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Ecotoxicological Profiling and Antibacterial Potency of a Series of 24 L-Phenylalanine Based SAILs

Dewi Kurnianingsih Arum Kusumahastuti

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TALLINN UNIVERSITY OF TECHNOLOGY
School of Science
Department of Chemistry and Biotechnology

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Supervisor: Academician Dr. Anne Kahru
Laboratory of Environmental Toxicology
National Institute of Chemical Physics and Biophysics
Estonia

Co-supervisors: Prof. Nicholas Gathergood
School of Science
Tallinn University of Technