungal ionic liquid (Fig. 3.12) indicated inhibition of ergosterol biosynthesis. This was in agreement with the observations of Schrekker and co-workers that imidazolium ionic liquids can interfere in the ergosterol biosynthesis resulting

in lower ergosterol in the cell membrane [159]. It was hypothesized that imidazolium ionic liquids can interrupt conversion of lanosterol to ergosterol by inhibiting lanosterol 14α-demethylase [159].

Generation and accumulation of intracellular ROS was prominent in *C. albicans* 10231 cells treated with [C_{16}MIM][Cl] (Fig. 3.13). In yeasts, ROS are majorly produced in the mitochondria [178]. High ROS levels are detrimental as they cause oxidative damage to intracellular molecules and cell membrane lipids [179]. Interestingly, ROS production is one of the mechanisms by which yeast cells senses mitochondrial dysfunction [178]. The mitochondrial dysfunction in *C. albicans* 10231 cells was assessed by $\Delta\psi_m$ and dehydrogenases activity. Rho123, a cationic and lipophilic dye used for quantifying $\Delta\psi_m$ because this dye can specifically stain negatively charged mitochondria [180]. A significant decrease in Rho123 staining by *C. albicans* 10231 upon treatment with antifungal ionic liquid (Fig. 3.14) indicated disruption of membrane potential. MTT assay indicated almost complete loss of dehydrogenases activity asserting mitochondrial dysfunction in ionic liquid treated *C. albicans* 10231 cells. Cell volume reduction, intracellular ROS production and mitochondrial dysfunction are often observed in apoptosis [181]. Additional studies are required to validate the role of apoptosis in cell death in presence of ionic liquids.

3.4.3. Clinical relevance

Fungal infections alone account for approximately 11.5 million life-threatening infections and 1.6 million deaths annually around the globe [150, 155]. Treatment of fungal infections is a challenge in clinical settings because of limited number of antifungal drugs for treating invasive infections, inefficacy

in preventing infections, difficulty in administering and combination of all these factors [182, 183]. This is amplified by emerging antifungal resistance and resistance conferred by fungal biofilms. Ionic liquids, unique class of compounds, are seen as promising assets for treating life-threatening fungal infections due to their structural diversity and tunable physical and chemical properties which contribute to the synthesis of a large number of compounds [163]. Imidazolium ionic liquids are promising because of their strong antifungal and biofilm inhibition activities. The other important attributes of these compounds are (i) broad spectrum activity on bacteria and fungi [97, 161, 162], and (ii) multiple cellular targets for exhibiting antifungal activity as demonstrated in this study. Antifungal compounds with multiple pharmaceutical targets are promising for evading resistance development, a menace in fungal therapy. Antifungal susceptibility testing and other *in vitro* assays can screen a large number of compounds, identify effective compounds and identify cellular targets. Effective ionic liquids are suitable for prospective applications in antifungal creams for topical applications, disinfection of surgical tools and treatment of dental lines in hospital settings. The results of *in vitro* assays are useful for guiding treatment choices [183] and complementation with *in vivo* studies is necessary for considering potential clinical applications.

Schrekker and co-workers evaluated the biocompatibility of imidazolium ionic liquids *in vitro* using L929 fibroblast cells of mice [184]. These tests revealed that cytotoxicity increases with an increase in alkyl chain length from –butyl to –decyl and –hexadecyl group. 1-butyl-3-methylimidazolium chloride was fully biocompatible with no detectable toxicity to fibroblast cells. Whereas, 1-decyl-3-methylimidazolium

chloride and 1-hexadecyl-3-methylimidazolium chloride were cytotoxic to fibroblast cells, respectively, at 50 to 500 µg ml^{-1} and 10 to 500 µg ml^{-1}. Thus, biocompatibility of –hexadecyl containing ionic liquid was reported to be good at <10 µg ml^{-1} with minimal toxicity to fibroblast cells suggesting that low concentrations of these compounds are still safe. Cytotoxicity data for 1-dodecyl-3-methylimidazolium iodide is not available, although it can be predicted to possess the toxicity between –decyl and –hexadecyl alkyl group containing imidazolium ionic liquids. The concentrations of 1-hexaydecyl-3-methylimidazolium chloride at 0.84 µg ml^{-1} and 1.69 µg ml^{-1} for antifungal and antibiofilm (for complete biofilm prevention) activities, respectively, are much below the reported toxic value of 10 µg ml^{-1} to fibroblast cells [184] and seems to be safe. Nevertheless, additional biocompatibility studies of antifungal ionic liquids are warranted for considering these compounds in prospective formulations. Therefore, further research should focus on (i) efficacy, (ii) formulation and co-administration with other compounds and (iii) cellular toxicity using *in vivo* models.

3.5. Conclusions

- The antifungal activities of 1-alkyl-3-methylimidazolium ionic liquids were found to be dependent on the structure of compounds chiefly on the alkyl side chain length and initial cell density. Ionic liquid with –butyl side chain did not inhibit the growth of *C. albicans*. However, ionic liquids with –dodecyl and –hexadecyl groups exhibited remarkable antifungal and antibiofilm activities against laboratory and drug resistant *C. albicans* strains.
- Biofilm formation by *C. albicans* was completely inhibited in the presence of < 5 µM of 1-hexadecyl-3-methylimidazolium chloride. Distinct biofilm

eradication potential activities were observed. Though ionic liquid with –dodecyl side chain exhibited biofilm cell killing, but it was unable to disturb the biofilm structure. In contrary, ionic liquid with –hexadecyl side chain exhibited biofilm eradication by disturbing the biofilm structure and causing biofilm dispersal.

- Shrinking of cells upon ionic liquid exposure was evident from microscopic observations. Live/Dead staining and leakage of intracellular contents revealed –hexadecyl side chain containing ionic liquid can permeabilise cell membrane and cause mortality in *C. albicans* cells.
- 1-hexaydecyl-3-methylimidazolium chloride also affected ergosterol biosynthesis, an important constituent in fungal cell membranes. Additionally, the antifungal ionic liquid induced oxidative stress and impacted mitochondrial functioning in *C. albicans* cells.

CHAPTER 4

Antimacrofouling potential of alkylimidazolium ionic liquids against macrofouling barnacle larvae

4.1. Introduction

Biofouling by marine invertebrates such as barnacles, mussels, hydroids, and tubeworms on submerged surfaces is a serious problem in power plant cooling water systems, shipping, aquaculture and fishing industries [185]. Antifouling (AF) formulations/paints with biocides such as tributyltin (TBT) are very successful for mitigation of biofouling in shipping industry. However, due to high toxicity towards other marine organisms [84], a global ban on the use of TBT in antifouling paints came into force on 1st January 2008 [186]. Currently, copper-based AF paints with co-biocides such as Irgarol 1051, Sea-Nine 211, diuron, chlorothalonil, zinc pyrithione (ZnPT) and copper pyrithione (CuPT) are being used [84, 185]. However, several studies have reported the acute toxicity of Cu-based compounds to non-target organisms and persistent nature of co-biocides in the marine environment [84, 187]. In view of this, alternative approaches including novel natural/synthetic compounds [188, 189, 190], biomimetic surfaces [191], surface-active polymers [192] and ultrasonic sound waves [193] are investigated for developing non-toxic environmentally benign antifouling methods. Incorporation of green chemicals of natural or synthetic origin in formulations/paints is promising for developing antibiofouling strategies with a minimal environmental impact.

Ionic liquids are attractive for antifouling due to huge synthetic repertoire (~10^{18}), tunability of structure, and broad-spectrum antimicrobial activity [194]. The alkylimidazolium ionic liquids showed potent antimicrobial and antibiofilm activities on bacteria, diatoms, phototrophic biofilms and fungal biofilms [97, 101, 108, 174]. Here, the antibiofouling activity of the ionic liquids was assessed using barnacle larvae. Barnacles are notorious macrofouling calcareous organisms in power plant

cooling water systems and maritime activities, settles on surfaces in their larval stage and continue the remaining life in sessile mode. The settling stage of barnacle larvae (cypris) is an ideal model often used in evaluating antifouling compounds or formulations. The effect of ionic liquids on survival, settlement and metamorphosis of barnacle larvae was investigated. The general toxicity of the tested ionic liquids to non-target organisms was assessed using brine shrimp (*Artemia salina*).

4.2. Materials and methods

4.2.1. Ionic liquids

Chemical structures of the tested compounds are given in Fig. 2.1 of Chapter 2. A 100 mM stock solution was prepared for each of the ionic liquid in autoclaved Milli Q water and stored at room temperature until further use.

4.2.2. Adult barnacle broods and release of nauplii

Larvae of fouling barnacle, *Amphibalanus* (= *Balanus*) *reticulatus* was used for experiments. For collecting adult barnacle broods, fibre reinforced plastic (FRP) coupons (10 cm X 15 cm) were suspended near the seawater intake point of madras atomic power station, Kalpakkam (12°33′ N and 80°11 ’E), INDIA. Fouled FRP coupons containing adult barnacles were transferred to the laboratory in seawater. FRP coupons were kept under the running water to remove epibionts and cleaned using nylon brush. Barnacle adults were air dried for 2 h and placed in autoclaved seawater to allow the release of nauplii. Barnacle larval culture, storage of cypris larvae and cypris assays were performed as described previously [195].

4.2.3. Barnacle larval culture

Nauplii (Stage II) were collected using pointed light source and Pasteur pipette for concentrating them in fresh autoclaved filtered seawater (AFSW). AFSW was prepared by filtering fresh seawater through 0.22 µm filter and autoclaving at 121 °C and 15 psi for 20 min. Larvae were reared in AFSW in a temperature controlled (26±1 °C) room with alternating 12 h light: 12 h dark cycles. After every 24 h, nauplii were concentrated and transferred into fresh AFSW. A daily dose of *Chaetoceros lorenzianus* (strain NIOT-18) at a concentration of ~2 × 10^5 cells/mL was fed to nauplius larvae in fresh AFSW, until the cypris stage. *C. lorenzianus* was regularly cultured in AFSW spiked with *f*/2 media components and used as the feed for barnacle larvae. Cyprid stage larvae were obtained in 6 to 7 days of culture. Cypris were collected and stored in the dark at 4 °C until use for settlement assays. Different stages of nauplius larvae were also harvested from different days of larval culture and were used for determining the effect of ionic liquids.

4.2.4. Effect of ionic liquids on survival and settlement of cypris larvae

Experiments were performed in 35 mm polystyrene Petri dishes (Tarsons, India). Different concentrations of ionic liquids were prepared in AFSW by 2-fold dilution method. Concentrations of ionic liquids ranging from 100 µM to 1.6 nM for [C_4MIM][Cl], 25 µM to 0.048 nM for [C_{12}MIM][I] and 25 µM to 0.024 nM for [C_{16}MIM][Cl] were prepared for determining cypris survival and settlement. All assays were performed using cypris larvae stored for 3 days at 4 °C. Around 15 cyprid larvae were placed in a Petri dish and immediately added with 4 mL of AFSW containing ionic liquids. Controls received AFSW alone without ionic liquids. Three replicates were used for each concentration. Petri dishes were covered with aluminium foils and incubated in temperature controlled (26±1 °C) dark chamber for 48 h [195].

At the end, cyprid larvae were scored for mortality and settlement using stereo microscope (Zeiss, India). Dose response curves were plotted for concentration against mortality or settlement. Mortality was expressed as LC_{50}, which is the minimum concentration required for killing of 50% of added larvae. Settlement inhibition was expressed as EC_{50}, which is the minimum concentration required to inhibit 50% settlement. From the ratios of LC_{50} to EC_{50}, therapeutic concentrations were determined [189] for each of the ionic liquid compound.

4.2.5. Effect of ionic liquids on survival and metamorphosis of nauplius larvae

Based on ease of identification under microscope, three different naupliar stages such as nauplius II, V and VI were harvested from culture flasks and used for determining the effect of ionic liquids on mortality and metamorphosis. Different concentrations of ionic liquids were prepared in AFSW by 2-fold dilution method and aliquoted to the Petri dishes containing nauplii. Around 15 nauplius larvae were added to each dish and three replicates were kept for each concentration. Petri dishes were also spiked with *C. lorenzianus* to allow growth and metamorphosis of nauplii. By repeated centrifugation, algal cells were adjusted such that, the addition of 0.5 mL of suspension to 4.5 mL of AFSW/ AFSW + ionic liquid would give a final concentration of ~2×10^5 cells mL^{-1}. 4 mL of these suspensions were added to each dish and were incubated for 24 h in 12 h light: 12 h dark cycle in a temperature controlled (26±1 °C) chamber. After incubation, larvae were scored for mortality and metamorphosis to next stage. Control nauplii were fed with algal cells without test compounds in AFSW.

4.2.6. Effect of ionic liquids on mortality of *A. salina* larvae

Toxicity evaluation of ionic liquids on *A. salina* larvae was performed as per Artoxkit M procedure [196], with minor modifications. *A. salina* cysts were incubated in filtered seawater under strong aeration and continuous illumination in a temperature controlled room (26±1 °C) for 36 h. Hatched larvae were collected in filtered seawater and used in the experiment. Around 15 larvae were added to 35 mm Petri dish and dispensed 4 mL aliquots of filtered seawater containing different concentrations of alkylimidazolium ionic liquids. Cypris EC_{50}, LC_{50} and 10-fold EC_{50}/LC_{50} concentrations of ionic liquids were prepared as described above and used in *A. salina* assay. Experimental controls received only filtered seawater and three replicates were used for each of the concentration. The dishes were incubated in temperature controlled dark room (26±1 °C) for 24 h. At the end of the incubation, dead larvae were counted for determining percentage mortality. Dose response curves were derived from the survival data of brine shrimp larvae across various concentrations of ionic liquids.

4.2.7. Data analysis

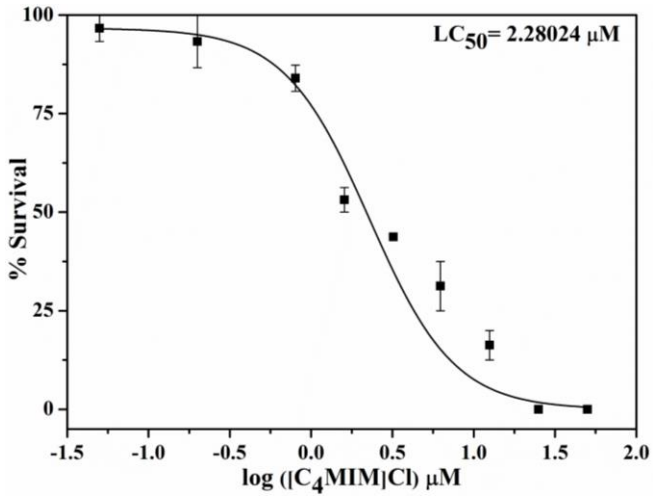
Data was processed and reported as mean ± standard deviation. EC_{50} and LC_{50} values were calculated from the dose response curves plotted using GraphPad prism software. Student's t-test was performed to compare test data points with corresponding controls.

4.3. Results

4.3.1. Effect of ionic liquids on cypris survival

There was a differential survival of larvae to the tested ionic liquids (Fig. 4.1). About 85% survivorship was observed in cypris larvae in the presence of up to 0.8 µM [C_4MIM][Cl]. Further increase in [C_4MIM][Cl] concentration up to 12.5 µM

resulted in progressive decline in cypris survivorship to 16%. At 25 µM of [C_4MIM][Cl], all the cypris larvae died resulting in 100% mortality. From the dose dependent survivorship curves, 48 h LC_{50} value for [C_4MIM][Cl] was determined to be 2.28 µM (Fig. 4.1). [C_{12}MIM][I] showed highest mortality in cypris larvae at much lower concentrations as compared to other two tested ionic liquids. For example, 40% mortality was caused by as low as 0.2 µM of [C_{12}MIM][I]. As the concentration increased to 0.8 and 1.6 µM, survival fraction was decreased to 20 and 12%, respectively. Complete mortality in cypris larvae was observed at 3.2 µM [C_{12}MIM][I]. This high sensitivity of cypris larvae towards [C_{12}MIM][I] led to a very low LC_{50} concentration of 0.35 µM. Mortality of cypris larvae in the presence of [C_{16}MIM][Cl] was much higher than [C_4MIM][Cl] and marginally less than [C_{12}MIM][I]. Cypris survival fractions were 78, 41 and 28%, respectively, upon exposure to 0.2, 0.8 and 1.6 µM of [C_{16}MIM][Cl]. Complete mortality in cypris larvae was recorded at 3.2 µM [C_{16}MIM][Cl]. The calculated LC_{50} value from the dose-response survivorship curve was 0.63 µM for [C_{16}MIM][Cl]. Based on the data, it is evident that the toxicity of ionic liquids to cypris larvae decreased in the following order: [C_{12}MIM][I]>[C_{16}MIM][Cl]>[C_4MIM][Cl].



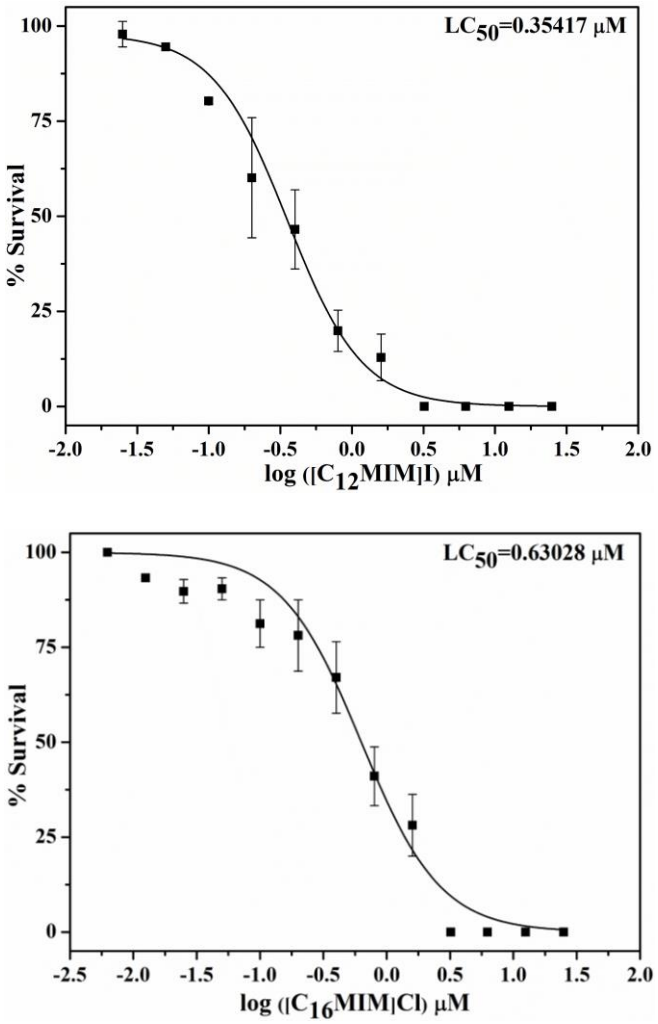


Fig. 4.1. Survival curves for cyprid larvae in presence of different concentrations of alkylimidazolium ionic liquids. Minimum concentration required to kill 50% of the added larvae was represented as LC_{50}.

4.3.2. Effect of ionic liquids on cypris settlement

Inhibition of cypris settlement was noticed at much lower concentrations of tested ionic liquids (Fig. 4.2). All three tested ionic liquids showed different degrees of inhibition in larval settlement. Settlement of cypris larvae was not inhibited in the presence of 1.64 to 6.25 nM of [C_4MIM][Cl]. A gradual decrease in settlement was observed with increasing concentration of [C_4MIM][Cl] from 12.5 to 390 nM. Complete inhibition in settlement of cypris larvae was observed at 0.8 µM of

[C_4MIM][Cl]. From the dose-response curve, the EC_{50} value on settlement inhibition was determined to be 80.2 nM for [C_4MIM][Cl]. Settlement of cypris larvae was unaffected in the presence of 0.024 nM [C_{12}MIM][I] and is similar to that of control. However, settlement of cypris larvae was inhibited in the presence of 0.048 to 12.5 nM in a dose-dependent manner. In the presence of 25 nM of [C_{12}MIM][I], none of the cypris larvae settled resulting in 100% inhibition in settlement. EC_{50} of [C_{12}MIM][I] was determined to be 0.39 nM for eliciting settlement inhibition. Effect of [C_{16}MIM][Cl] on larval settlement was lower than [C_{12}MIM][I] and much higher than [C_4MIM][Cl]. Cypris settlement was not significantly affected in the presence of lower concentrations (0.048 to 0.195 nM) of [C_{16}MIM][Cl]. A dose-dependent inhibition in larval settlement was noticed at higher concentrations of [C_{16}MIM][Cl]. Complete inhibition in settlement of cypris larvae was observed at concentrations higher than 25 nM. The EC_{50} value for [C_{16}MIM][Cl] was determined to be 1.56 nM which is slightly lower than [C_{12}MIM][I] and much higher than [C_4MIM][Cl]. Settlement and metamorphosis of settled larvae was seen in control and in the presence of picomolar concentrations of all three ionic liquids (Fig. 4.3). However, unsettled but healthy cypris larvae were noticed in the presence of nanomolar concentrations of ionic liquids. As depicted in the quantitative data, dead cypris larvae were seen in the presence of micromolar concentrations of ionic liquids (Fig. 4.3).

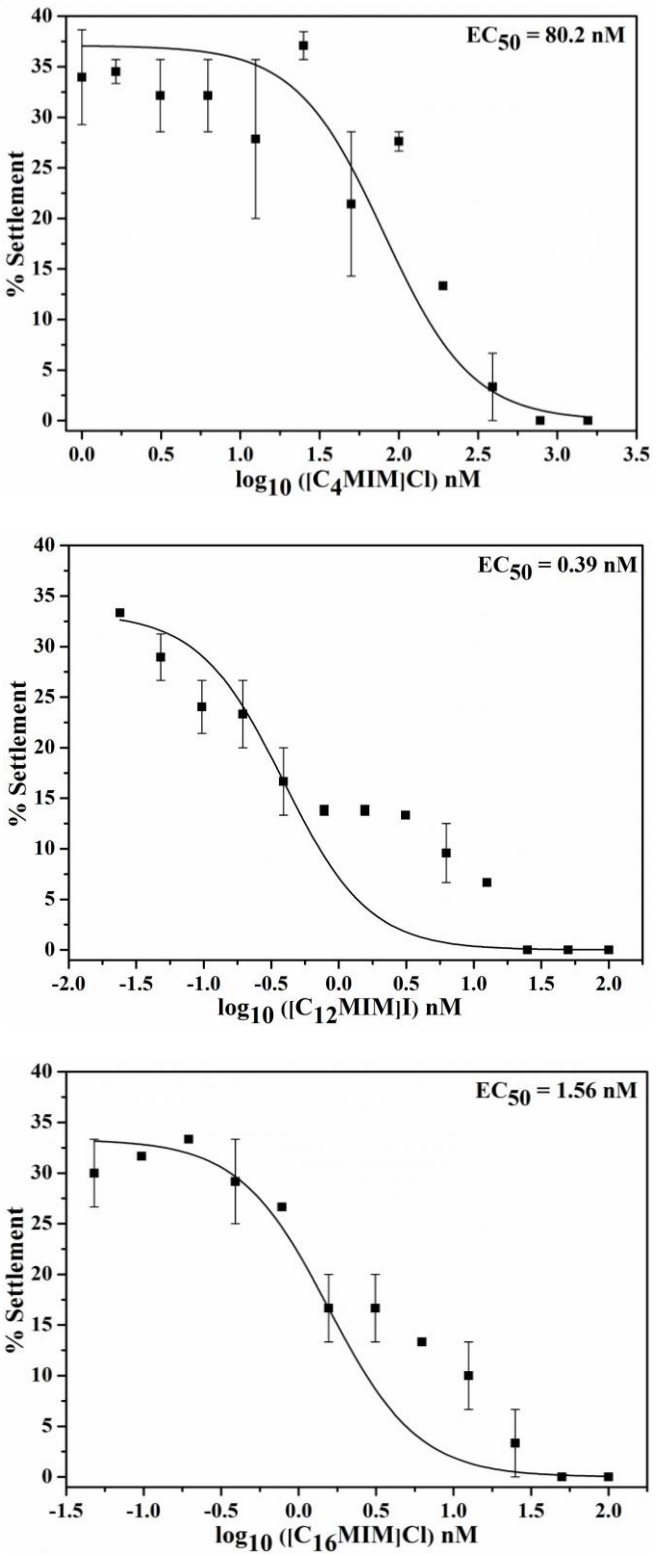


Fig. 4.2. Settlement of cyprid larvae in presence of alkylimidazolium ionic liquids. EC_{50} is the concentration that prevented settlement of 50% of cypris larvae.

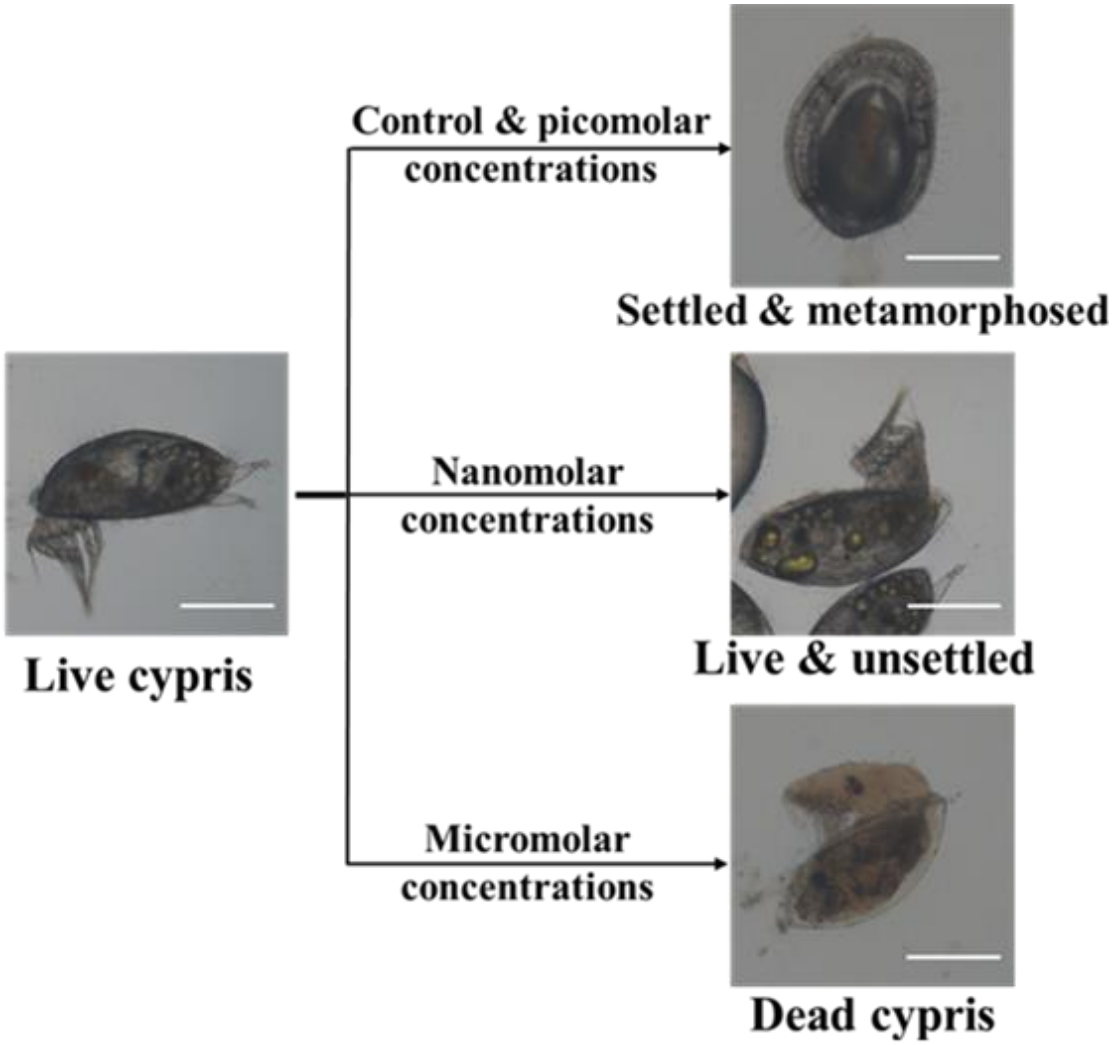


Fig. 4.3. Stereo zoom microscopic images of various physiological forms of larvae in presence of different concentrations of ionic liquids. In seawater controls and picomolar concentrations of ionic liquids, incubated cypris were alive, settled and metamorphosed. In presence of nanomolar concentrations of tested compounds, larvae were alive but settlement was impaired. At micromolar concentrations, dead larvae were observed. Scale bar = 250 µm.

4.3.3. Therapeutic ratios of the tested ionic liquids

Therapeutic ratios (T_R) for all the three tested alkylimidazolium ionic liquids were estimated from the cypris mortality (LC_{50}) and settlement inhibition (EC_{50}) assays. Ratio of LC_{50} to EC_{50} were calculated and presented in Table 4.1. Among the three tested ionic liquids, $[C_{12}MIM][I]$ and $[C_{16}MIM][Cl]$ had higher T_R values of 907

and 403, respectively. The other ionic liquid, [C_4MIM][Cl] had the least T_R value of 28 among the tested ionic liquids.

Table 4.1. The toxicity (LC_{50}) and antifouling effectiveness (EC_{50}) of ionic liquids. EC_{50}: minimum concentration for 50% inhibition of larval settlement; LC_{50}: the 50% lethal dose; LC_{50}/EC_{50}: therapeutic ratio.

Compound	LC_{50}		EC_{50}		LC_{50}/EC_{50}
	µM	µg L^{-1}	µM	µg L^{-1}	
[C_4MIM][Cl]	2.28	400	0.0802	14	28.5
[C_{12}MIM][I]	0.354	133	0.00039	0.147	907.6
[C_{16}MIM][Cl]	0.63	216	0.00156	0.535	403.8

*Suggested reference values for potential non-toxic antifouling compounds: EC_{50}: < 25 mg L^{-1}; therapeutic ratio (LC_{50}/EC_{50}): >15.

4.3.4. Effect of ionic liquids on survival and metamorphosis of nauplii

Easily distinguishable naupliar larval stages such as nauplius II, V and VI were chosen for determining the effect of ionic liquids on survival and metamorphosis. Presence of alkylimidazolium ionic liquids had profound influence on the survival and metamorphosis of various naupliar stages (Fig. 4.4). Survival of naupliar larvae was near normal at about 100% under larval culture conditions in the absence of ionic liquids. The percentage metamorphosis was high at 93.5 ± 4.4% in nauplius II to III. However, lower metamorphosis percentages of 40 ± 6 and 57 ± 3% were recorded in nauplius V to VI and VI to cypris, respectively. These metamorphosis percentages were normalised to 100% and compared with the metamorphosis success observed in presence of ionic liquids. The effect of 0.031 to 90 µM [C_4MIM][Cl] on survival and metamorphosis are shown in Fig. 4.4A and 4.4B, respectively. Similar survivorship was observed for all the three naupliar larval stages in presence of [C_4MIM][Cl] (Fig. 4.4A). At 67.5 µM, more than 85% mortality was observed in all naupliar stages. Complete killing of naupliar larvae with no survival was observed at 90 µM of

[C_4MIM][Cl]. Metamorphosis of nauplius larvae was strongly inhibited in the presence of [C_4MIM][Cl]. About 25% of the nauplius larvae metamorphosed into the next stage in the presence of 1.125 µM. Metamorphosis of nauplius V and VI larvae was completely prevented in the presence of as low concentration as 9 µM [C_4MIM][Cl].

Effect of [C_{12}MIM][I] on survival and metamorphosis of naupliar larval stages was tested in the range of 0.0035 to 2.25 µM (Fig. 4.4). Around 15% survival was observed at 0.9 µM and complete mortality at 2.25 µM. Inhibition in metamorphosis of nauplius larvae was noticed at these tested concentrations. Nauplius larvae did not metamorphose in the presence of 0.45 µM [C_{12}MIM][I]. In the case of [C_{16}MIM][Cl], tested concentrations were in the range of 0.0035 to 4.5 µM. Complete mortality of nauplius (II, V and VI) was observed in presence of 4.5 µM of [C_{16}MIM][Cl] (Fig. 4.4E). The lethal or effective concentrations of [C_{16}MIM][Cl] were closer to [C_{12}MIM][I] concentrations, but much less than [C_4MIM][Cl]. For example, 50% of the nauplius larvae were killed in the presence of 0.05 µM of [C_{16}MIM][Cl]. The metamorphosis of nauplius larvae was strongly interrupted by [C_{16}MIM][Cl]. None of the nauplius larvae metamorphosed to the next stage in the presence of 0.9 µM of [C_{16}MIM][Cl] (Fig. 4.4F). The results indicate that alkylimidazolium ionic liquids have more profound impact on naupliar metamorphosis than survival. The concentrations required for causing complete mortality and metamorphosis inhibition in nauplius larvae were somewhat similar for [C_{12}MIM][I] and [C_{16}MIM][Cl]. However, substantially higher concentrations of [C_4MIM][Cl] were required for causing complete mortality and metamorphosis inhibition.

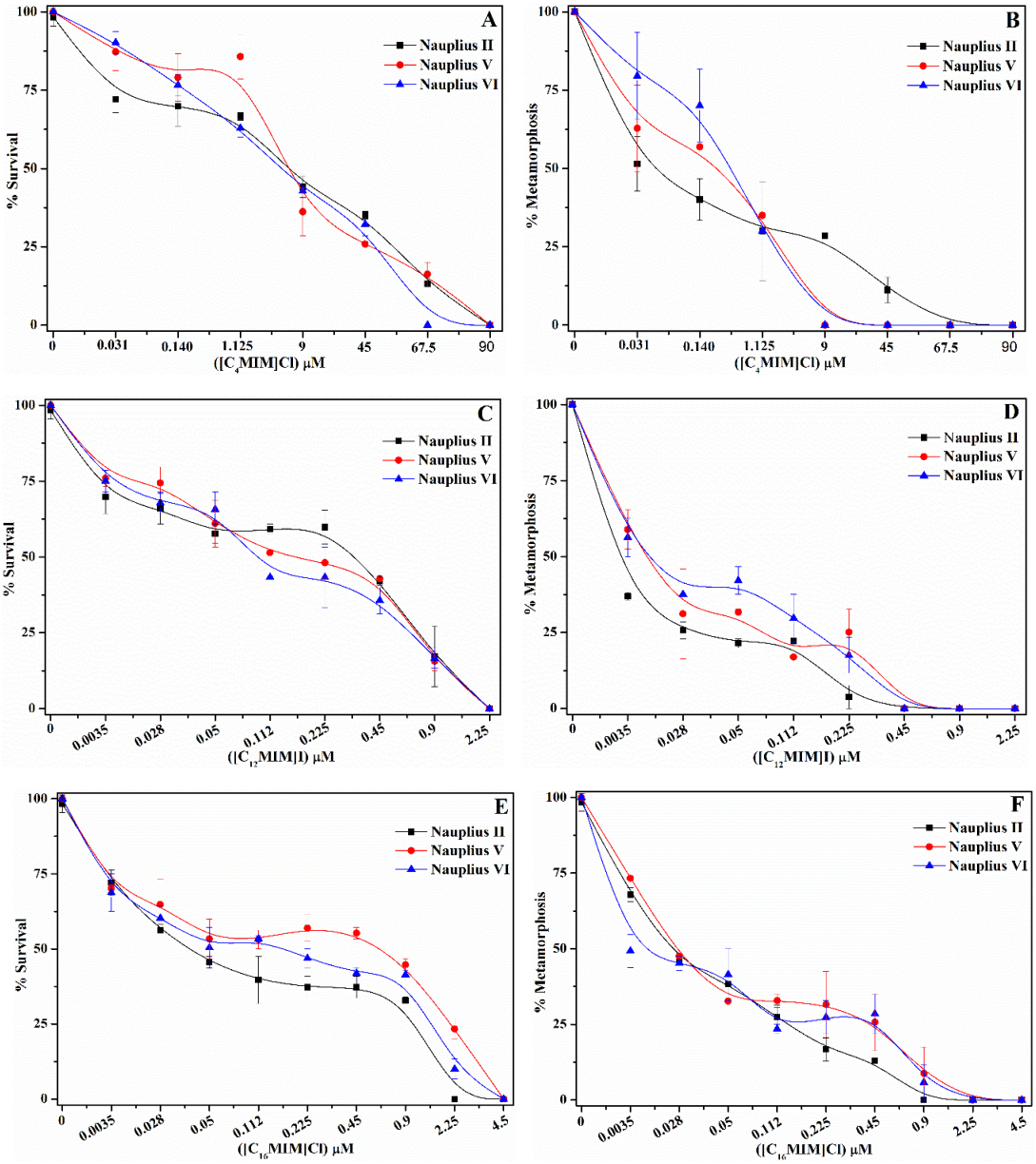


Fig. 4.4. Survival and metamorphosis in various stages of barnacle larvae in presence of ionic liquids. Assay lasted for 24 h with 10^6 cells mL^{-1} *C. lorenzianus* as the feed in the sterile filter seawater. Survival (A) and metamorphosis (B) in presence of [C_4MIM][Cl]. Survival (C) and metamorphosis (D) in presence of [C_{12}MIM][I]. Survival (E) and metamorphosis (F) in presence of [C_{16}MIM][Cl].

4.3.5. Toxicity of ionic liquids to *A. salina* larvae

EC_{50} and LC_{50} concentrations of each of the three ionic liquids on cypris larvae and their 10-fold concentrations were evaluated for determining the toxicity to

Artemia larvae. Healthy *Artemia* larvae were observed in control and in the presence of LC_{50}, EC_{50} and 10X EC_{50} concentrations of ionic liquids (Fig. 4.5A). However, *Artemia* larvae were dead upon exposure to 10X LC_{50} of ionic liquids (Fig. 4.5B).

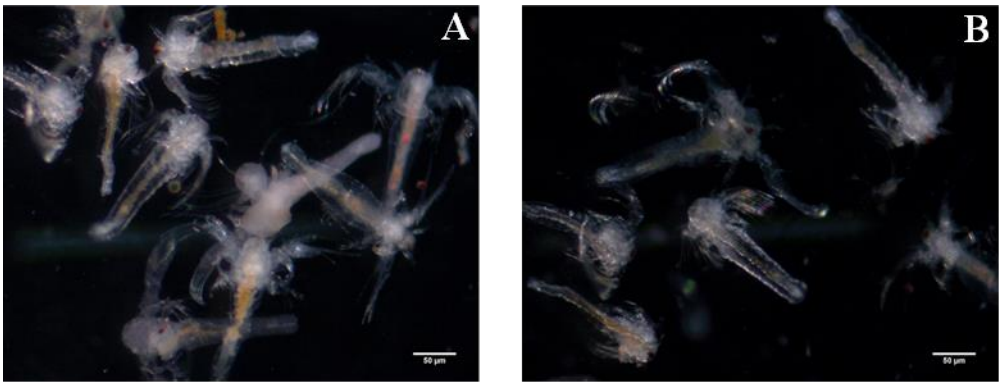
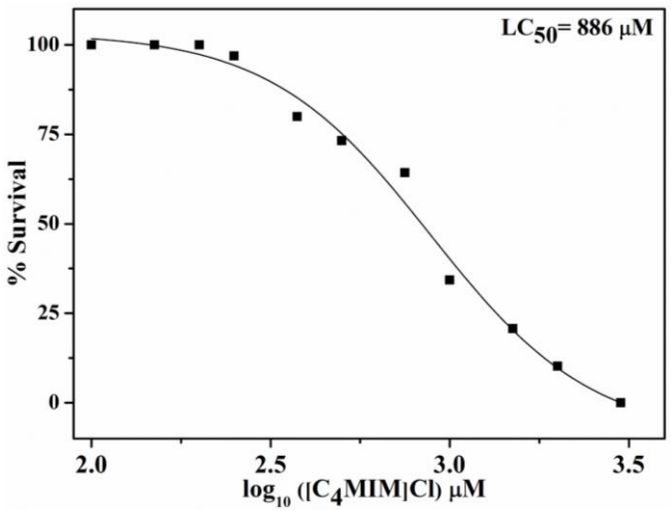


Fig. 4.5. Stereo zoom microscopic images of live and dead *Aretmia salina* larvae. A) Live *A. salina* larvae in seawater controls and in the presence of ionic liquids ([C_4MIM][Cl]: 0.08 to 22.8 µM; [C_{12}MIM][I]: 0.00039 to 0.354 µM and [C_{16}MIM][Cl]: 0.00156 to 0.63 µM). B) Dead *A. salina* larvae in presence of 10X LC_{50} (against cypris larvae) of [C_{12}MIM][I] (3.54 µM) and [C_{16}MIM][Cl] (6.3 µM). Scale bar = 50 µm.

The toxicity data of ionic liquids to *Artemia* larvae was summarized in Table 4.2. [C_4MIM][Cl] had no effect on the mortality of *Artemia* larvae. Survival of larvae at all the four tested concentrations (LC_{50}, 10X of LC_{50}, EC_{50} and 10X of EC_{50}) was similar to that of control. [C_{12}MIM][I] was not toxic to *Artemia* larvae at lower concentrations. Survival of *Artemia* larvae was not impacted by the presence of LC_{50}, EC_{50} and 10X of EC_{50} concentrations. However, a significant mortality ($P < 0.001$) was observed in the presence of 10-fold LC_{50} concentrations [C_{12}MIM][I]. Most of the *Artemia* larvae died upon exposure to 10-fold LC_{50} concentration of [C_{16}MIM][Cl]. But, mortality of *Artemia* larvae was not evident in the presence of lower concentrations (LC_{50}, EC_{50} and 10X of EC_{50}) of [C_{16}MIM][Cl]. From the dose response curves (Fig. 4.6) of brine shrimp tests, the LC_{50} values for [C_4MIM][Cl], [C_{12}MIM][I] and [C_{16}MIM][Cl] were estimated to be 886, 3.365 and 3.355 µM, respectively.

Table 4.2. The toxicity of alkylimidazolium ionic liquids to non-target organism, *Artemia salina*. EC_{50} and LC_{50} of ionic liquids for barnacle cyprid larvae and their 10-fold concentrations on brine shrimp larval mortality were shown. (***: $p < 0.001$, ****: $p < 0.0001$).

Test	Concentration (µM)	% mortality (± SD)
[C_4MIM][Cl]		
EC_{50}	0.08	11.95 (± 3.50)
EC_{50} 10X	0.8	16.34 (± 3.61)
LC_{50}	2.28	17.85 (± 7.57)
LC_{50} 10X	22.8	15.27 (± 8.39)
[C_{12}MIM][I]		
EC_{50}	0.00039	10.83 (± 2.96)
EC_{50} 10X	0.0039	6.84 (± 0.42)
LC_{50}	0.354	16.66 (± 3.36)
LC_{50} 10X	3.54	45.69 (± 10.97) (***)
[C_{16}MIM][Cl]		
EC_{50}	0.00156	10.69 (± 3.16)
EC_{50} 10X	0.0156	10.71 (± 2.52)
LC_{50}	0.63	2.96 (± 3.14)
LC_{50} 10X	6.3	93.33 (5.44) (****)
Control	0	12.28 (± 4.95)



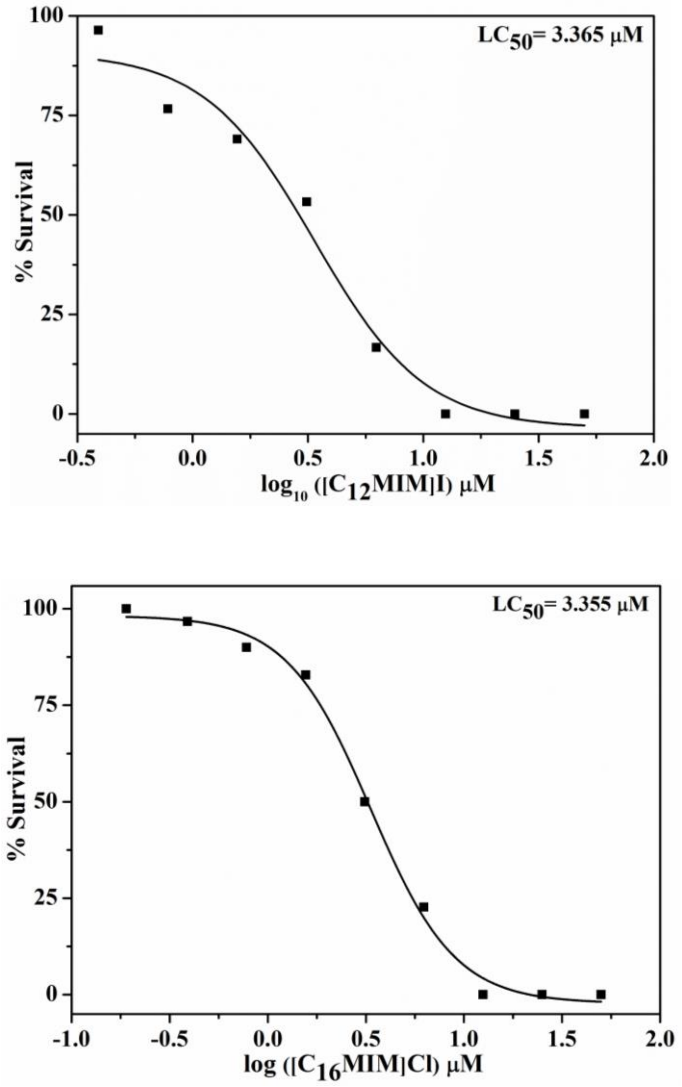


Fig. 4.6. Survival curves for *Artemia salina* larvae in presence of different concentrations of alkylimidazolium ionic liquids. Minimum concentration required to kill 50% of the added larvae was represented as LC_{50}.

4.4. Discussion

Development of novel antifouling compounds for effective prevention of barnacle fouling with relatively less environmental impact is desired due to ban on tributyltin and environmental concerns of copper-based formulations and booster biocides [197, 198]. In this study, alkylimidazolium ionic liquids, an important class of ionic liquids were evaluated for their ability to prevent settlement of larvae of barnacle, a dominant biofouling organism in seawater cooling system of Madras

Atomic Power Station and other maritime activities. Rapid settlement on immersed substratum by barnacle cyprids [199], synchronous rearing of mass cultures, barnacles being dominant and important fouling species around the world [200] makes it as a relevant model for antimacrofouling studies. Different classes of ionic liquids with modifications in cation/anion components have received immense interest for developing antimicrobial or antibiofilm agents [101, 108]. Due to the possibility of synthesizing large number of compounds by various cation and anion combinations, designer ionic liquids can be synthesized based on the desired application [92]. Activity of a particular ionic liquid is dependent on the constituent cation, anion and substituent groups [201]. The alkylimidazolium ionic liquids used in the current study contained halides (chloride or iodide) as anion. These anions (i.e. chloride and iodide) are inherently present in higher concentrations in seawater, in which all the experiments were carried out. Therefore, effect of anion on the biological activity was negligible and not considered. Biological activity of the tested ionic liquids is solely attributed to the cation (imidazolium) and the alkyl substituent group (n: 4, 12 and 16).

Barnacle life cycle includes pelagic larval (i.e. nauplius and cypris) and sessile adult phases. Adult barnacles release nauplius larvae which pass through six naupliar stages and one non-feeding cypris stage which finally settles on a surface to start sessile life. Laboratory assays with cyprid larvae showed dose-dependent mortality upon exposure to all three ionic liquids. LC_{50} values were found to be 0.354 µM and 0.63 µM, respectively, for $[C_{12}MIM][I]$ and $[C_{16}MIM][Cl]$ ionic liquids. Surprisingly, $[C_4MIM][Cl]$ with butyl alkyl side chain was also found to be lethal to cyprid larvae with an LC_{50} value of 2.28 µM. This is in contrast to the fact that alkylimidazolium ionic liquids with butyl (C_4) side chain are mostly non-toxic to bacteria and

microalgae [98, 139]. However, other studies reported that [C_4MIM][Cl] does exhibit toxicity on seedlings, plants and human cell lines albeit at much higher concentrations [202, 203]. This study clearly indicates that barnacle cyprid larvae are highly sensitive for exposure to all three tested ionic liquids. But, the activity of alkylimidazolium ionic liquid compounds with either dodecyl or hexadecyl side chains was much higher than that of butyl side chain.

As compared to mortality, inhibition of cypris settlement was achieved at much lower concentrations. This is in agreement with other potential antifouling agents (i.e. phenolic compounds, butenolide derivatives, 2,5-Diketopiperazines derivatives) that inhibited cypris settlement at lower concentrations than those required for causing mortality in larvae [204, 205, 206]. Among the tested ionic liquids, [C_{12}MIM][I] showed the highest activity on cyprid larvae with lower LC_{50} and EC_{50} values. Whereas, [C_4MIM][Cl] showed least activity among the three ionic liquids. The activity of [C_{16}MIM][Cl] was slightly lower than that of [C_{12}MIM][I]. In case of bacteria and microalgae, it has been reported that the toxicity of alkylimidazolium ionic liquids increases with an increase in alkyl side chain length [139, 207].

Li and co-workers reported enhanced inhibition of cypris settlement by increasing the length of alkyl amide side chain from 6 carbons to 10 carbons in synthetic butenolides [205]. Further increase in side chain length from 12 carbons to 16 carbons showed a decrease in activity. Another study reported that the killing of greater wax moth (*Galleria mellonella*) larvae increased with increase in alkyl side chain from –butyl to –octyl [208]. However, the activity of compounds decreased with further increase in alkyl side chain from –decyl to -hexadecyl. Data obtained in the

present study indicated that the antifouling activity of alkylimidazolium ionic liquids increased substantially with increase in alkyl side chain from -butyl to -dodecyl. However, $[C_{12}MIM][I]$ with -dodecyl side chain was found to be more potent than $[C_{16}MIM][Cl]$ in killing or preventing settlement of barnacle larvae. This shows that parameters other than alkyl chain length are contributing to activity of ionic liquids. This effect appears to be similar to the cut-off-effect referred in previous studies [208]. It is likely that, parameters such as lipophilicity and bioavailability of ionic liquids or some other unknown parameters other than alkyl chain length contribute to the biological activity of ionic liquids on larvae.

The EC_{50} concentrations obtained for all the three tested ionic liquids (14, 0.147 and 0.535 µg L^{-1} for $[C_4MIM][Cl]$, $[C_{12}MIM][I]$ and $[C_{16}MIM][Cl]$ ionic liquids) were much less than the reference value (25 mg L^{-1}) suggested for a potential antifouling agent [189]. This is an indication that the tested compounds are very effective; hence very small concentrations of the tested compounds are sufficient to inhibit the settlement of cyprid larvae. In addition, the therapeutic ratios (T_R) of all three tested compounds were found to be much higher than 15, a requirement set by the US Navy for qualifying effective antifouling agent [209]. T_R is a measure of antifouling efficacy of the compound in relation to its toxicity and calculated from ratio of LC_{50} to EC_{50}. Higher T_R values are desired for developing potential non-toxic antifouling compounds. The calculated T_R value for least active C_4 bearing ionic liquid was 28.5. However, the T_R value was very high at > 900 for most potent C_{12} ionic liquid. These results indicate that, the concentrations required for microfouling (biofilm) control [97] were generally high and those concentrations could very well inhibit macrofouling. Additionally, low concentrations of these compounds could be

applied to specifically inhibit larval settlement and consequent macrofouling at desired locations of power plant cooling water systems.

When three of the ionic liquids were evaluated independently, survival and metamorphosis of different stages (II, V and VI) of nauplius followed similar trend (Fig. 4.4). Nauplius being actively swimming and feeding stages, microalgae feed was provided in the assays. The results in chapter 2 indicated the interaction of alkylimidazolium ionic liquids with algal cell membrane and this could lead to reduction in supplied ionic liquid concentration in the assay. To compensate for this, micromolar concentrations of ionic liquids were used in the assays. The tested ionic liquids had the similar activity pattern on naupliar stages as seen in the case of cypris. The effect of tested ionic liquids on mortality and metamorphosis of nauplius larvae decreased in the following order: $[C_{12}MIM][I]>[C_{16}MIM][Cl]>[C_4MIM][Cl]$. Both $[C_{12}MIM][I]$ and $[C_{16}MIM][Cl]$ were found to be more potent in eliciting mortality and interrupting metamorphosis in nauplius larvae. For $[C_4MIM][Cl]$, higher concentrations were required for killing or interrupting metamorphosis in nauplius larvae. In general, interruption of metamorphosis of nauplius larvae required much lower concentrations of tested compounds than those required for killing the nauplius larvae. At sub lethal concentrations, nauplius remained in the same stage without metamorphosing to the next. Although differential response with concentration of ionic liquids, survival and metamorphosis of nauplius followed similar trend with cyprid survival and settlement with $[C_{12}MIM][I]$ being more effective.

Lower toxicity towards non-specific organisms is an important criterion for potential antifouling agents. *A. salina* is routinely used for evaluating general toxicity of antifouling agents to non-target organisms [210, 211]. Besides their cosmopolitan

distribution in marine environments, small body size, high fecundity, adaptability to varied nutrient resources being a non-selective feeder, survival under varied environmental conditions (i.e. temperature, salinity), ease of enumeration under bright field microscope and commercial availability of cysts makes brine shrimp a common model organism for toxicity testing [212]. The concentrations of ionic liquids at which they inhibited the cyprid settlement (EC_{50}) and survival (LC_{50}) had no effect on mortality of shrimp larvae. Even the 10-fold concentrations of respective EC_{50} values for cypris larvae had no effect on survival of *A. salina* larvae. Significant toxicity to *A. salina* larvae was observed only at 10-fold LC_{50} concentrations of [C_{12}MIM][I] and [C_{16}MIM][Cl]. [C_4MIM][Cl] had no effect on *A. salina* larvae survival even at 10X of LC_{50}. Dose response curves revealed a very high LC_{50} value of 886 µM for [C_4MIM][Cl] against *Artemia* larvae indicating its non-toxicity. The other two ionic liquids ([C_{12}MIM][I] and [C_{16}MIM][Cl]) exhibited a similar LC_{50} value of 3.3 µM. The toxicity of alkylimidazolium ionic liquids on *A. salina* larvae was in corroboration with previous studies that the toxicity increases with an increase alkyl chain length [139, 202, 203, 207]. However, the LC_{50} values are much higher for brine shrimp than the cypris LC_{50} and EC_{50} concentrations.

TBT-experience highlights that environmental fate is an important consideration to be taken into account while contemplating for antifouling compounds [84]. Non-toxic or environmentally benign antifouling compounds should either produce biologically non-active compounds or short-lived ones that do not persist long enough to cause impact on non-target organisms or the environment [213]. The alkyl side chains (e.g., -hexyl and –octyl) of alkylimidazolium ionic liquids are prone to biodegradation, thereby releasing imidazole [214]. But, biodegradation of –butyl

side chain containing imidazolium ionic liquids was not observed. However, several studies showed alkylimidazolium ionic liquids with short alkyl side chains (e.g., -ethyl, -butyl) exhibits significantly lower biological activity as compared to alkylimidazolium ionic liquids with longer alkyl chains like –dodecyl or –hexadecyl groups. Based on activated sludge respiratory inhibition assays and specific studies against bacteria, microalgae, plants, animals, the –butyl side chain containing ionic liquid was termed as "practically harmless" [201]. Moreover, imidazole and its derivatives are the secondary metabolites produced by marine organisms [215], thus naturally available in the marine environment. Hence, alkylimidazolium ionic liquids presented in this study may not pose environmental issue at this reported concentrations. Nevertheless, additional research should address the fate and half-life of alkylimidazolium ionic liquids in the marine environment.

The results suggest that alkylimidazolium ionic liquids are very effective in preventing settlement of barnacle larvae at nanomolar concentrations. High therapeutic ratios of the tested ionic liquids qualify them as potential non-toxic antifouling compounds. The toxicity assay of *Artemia* showed that the effective concentrations of ionic liquids for preventing barnacle larval settlement are benign and non-toxic. The effective antifouling activity coupled with minimal ecotoxicity on non-specific organisms could further future work on preparation of ionic liquid formulations/coatings for biofouling control.

4.5. Conclusions

- The tested alkylimidazolium ionic liquids with -butyl, -dodecyl and -hexadecyl side chains were found to exhibit antimacrofouling potential. The compounds were found to cause mortality in cyprid larvae at micromolar concentrations and

prevent settlement at nanomolar concentrations. The killing and anti-settlement activity of the tested ionic liquids decreased in the following order: $[C_{12}MIM][I]>[C_{16}MIM][Cl]>[C_4MIM][Cl]$.

- Relatively non-toxic alkylimidazolium ionic liquid with -butyl side chain prevented settlement of cypris larvae at concentrations as low as 80 nM.
- High therapeutic ratios (T_r = 28, 907 and 403) of the tested ionic liquids qualify them as potential candidates for developing non-toxic antifouling formulations/paints.
- Alkylimidazolium ionic liquid compounds affected the survivorship and inhibited the metamorphosis of different naupliar larval stages of barnacle. The effect of the tested ionic liquids on killing and metamorphosis of nauplius larvae decreased in the following order: $[C_{12}MIM][I]>[C_{16}MIM][Cl]>[C_4MIM][Cl]$. Interruption of metamorphosis of nauplius larvae required much lower concentrations of tested compounds.
- The activity (killing, metamorphosis inhibition, settlement inhibition) of alkylimidazolium ionic liquids increased with an increase in alkyl chain length from -butyl to -dodecyl group. However, $[C_{12}MIM][I]$ with -dodecyl alky chain was found to be more potent than $[C_{16}MIM][Cl]$ with -hexadecyl alkyl chain in exhibiting antifouling activity.
- The concentrations of all the three ionic liquids which affect the settlement and survival of cypris were not lethal to larvae of non-target organism, *A. salina*.

CHAPTER 5

Alkylimidazolium ionic liquids against natural, multispecies bacterial biofilms: Studies under static and dynamic conditions

5.1. Introduction

Alkylimidazolium ionic liquids have emerged as potential antimicrobial and antibiofilm agents [91]. However, majority of these studies have used monoculture (single species) biofilms and defined culture conditions for efficacy evaluation [98, 110]. But, the majority of biofilms formed in industrial and medical settings are polymicrobial in nature [3, 42]. Heterotrophic biofilms developed in natural waters often constitutes multiple genera from Gram-positive and Gram-negative bacteria. The polymicrobial biofilms (or multispecies biofilms), harbor more structural complexity than monoculture biofilms and exhibits higher tolerance to antimicrobials [216]. Multispecies biofilms possess higher microbial diversity, complex extracellular polymeric substances matrix, distinct organization and metabolism [217, 218, 219]. Most of the previous work on antimicrobial ionic liquids was performed using defined cultures and under static conditions. Therefore, determining the efficacy of antimicrobial ionic liquids on multispecies biofilms under dynamic conditions is essential for contemplating prospective applications. The efficacy of alkylimidazolium ionic liquids on biofilm formation in natural waters (freshwater and seawater) was determined in laboratory assays. Subsequently, the efficacy of potential antimicrobial ionic liquid on biofilm formation in natural waters was determined under dynamic flow condition using laboratory scale re-circulating system.

5.2. Materials and methods

5.2.1. Alkylimidazolium ionic liquids

The chemical structures of the ionic liquids tested in this study are previously provided (Fig. 2.1). A 100 mM stock solution of each of these ionic liquids were prepared in sterile, ultrapure water and stored at room temperature until further use.

5.2.2. Characterisation of freshwater and seawater

The effect of ionic liquids on prevention of multispecies biofilms was studied in freshwater and seawater, respectively, used in the fast breeder test reactor (FBTR) and Madras Atomic Power Station (MAPS). Freshwater was collected from the open freshwater reservoir, a water source for the FBTR cooling water system [118]. Seawater was collected from the intake well of the MAPS cooling water system [124]. Water collected from the FBTR and MAPS cooling water systems were analyzed for determining total viable counts (TVC) and general water quality parameters. Freshwater and seawater samples were spread-plated on nutrient agar and Zobell marine agar, respectively, for determining TVC. Ammonium and phosphorus were determined spectrophotometrically according to standard methods using indophenol blue and ascorbic acid reagents, respectively [220]. Monovalent and divalent metal cations were estimated by ICP-AES (Horiba, Japan) using respective reference standards. Dissolved oxygen (DO) was determined using DO meter (Hach, USA). Turbidity, salinity and pH were measured using standard laboratory instruments.

5.2.3. Optimisation of biofilm formation from natural waters

Formation of multispecies biofilms in natural waters was determined under different nutrient amendment conditions. Biofilm formation was studied in (i) plain water, (ii) spiking with 0.2% (w/v) glucose or 0.2% (w/v) acetate and (iii) spiking with 0.2% (w/v) glucose or 0.2% (w/v) acetate along with 10 mg/l inorganic phosphate. Water without and with added nutrients was dispensed as 200 µl aliquots in 96-well sterile, flat bottom, polystyrene microtiter plates (Tarsons, India). The microtiter plates were incubated at 30 ºC and 100 rpm for 72 h in a temperature controlled orbital shaker. At the end of incubation, the wells were processed for

quantifying biofilm by crystal violet staining [97]. Briefly, liquid was discarded from wells and rinsed with ultrapure water/ filter sterile seawater. Subsequently, 200 µl of 0.1% crystal violet was transferred to each well and incubated for 10 min. The CV solution was discarded and the wells were rinsed thoroughly with ultrapure water. After drying, biofilm-bound CV was eluted into 200 µl 33% glacial acetic acid. Absorbance of CV was measured at 570 nm using multimode reader (BioTek®, United States). Appropriate dilutions were made for measuring when absorbance readings were above 2. After determining optimum nutrient amendment, biofilm growth was determined at different incubation periods of 24, 48 and 72 h.

5.2.4. MIC and MBC of ionic liquids against natural waters

A two-fold serial dilution method was used for preparing different concentration of ionic liquids in nutrient amended freshwater and seawater. Ionic liquid concentrations were prepared in the range of 500 to 7.8 µM for [C_4MIM][Cl], 500 to 0.12 µM for [C_{12}MIM][I] and 500 to 0.06 µM for [C_{16}MIM][Cl] in nutrient amended water. The ionic liquid solutions were transferred as 200 µl aliquots into microtiter plate and incubated as mentioned above. Control wells received only the water sample without ionic liquids. After incubation, growth in the wells was determined by measuring absorbance at 600 nm using a microplate reader (BioTek®, United States). The lowest concentration of ionic liquid that prevented the growth (measured as absorbance at 600 nm) was represented as minimum inhibitory concentration (MIC). After overnight incubation, the suspensions from selected microtiter wells were plated on nutrient agar plates and incubated at 30 ºC for 48 h. The lowest concentration at which no colonies appeared was considered as minimum bactericidal concentration (MBC).

5.2.5. Activity of ionic liquids against natural multispecies biofilms

Alkylimidazolium ionic liquids were prepared in the range of 500 to 15.6 µM for [C_4MIM][Cl], 500 to 0.25 µM for [C_{12}MIM][I] and 250 to 0.06 µM for [C_{16}MIM][Cl] by two-fold dilution method in nutrient amended natural water (freshwater and seawater). A 200 µL of ionic liquid solutions were aliquoted into microtiter plate in five replicates. Control wells did not receive any ionic liquids. Plates were incubated as mentioned previously. After the incubation, biofilm formation was quantified by CV staining and presented as % inhibition in the presence of ionic liquids.

To visualise biofilm formation, glass slides were immersed in ionic liquid containing waters in 6 well polystyrene plates. Sterile glass slides (2.5 X 2.5 cm) were immersed in 4 mL of selected ionic liquids solutions for 72 h. After the incubation, the slides were washed with phosphate buffered saline or sterile seawater to remove the loosely attached cells. Subsequently, the slides were stained with Syto 9 and visualised under epi-florescence microscope through FITC filter.

Effect of ionic liquids on established biofilms was estimated by challenging the pre-formed biofilms (72 h) with different concentrations of ionic liquids. 72 h biofilms were cultivated as explained previously from nutrient spiked natural freshwater and seawater samples, without adding ionic liquids. In sterile PBS or filter sterile seawater, different concentrations of ionic liquids were prepared in the range of 1000 to 15.6 µM each for [C_4MIM][Cl], [C_{12}MIM][I] and [C_{16}MIM][Cl]. These suspensions were added to the microtiter wells in five replicates each for every concentration. PBS alone or sterile seawater was added for the controls. Plates were incubated at 30 ºC for 24 h at 100 RPM in a temperature controlled orbital shaker.

After the 24 h challenge period, biofilm eradication was estimated by CV assay. Biofilm remaining in ionic liquid challenged wells was compared with the control wells which received PBS or sterile seawater alone.

5.2.6. Antibiofilm activity of ionic liquid in dynamic recirculation system

Laboratory scale, dynamic recirculation system was operated with a module which has provision for holding coupons and glass slides. Fibre reinforced plastic (FRP) coupons in the size of 8.5 X 5.5 cm and glass slides with a size of 2.5 X 5.5 cm were inserted in the module. Module was connected to a reservoir through silicon tubing. Reservoir was filled with 1500 mL natural freshwater or seawater spiked with/ without ionic liquid. The potent ionic liquid, $[C_{16}MIM][Cl]$ which showed the highest activity against natural biofilms in multiwell plate biofilm assays was selected for activity evaluation in dynamic recirculation system. Water from the reservoir was pumped through the coupon module with the aid of peristaltic pump at a flow rate of 300 mL per minute. Water flows through the coupons and reaches back reservoir through outlet port. Two modules were operated simultaneously with one serving as the control and the other spiked with ionic liquid as the treatment.

With natural freshwater, control reservoir received water alone and treatment reservoir received 100 µM $[C_{16}MIM][Cl]$. In the case of natural seawater, 250 µM $[C_{16}MIM][Cl]$ was tested. System was operated for a period of 12 d under the similar flow conditions at room temperature. At the end of operation, biofilm from FRP coupons and glass slides were processed for biofilm quantification and visualisation. Biofilm developed on FRP coupons from freshwater or seawater module was scraped into sterile PBS or sterile seawater using a nylon brush. The collected biomass was concentrated and net weight was estimated. Biofilm biomass from control and ionic

liquid treated coupons was quantified through XTT and fluorescein diacetate (FDA) assays as previously described [166, 221]. Briefly, freshly prepared 0.5 mg/ mL XTT in PBS or sterile seawater was incubated with biofilm biomass for 2 h at 37 ºC. At the end of incubation, biomass was pelleted and reduced XTT (as purple formazan) as intracellular crystals was extracted using DMSO. After 10 min of extraction, biomass was separated out through centrifugation and the supernatant was measured for absorbance at 510 nm. Similarly, a final working concentration of 0.25 mg/ mL FDA was prepared in saline or sterile seawater and incubated with biofilm biomass at 37 ºC. At the end of 1 h, biomass was pelleted out and the supernatant containing fluorescein was estimated through absorbance at 490 nm. The obtained absorbance for both formazan and fluorescein were normalised to the surface area of the coupon from which the biofilm was obtained. Slides taken out from the module were stained with Syto 9 for 15 min and directly imaged under fluorescence microscope for biofilm visualisation.

5.2.7. Statistical analysis

Obtained data from various replicates of the experiments were processed and presented as mean ± standard deviation (SD). Statistical significance between control and treatment samples was determined from Student's t-test and were considered to be significant at P-values <0.05, <0.01, and <0.001.

5.3. Results

5.3.1. Characterisation of freshwater and seawater

The water quality of natural waters (freshwater and seawater) used for various biofilm assays is shown in Table 5.1. The ammonium and inorganic phosphate concentrations were low at ~1 mg/l. The seawater was saline (35 ppt) and had higher

concentration of metal cations such as sodium, potassium, calcium and magnesium. The salinity and concentration of metal cations are low in freshwater. The microbial load was determined to be 200 to 300 cfu/ mL and 30,000 to 40,000 cfu/ mL, in freshwater and seawater, respectively.

Table 5.1. Characterisation of freshwater and seawater used for the experiments.

Parameter (mg/L)	Seawater	Freshwater
Ammonium- N	1.03	-
Phosphorus- P	0.89	0.62
Calcium	359	76
Magnesium	1189	50
Sulphur	11.5	2.0
Sodium	7455.2	293
Potassium	353	60
Strontium	6	-
Dissolved oxygen	7.2 ± 0.2	6.8 ± 0.3
Turbidity	4.53 ± 2.3 NTU	3.8 ± 1.1 NTU
Salinity	35 g L^{-1}	0.6 g L^{-1}
pH	8.1 ± 0.1	7.8 ± 0.1
Microbial load	3-4 x10^4 CFU ml^{-1}	2-3 x10^2 CFU ml^{-1}

5.3.2. Biofilm formation in natural waters

Fig. 5.1 describes the results of biofilm formation in natural waters under different nutrient amendment conditions. Biofilm formation was found to be poor in plain natural waters after 3 days of incubation (Fig. 5.1A and 5.1C). Hence, the natural waters were amended with carbon (glucose or acetate), nitrogen and phosphorus sources for improving biofilm formation. Amendment of natural waters with glucose or acetate has marginally increased biofilm formation. But, the biofilm yield was higher in acetate amended condition. Addition of nitrogen and phosphorus along with carbon source improved the biofilm formation significantly. Maximum biofilm was formed when the natural waters were amended with acetate, ammonia and phosphorus. The biofilm formation was found to be much higher in seawater than that

of freshwater under all the nutrient amendment conditions. Moreover, independent experiments revealed that, inorganic phosphate is a critical nutrient for increased biofilm formation in both freshwater and seawater. Time course experiment revealed maximum biofilm formation within 72 h incubation (Fig. 5.2B and 5.2D). Based on these results, amendment of natural waters with nutrients (acetate and phosphorus) and 72 h incubation were chosen for further experiments on determining the efficacy of alkylimidazolium ionic liquids.

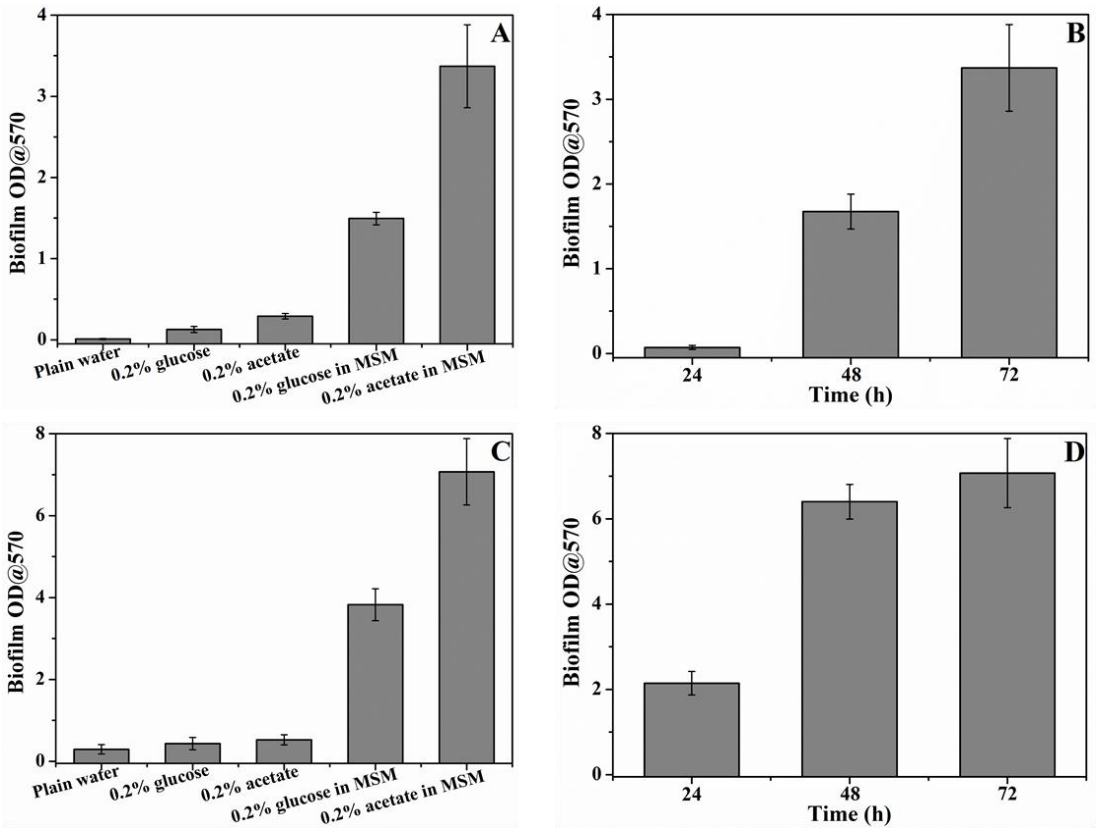


Fig. 5.1. Standardisation of biofilm formation in natural waters. A & B) Freshwater. C & D) Seawater. A & C) Biofilm formation under different medium conditions. B & D) Biofilm formation at different incubation times.

5.3.3. Growth inhibition and bactericidal concentrations of ionic liquids

Freshly collected water samples (freshwater and seawater) were spiked with nutrients and three different ionic liquids in the concentration range mentioned previously. Growth inhibition was represented as minimum inhibitory concentrations (MIC), a concentration which completely inhibits the visible growth. The concentrations at which no bacterial colonies were appeared on agar media plates is termed as minimum bactericidal concentrations (MBC).

Table 5.2: MIC and MBC values for different ionic liquids to the freshwater and seawater microbes after 72 h incubation in 0.2% acetate containing minimal salts media.

Microbial source	[(C_4MIM)]Cl (µM)		[(C_{12}MIM)]I (µM)		[(C_{16}MIM)]Cl (µM)	
	MIC	MBC	MIC	MBC	MIC	MBC
Freshwater	>500	>500	32	125	8	16
Seawater	>500	>500	250	500	125	125

The MIC and MBC values were determined for the three alkylimidazolium ionic liquids in natural waters. The data in Table 5.2 indicated that complete inhibition in microbial growth was not observed even with 500 µM of [C_4MIM][Cl]. The MIC and MBC values for [C_4MIM][Cl] were higher than 500 µM. For [C_{12}MIM][I], inhibition in bacterial growth was achieved with 32 µM and 250 µM, respectively, in freshwater and seawater. The MBC values were also different at 125 and 500 µM in freshwater and seawater, respectively. Alkylimidazolium ionic liquid with -hexadecyl alkyl group [C_{16}MIM][Cl] elicited growth inhibition at much lower concentrations. The MIC values of [C_{16}MIM][Cl] were at 8 and 125 µM in freshwater and seawater, respectively. The MBC values were also lower at 16 and 125 µM in freshwater and seawater. Growth inhibition in seawater required relatively higher concentration of ionic liquid than freshwater.

5.3.4. Effect of ionic liquids on multispecies biofilms formation in microtiter plates

Fig. 5.2 to 5.5 describes biofilm formation in the presence of different concentrations of three different alkylimidazolium ionic liquids in freshwater and seawater. Similar to growth inhibition, [C_4MIM][Cl] did not show inhibitory action on biofilm formation at the maximum tested concentration of 500 µM in freshwater (Fig. 5.2A) as well as seawater (Fig. 5.4A). Microscopic evidence also shows that [C_4MIM][Cl] had no impact on biofilm formation in natural waters (Fig. 5.3 and Fig. 5.5). Biofilm formation was affected only in the presence alkylimidazolium ionic liquids containing –dodecyl or –hexadecyl alkyl groups. Complete inhibition in biofilm formation in freshwater and seawater was achieved by 32 µM and 250 µM of [C_{12}MIM][I], respectively. The quantitative data was also supported by the microscopic images with negligible biofilm growth in freshwater and seawater at the respective concentrations of [C_{12}MIM][I] (Fig. 5.3 and Fig. 5.5). The ionic liquid with –hexadecyl alkyl group showed the best antibiofilm activity among the three tested compounds. Biofilm formation in freshwater was retarded with 8 µM [C_{12}MIM][I]. But, complete inhibition in biofilm formation in seawater required 125 µM [C_{12}MIM][I]. These results were corroborated by microscopic images showing negligible attachment and growth in freshwater (Fig. 5.3) and seawater (Fig. 5.5). The data clearly suggest that [C_4MIM][Cl] is ineffective for preventing biofilm formation. Whereas, alkylimidazolium ionic liquids with –dodecyl and –hexadecyl alkyl groups were able to retard biofilm formation in freshwater and seawater in concentration dependent manner. The compound with longest alkyl group ([C_{16}MIM][Cl]) showed highest antibiofilm activity. Whereas, [C_{12}MIM][I] showed the intermediate activity,

requiring relatively higher concentrations for achieving similar level of antibiofilm activity.

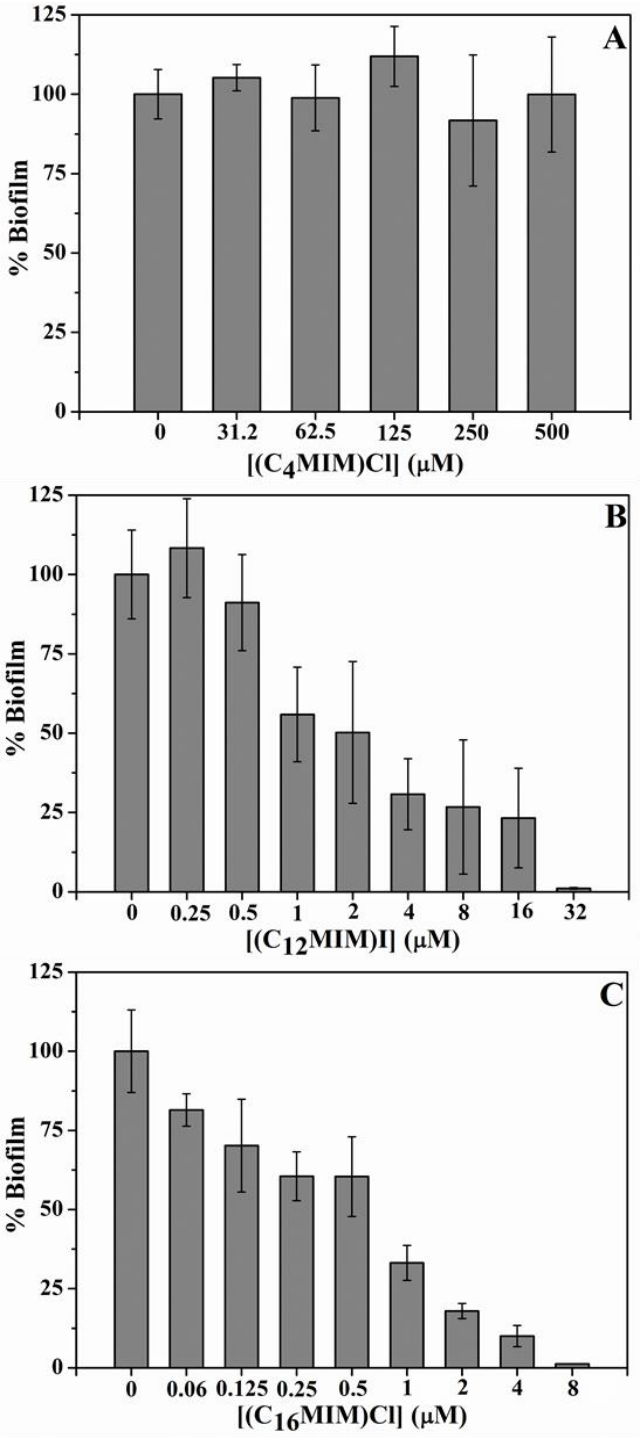


Fig. 5.2. Biofilm formation from freshwater in presence of ionic liquids. Nutrient amended freshwater in microtiter plates in the presence of different concentrations of alkylimidazolium ionic liquids and biofilm was quantified by CV assay. A) $[C_4MIM]Cl$. B) $[C_{12}MIM]I$ and C) $[C_{16}MIM]Cl$.

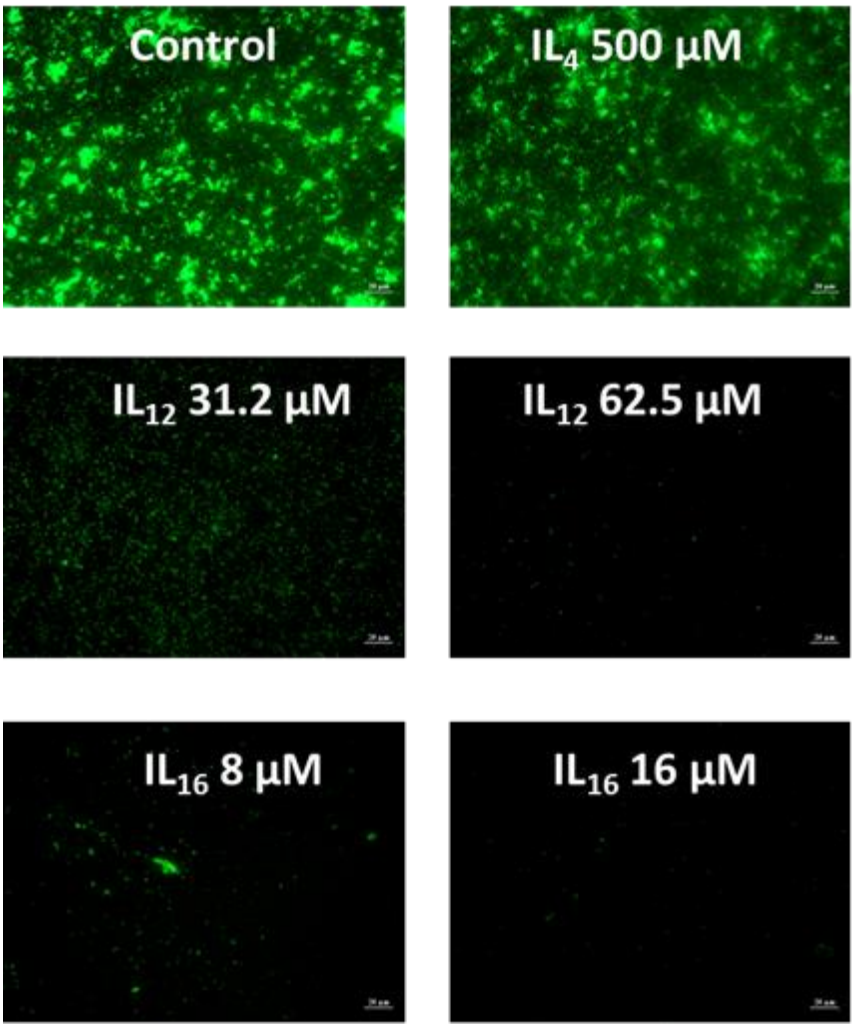


Fig. 5.3. Fluorescence microscopic images of biofilms formed on glass slides in nutrient amended freshwater in the presence of different alkylimidazolium ionic liquids. The biofilms were stained using Syto 9 for visualization.

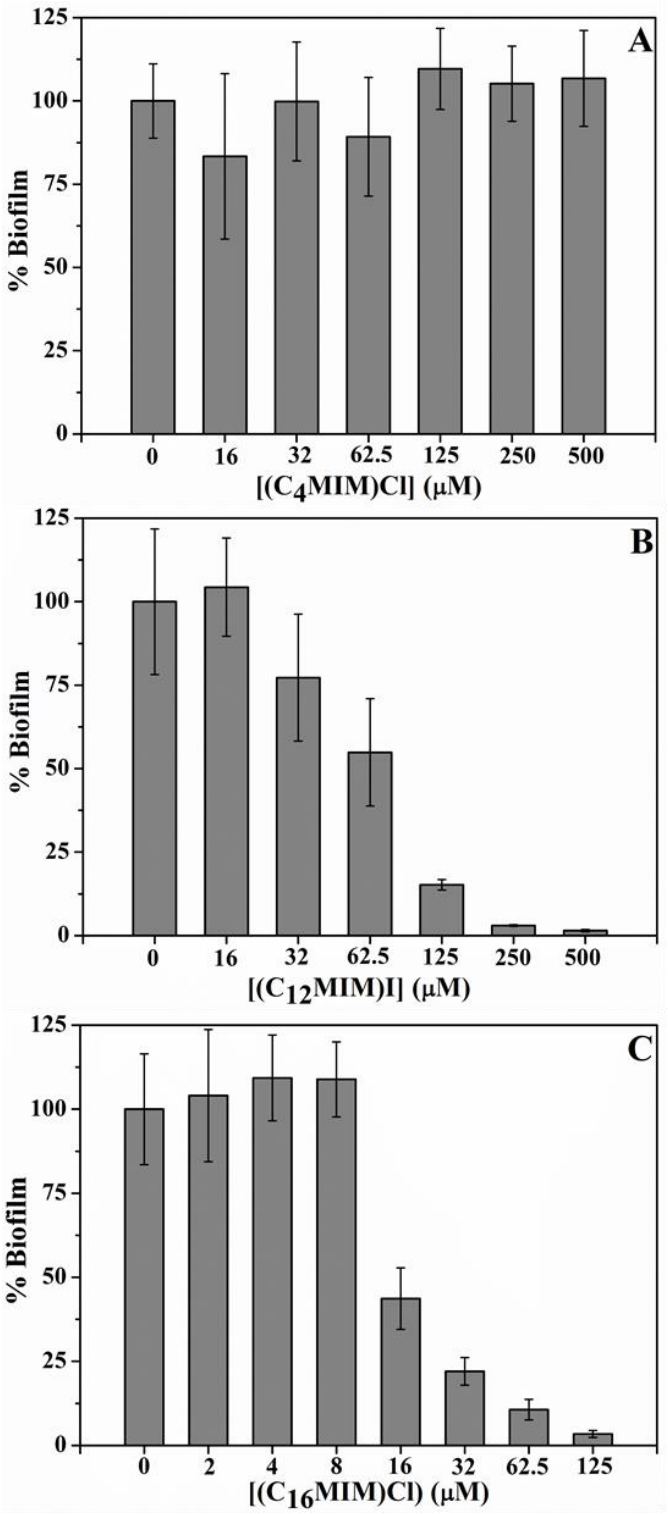


Fig. 5.4. Biofilm formation from seawater in presence of ionic liquids. Nutrient amended seawater in microtiter plates in the presence of different concentrations of alkylimidazolium ionic liquids and biofilm was quantified by CV assay. A) $[C_4MIM]Cl$. B) $[C_{12}MIM]I$ and C) $[C_{16}MIM]Cl$.

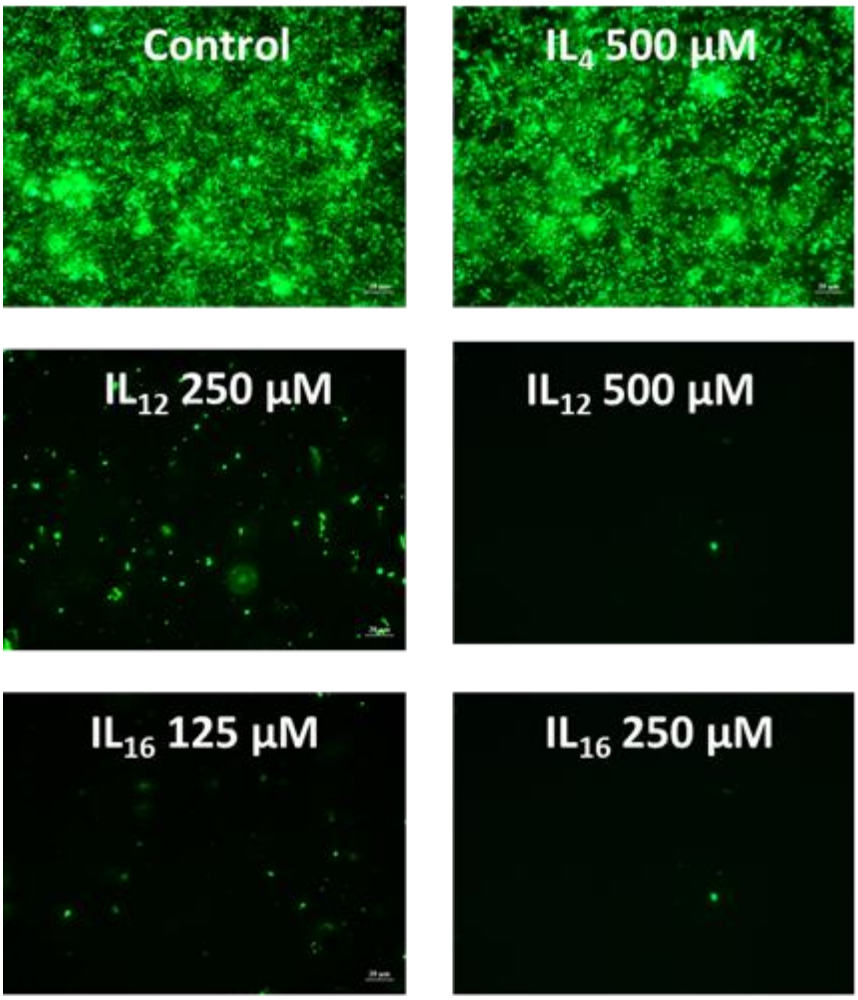


Fig. 5.5. Fluorescence microscopic images of biofilms formed on glass slides in nutrient amended seawater in the presence of different alkylimidazolium ionic liquids. The biofilms were stained with Syto 9 for visualization.

Fig. 5.6 describes the effect of alkylimidazolium ionic liquids on the biofilms already developed (pre-formed biofilms) in freshwater and seawater. Among the three tested compounds, only [C_{16}MIM][Cl] was able to dislodge the biofilms developed in freshwater. The disassembly of biofilms was also found to be concentration dependent. Maximum dispersal activity was achieved using 125 µM and higher. Other ionic liquids [C_4MIM][Cl] and [C_{12}MIM][I] had no major dispersal activity on pre-formed biofilms up to a tested concentration of 1000 µM. Similarly, [C_4MIM][Cl] and [C_{12}MIM][I] had no major dispersal activity on biofilms formed in seawater. Higher

biofilm dispersal activity was shown by [C_{16}MIM][Cl] at 250 µM. Dispersal of biofilms formed in seawater required relatively higher concentrations of [C_{16}MIM][Cl] than freshwater biofilms. Data clearly shows that an ionic liquid with long alkyl group ([C_{16}MIM][Cl]) is suitable for achieving biofilm disassembly/dispersal.

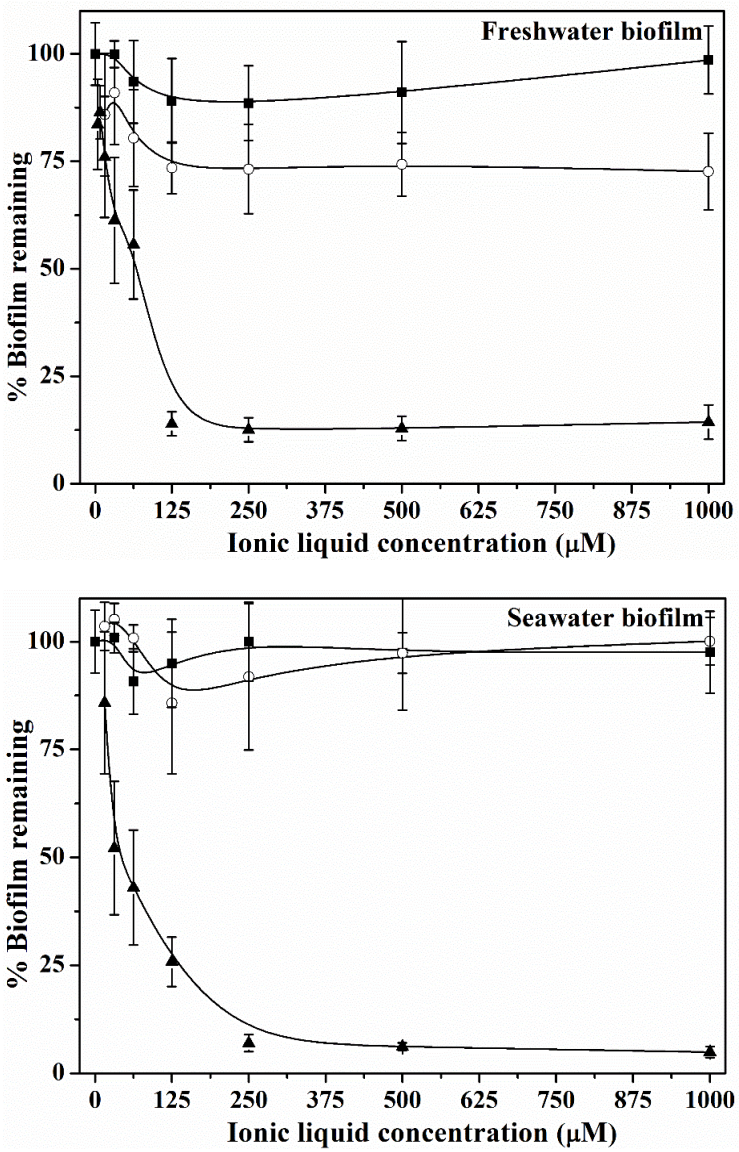


Fig. 5.6. Biofilm dispersal activity of ionic liquids. On pre-formed biofilms developed in nutrient amended freshwater and seawater, different concentrations of ionic liquids were challenged and dislodgement was estimated. ■- [C_4MIM]Cl, ○- [C_{12}MIM]I, ▲- [C_{16}MIM]Cl).

5.3.5. Effect of ionic liquids on multispecies biofilms formation in a dynamic recirculation system

The potent ionic liquid [C_{16}MIM][Cl], which showed the highest activity against natural biofilms in microtiter plates was further evaluated in a model re-circulating system. Fig. 5.7 illustrates the schematic diagram of the model flow cell with provision for placing coupons and glass slides was used for evaluating antibiofilm efficacy of ionic liquid.

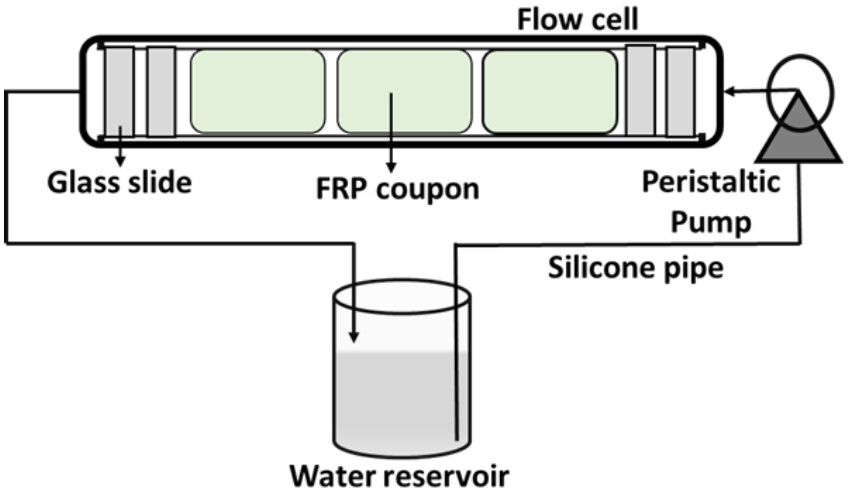


Fig. 5.7. Schematic diagram of flow cell used for biofilm development in natural waters under dynamic conditions.

The flow cell was operated for 12 days in re-circulating mode for determining biofilm formation. Complete inhibition in biofilm growth was achieved at 100 µM [C_{16}MIM][Cl] in freshwater conditions. Both quantitative data and microscopic observations showed near complete retardation of biofilm formation in the presence of ionic liquid (Fig. 5.8). The biomass accumulated on the FRP coupons was found to be around 1.69 and 0.33 mg/ cm^2 in control and [C_{16}MIM][Cl] spiked freshwater, respectively. The negligible biofilm growth in the ionic liquid spiked freshwater was

also indicated by lower MTT reduction to formazan and FDA hydrolysis to fluorescein by the biomass accumulated on FRP coupons.

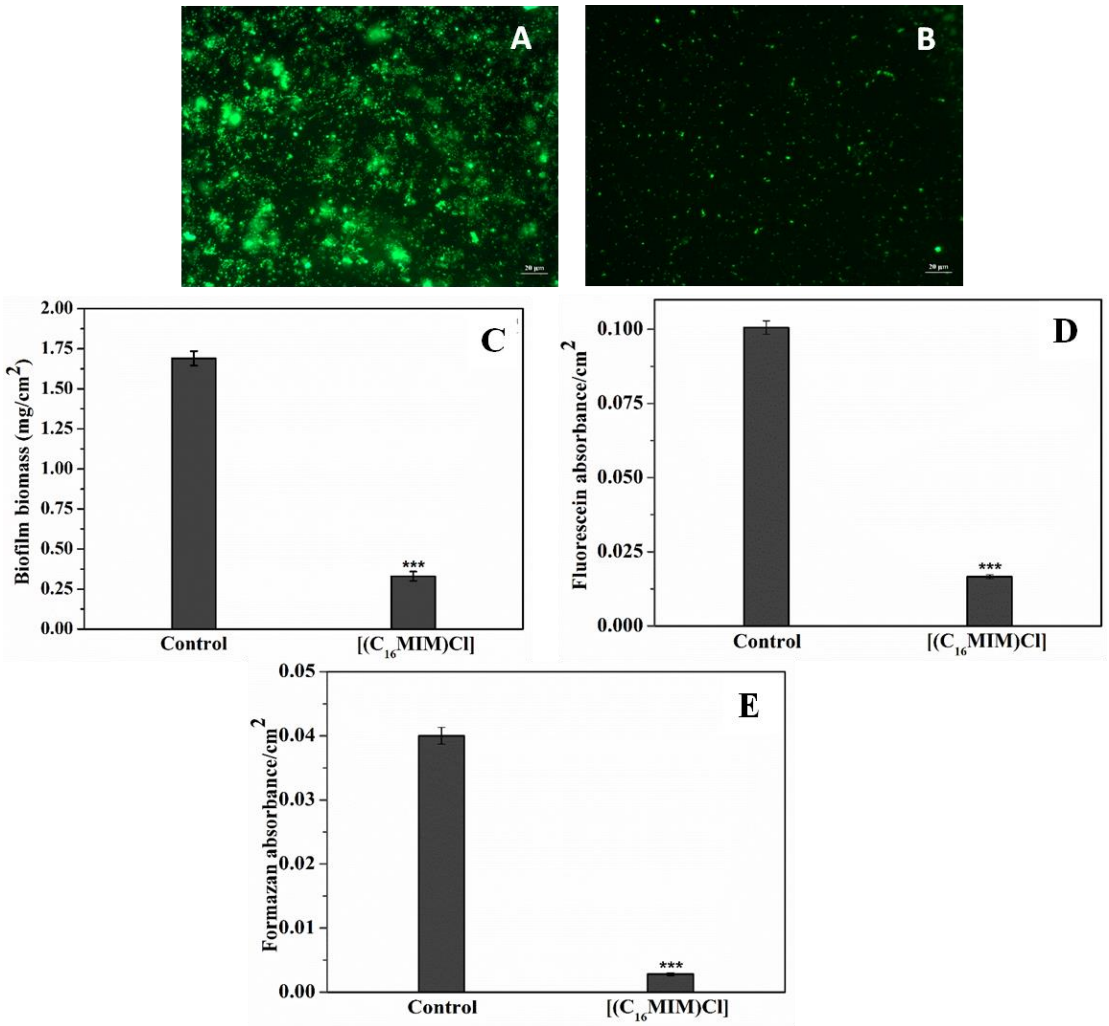


Fig. 5.8. Natural freshwater biofilm formation in flow cell in presence of ionic liquid. Fluorescence microscopic images of freshwater biofilms formed on glass slides exposed to natural waters (A) and natural waters spiked with ionic liquid (B) in flow cell reactor. Biofilm formed on FRP coupons was quantified by biofilm mass (C), FDA hydrolysis yielding fluorescein (D) and XTT reduction to formazan (E).

Similar level of biofilm inhibition was achieved in seawater operated flow cell where the module was operated with 250 µM spiked seawater. Syto 9 staining of biofilms developed on glass slides kept in control flow cell showed uniform biofilm

development. But, in [C_{16}MIM][Cl] spiked flow cell, biofilm formation was severely retarded (Fig. 5.9). The microscopic visualization results were corroborated by quantitative data on the biofilm biomass, FDA hydrolysis to fluorescein and XTT reduction to formazan by the biofilm developed on FRP coupons in control and treated flow cells. Overall, the data suggest that [C_{16}MIM][Cl] can cause near complete retardation in formation of multispecies biofilms under natural water conditions.

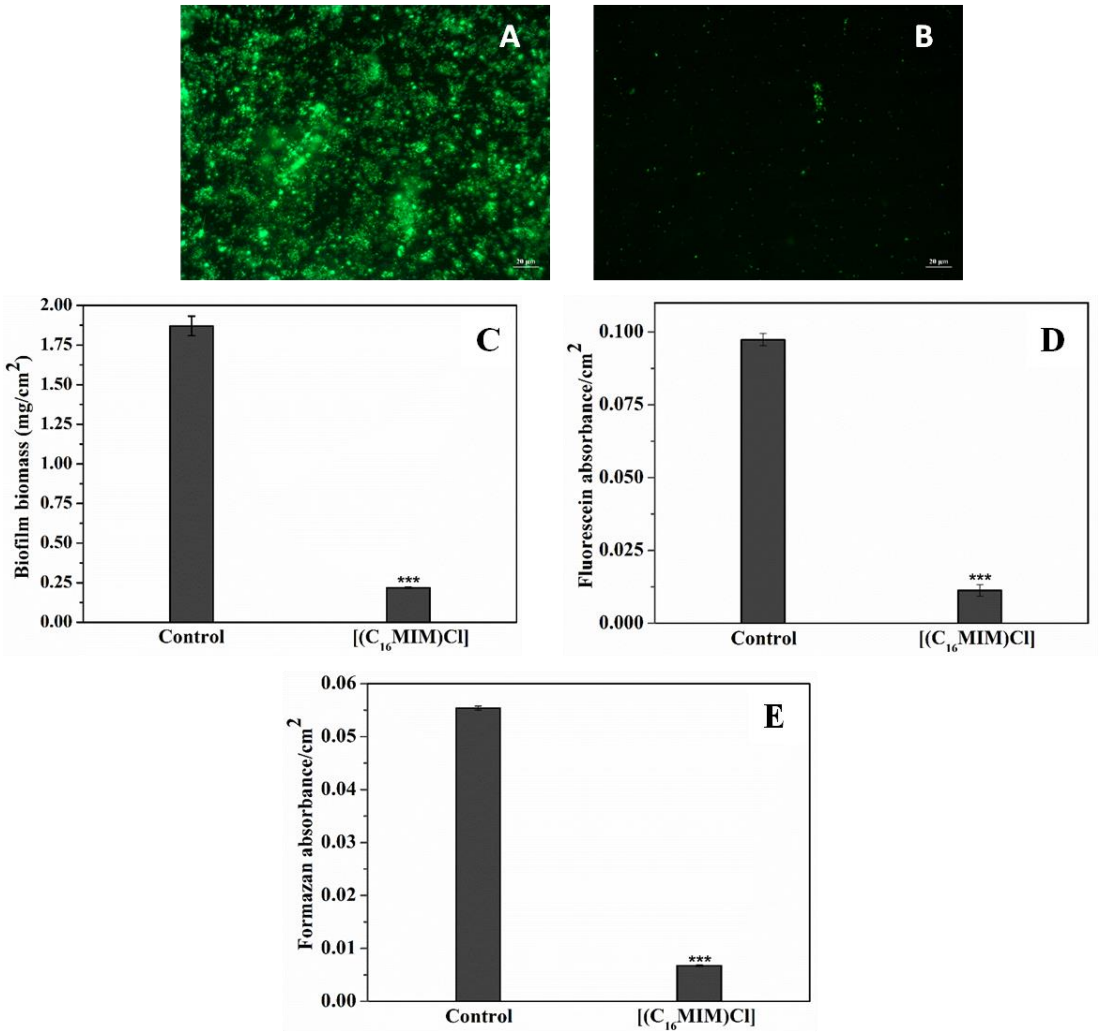


Fig. 5.9. Natural seawater biofilm formation in flow cell in presence of ionic liquid. Fluorescence microscopic images of seawater biofilms formed on glass slides exposed to natural waters (A) and natural waters spiked with ionic liquid (B) in flow cell reactor. Biofilm formed on FRP coupons was quantified by biofilm mass (C), FDA hydrolysis yielding fluorescein (D) and XTT reduction to formazan (E).

5.4. Discussion

In the recent past, ionic liquids have gained prominence for biomedical and pharmaceutical applications [201]. The antimicrobial activities are being increasingly reported for diverse classes of ionic liquid compounds [98, 222, 223]. Some of the ionic liquid compounds were found to be effective for preventing bacterial and fungal biofilms suggesting prospective applications in controlling biofilms in medical and industrial settings [107]. Although these studies were carried out mainly using monoculture biofilms, they shed light on structure-activity relation, broad-spectrum activity on several Gram-positive and Gram-negative bacteria and mechanism of action [97, 110]. But, the biofilms formed in environmental, industrial and medical settings constitute more than one organism (polymicrobial) and complex extracellular matrix [4]. Biofilms, in particular the polymicrobial biofilms confer higher tolerance and resistance to antimicrobials due to diverse organisms, interspecies interactions, complex biomolecular matrix [216]. Although single culture antibiofilm studies have yielded valuable information, generation of quantitative data on efficacy of ionic liquids on multispecies biofilms is desired for contemplating prospective applications in biofilm control. This study was aimed at bridging that knowledge gap, thereby focused on the efficacy of alkylimidazolium ionic liquids on multispecies biofilms formed in natural waters.

For developing multispecies biofilms, aliquots of natural waters (freshwater and seawater – a source for cooling water systems for electric power stations) were transferred to the wells of microtiter plates and incubated for up to 72 h. Biofilm formation under these conditions was limited due to the experimental limitations like static condition and limited incubation period. Extended incubation was not possible

due to evaporation losses leading to errors in quantification. Thus, nutrient amendment of natural waters was considered for increasing the biofilm formation. Among the carbon sources tested, acetate was found to be suitable for giving good biofilm yields within 3 d incubation. Additionally, independent experiments showed that phosphorus is key limiting factor (data not shown) and spiking of natural waters with inorganic phosphorus and acetate yield higher biofilm adequate for efficacy experiments (Fig. 5.1). Interestingly, biofilm formation in seawater was nearly 2-fold higher as compared to freshwater (Fig. 5.1) and related to high microbial load and divalent cations (Table 5.1). It is well known that divalent cations such as calcium and magnesium contribute to enhanced adhesion, extracellular matrix production and biofilm formation in marine bacteria [141].

The inhibitory and bactericidal concentrations followed similar trend for microbial growth in natural freshwater and seawater, although a distinction was noticed between MIC and MBC values (Table 5.2). Alkylimidazolium ionic liquid with -butyl side chain containing [(C_4MIM)]Cl ionic liquid was not inhibitory to the microbial growth even at the highest tested concentration (500 µM). This was in agreement with previous studies that alkylimidazolium ionic liquids with short alkyl side chains from C_3 to C_5 were not suitable for inhibiting the microbial growth [98]. The alkylimidazolium ionic liquid with –dodecyl group ([(C_{12}MIM)]I) was inhibitory and bactericidal to microbial growth in both freshwater and seawater. However, preventing microbial growth in seawater required higher concentrations and related to higher microbial load. In the case of [C_{16}MIM][Cl], much lower concentrations were enough to achieve inhibitory action on microbial growth in both freshwater and seawater. These data was in agreement with previously established observations that

the antimicrobial activity is related to an increase in alkyl chain length for alkylimidazolium ionic liquids [98, 223].

The efficacy of alkylimidazolium ionic liquids to prevent multispecies biofilm in natural waters was dependent on alkyl side chain length, concentration and type of water (freshwater or seawater). Effect of alkyl side chain length in deciphering antibiofilm activity against single culture biofilms of *S. aureus*, *P. aeruginosa*, *E. coli*, *K. aerogenes* indicated the increase in activity with increase in side chain length. Alkylimidazolium ionic liquids with alkyl group from C_{10} to C_{16} showed effective antibiofilm activity on monoculture biofilms of these organisms [103, 107]. Imidazolium, pyrrolidinium and piperidinium ionic liquids with –dodecyl side chains were more effective for retarding biofilm formation by Gram-positive bacteria than Gram-negative bacteria [97, 224]. Bacterial biofilms formed in natural waters are polymicrobial and comprises of multiple genera from Gram-positive and Gram-negative bacteria. Therefore, systematic analysis is required to determine the concentrations that are required to inhibit biofilm formation. Similar to growth inhibition, biofilm formation in natural waters was not impeded in freshwater (Fig. 5.2A and Fig. 5.3) and seawater (Fig. 5.4A and Fig. 5.5) in the presence of up to 500 µM $[C_4MIM]Cl$. $[C_{12}MIM]I$ was effective in retarding biofilm formation in freshwater (Fig. 5.2B). But, relatively higher concentrations were required to cause complete inhibition of biofilms in seawater (Fig. 5.4B). $[C_{16}MIM]Cl$ was highly effective in preventing biofilm formation in both freshwater (Fig. 5.2C) and seawater (Fig. 5.4C). Prevention of biofilm formation in seawater required comparatively higher concentration of respective ionic liquid compound than freshwater. $[C_{16}MIM]Cl$ was

able to cause prevention of multispecies biofilms in natural waters due to broad spectrum activity.

In addition to antibiofilm activity, effect of antimicrobials on established biofilms is an important characteristic for a compound to be an effective biofilm control agent. Environmental or medical biofilms often precede the treatment and eradication of these pre-formed biofilm is an important aspect for the normal functioning of equipment or to treat persistent infections. Additionally, the cells embedded in biofilm-matrix of pre-formed biofilms are more resistant and not accessible for antimicrobial treatment, thus requiring higher concentrations of antimicrobials. Carson et al. reported that up to 16 times higher MIC values for 1-octyl to 1-tetradecyl-3-methylimidazolium ionic liquids are required for killing the biofilm-bacteria [107]. Alkylimidazolium ionic liquids with –butyl and -dodecyl alkyl groups did not show any biofilm dispersal activity on multispecies biofilms (Fig. 5.6). $[C_{16}MIM]Cl$ showed biofilm dispersal activity on multispecies biofilm formed in both freshwater and seawater. But, higher concentration was needed to exhibit effective biofilm dispersal on biofilms formed in seawater.

For prospective application of alkylimidazolium ionic liquids against naturally occurring multispecies biofilms, a flow cell reactor [225] was operated with freshwater or seawater in a recirculation mode to simulate recirculating cooling water system. Similar setup was used by Simoes et al. to evaluate the antibiofilm activity of cationic surfactant, CTAB against *Pseudomonas fluorescens* biofilms on stainless-steel slides [226]. Based on the data from microtiter plate experiments, the potent ionic liquid $[C_{16}MIM]Cl$ was chosen at fixed concentrations of 100 µM and 250 µM, respectively, for freshwater and seawater. Data showed that formation of multispecies

biofilms was completely inhibited during 12 d of operation in the presence of [C_{16}MIM]Cl in freshwater or seawater. These results show that biofilm formation can be prevented under natural waters under dynamic flow conditions.

5.5. Conclusions

- Biofilm formation in natural waters was enhanced by amendment with acetate and inorganic phosphate. This allowed quantifying biofilm formation in control and treatment conditions in microtiter plates with up to 3 days incubation.
- Alkylimidazolium ionic liquids with -dodecyl and -hexadecyl alkyl groups were inhibitory to microbial growth and polymicrobial freshwater and seawater biofilms.
- The potent ionic liquid, [C_{16}MIM]Cl exhibited biofilm dispersal activity on multispecies biofilms formed in freshwater and seawater.
- Generally, the biofilm growth was more pronounced in seawater than freshwater under similar experimental conditions. Therefore, inhibition and biofilm-dispersal activity under seawater conditions required higher concentration of ionic liquid than those required in freshwater.
- [C_{16}MIM]Cl exhibited strong antibiofilm activity and prevented formation of multispecies biofilms in freshwater and seawater under dynamic flow conditions.

ANNEXURE

A1. Constituents of Guillard's *f*/2 media

A) *f*/2 stock solutions: All the stocks were prepared in distilled water.

Table 1: *f*/2 Stock Solution Chemical Composition

Components	**Concentration**	**Quantity**
$NaNO_3$	75 g/L	1 mL
$NaH_2PO_4\ H_2O$	5 g/L	1 mL
$Na_2SiO_3 \bullet 9H_2O$	30 g/L	1 mL
Trace Metal Solution	See table 2	1 mL
Vitamin Solution	See Table 3	0.5 mL

B) Trace metal solution: Following stocks of independent trace metals were prepared in distilled water. Mentioned volumes or weights of these stocks were added to 950 mL distilled water. After dissolution, final volume was brought to 1000mL with distilled water.

Table 2: Trace Metal Composition

Components	**Concentration**	**Quantity**
$FeCl_3\ 6(H_2O)$	---	3.15 g
$Na_2(EDTA)\ 2(H_2O)$	---	4.36 g
$CuSO_4\ 5(H_2O)$	9.8 g/L	1 mL
$Na_2MoO_4\ 2(H_2O)$	6.3 g/L	1 mL
$ZnSO_4\ 7(H_2O)$	22.0 g/L	1 mL
$CoCl_2\ 6(H_2O)$	10.0 g/L	1 mL
$MnCl_2\ 4(H_2O)$	180.0 g/L	1 mL

C) Vitamin solution: Independent stocks of following vitamin solutions were prepared in distilled water. Mentioned volumes or quantity was added to 950 mL distilled water and final make up to 1000 mL with distilled water.

Table 3: Vitamin Composition

Components	**Concentration**	**Quantity**
Thiamine HCl (vitamin B1)	---	200 mg
Biotin (Vitamin H)	0.1 g/L	10 mL
Cyanocobalamin	1 g/L	1 mL

Working solution: All the three stock solutions with the quantity mentioned in Table 1 were added to 950 mL filter sterile seawater. A final make up volume to 1000 mL was done with filter sterile seawater. Required volume was dispensed into flasks, plugged with cotton stoppers and sterilised by autoclaving.

(R.R.L. Guillard, 1975)

A2. Constituents of BG11 media

A) Stock solutions for BG-11:

Stock 1:	**Concentration**
Na_2Mg EDTA	0.1g/liter
Ferric ammonium citrate	0.6g/liter
Citric acid. H_2O	0.6g/liter
$CaCl_2$. $2H_2O$	3.6g/liter
Stock 2:	
$MgSO_4$. $7H_2O$	7.5g/liter
Stock 3:	
K_2HPO_4	3.05g/liter
Stock 4 (Microelements):	
H_3BO_3	2.86g/liter
$MnCl_2$. $4H_2O$	1.81g/liter
$ZnSO_4$. $7H_2O$	0.222g/liter
$CuSO_4$. $5H_2O$	0.079g/liter
$NaMoO_4$. $2H_2O$	0.391g/lite
$COCl_2$. $6H_2O$	0.050g/liter

Filter sterilised or autoclaved.

B) For BG11 medium following stock solutions were combined: 10mL each of stocks 1, 2, 3 and 1 mL of stock 4 to 800 mL distilled water. Additionally, 1.5 g $NaNO_3$ and 20 mg Na_2CO_3 were added and pH was adjusted to 7.5 using 0.1N HCl. This suspension was made up to 1000 mL with distilled water, sterilised by autoclaving and dispensed as per the requirement.

(http://microbiology.ucdavis.edu/meeks/BG11medium.html)

A3. Media constituents of Potato Dextrose Agar (For 1 Liter)

Component	**Weight (g)**
Dextrose	20
Potato extract	4 (equivalent to 200 g of potato infusion)
Bacto agar	15

39 g of commercial powder containing above constituents were dissolved and a final make up to 1000 mL was made with distilled water. Suspension was sterilised by autoclaving and poured into sterile Petri dishes while still hot in laminar hood. Plates were allowed to cool and solidify before using for experiments.

(https://www.mycrobe.org/blog/2018/7/6/potato-dextrose-agar-pda)

A4. Media constituents of Potato Dextrose Broth (For 1 Liter)

Component	**Weight (g)**
Dextrose	20
Potato extract	4 (equivalent to 200 g of potato infusion)

24 g of commercial powder containing above constituents were dissolved and a final make up to 1000 mL was made with distilled water. Suspension was sterilised by autoclaving and dispensed into sterile flasks as per the requirement.

(https://www.himedialabs.com/TD/M403.pdf)

A5. Constituents of Rosewell Park Memorial Institute (RPMI 1640) media

Component	Weight (mg)
Calcium nitrate tetrahydrate	100
Magnesium sulphate anhydrous	48.84
Potassium chloride	400
Sodium chloride	6000
Sodium phosphate dibasic anhydrous	800
Glycine	10
L-Arginine hydrochloride	241
L-Asparagine	50
L-Aspartic acid	20
L-Cystine dihydrochloride	65.2
L-Glutamic acid	20
L-Glutamine	300
L-Histidine hydrochloride monohydrate	20.96
L-Hydroxyproline	20
L-Isoleucine	50
L-Leucine	50
L-Lysine hydrochloride	40
L-Methionine	15
L-Phenylalanine	15
L-Proline	20
L-Serine	30
L-Threonine	20
L-Tryptophan	5
L-Tyrosine disodium salt	28.83
L-Valine	20
Choline chloride	3
D-Biotin	0.2

D-Ca-Pantothenate	0.25
Folic acid	1
Niacinamide	1
Pyridoxine hydrochloride	1
Riboflavin	0.2
Thiamine hydrochloride	1
Vitamin B12	0.005
i-Inositol	35
p-Amino benzoic acid (PABA)	1
D-Glucose	2000
Glutathione reduced	1
MOPS Buffer, Free acid	34500
Phenol red sodium salt	5.3

Above constituents containing in a 44.6 g commercial powder was dissolved in 900 mL distilled water. Using 1N NaOH, pH was adjusted to 7.0 and the final volume was made up to 1000 mL with distilled water. This suspension was filter sterilised with 0.22 µ syringe filter into an autoclave sterile bottle in laminar hood. Obtained sterile RPMI 1640 was stored at 4 ºC in dark, until use. This media was dispensed into sterile flasks, as per the requirement.

(https://himedialabs.com/TD/AT180.pdf)

A6. Composition of Phosphate Buffered Saline

Component	Weight (g)
NaCl	8
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.245

Above contents were dissolved in 900 mL distilled water. pH was adjusted to 7.4. Contents were made up to 1000 mL with distilled water. Sterilised through autoclaving. Stored at room temperature until use.

(https://www.aatbio.com/resources/buffer-preparations-and-recipes/pbs-phosphate-buffered-saline)

A7. Media constituents of Nutrient Agar

Component	Weight (g)
Peptone	5
Yeast extract	1.5
Beef extract	1.5
NaCl	5
Agar	15

28 g of commercial powder containing above constituents were dissolved in 900 mL distilled water. The final volume was made up to 1000 mL with the distilled water. Solution was sterilised by autoclaving and poured into sterile Petri dishes while still hot. Plates were allowed to cool and solidify before using for experiments.

(https://microbenotes.com/nutrient-agar-principle-composition-preparation-and-uses/)

A8. Media constituents of Zobell Marine Agar

Component	Weight (g)
Peptone	5.000
Yeast extract	1.000
Ferric citrate	0.100
Sodium chloride	19.450
Magnesium chloride	8.800
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15

55.25 g of the commercial powder with above listed constituents was dissolved in 900 mL distilled water. Final volume was made to 1000 mL with distilled water. Contents were sterilised by autoclaving. Medium is poured into sterile Petri dishes while still hot and allowed to cool and solidify before using for experiments.

(https://himedialabs.com/td/m384.pdf)

Highlights of the thesis

1. 1-Alkyl-3-methylimidazolium ionic liquids with different alkyl groups ($[CnMIM]^+[X]^-$, n=4, 12 and 16) effectively inhibited monoculture (*Navicula* sp.) and mixed species phototrophic biofilms.
2. Antimicrobial and antibiofilm activities were dependent on alkyl side chain length, with –hexadecyl side chain containing $[C_{16}MIM][Cl]$ exhibiting highest biological activity.
3. $[C_{16}MIM][Cl]$ effectively inhibited biofilm formation and dispersed the preformed biofilms of laboratory and clinical isolates of *Candida albicans*.
4. Multiple cellular targets such as membrane damage, leakage of intracellular contents, reduction in ergosterol synthesis, mitochondrial dysfunction, and oxidative stress were observed in *C. albicans* cells challenged with ionic liquid.
5. All the three tested ionic liquids showed larvicidal activity (mortality, metamorphosis interruption and settlement inhibition) on barnacle larvae.
6. Ionic liquids strongly inhibited cypris settlement at nanomolar concentrations and showed higher therapeutic ratios, indicating their use in developing prospective environmentally benign antifouling formulations.
7. The effective concentrations of ionic liquids for preventing barnacle larval settlement were not lethal to non-target organism, *Artemia salina*.
8. $[C_{16}MIM][Cl]$ strongly inhibited formation of natural and multispecies bacterial biofilms in freshwater and seawater in static and dynamic recirculation experimental systems.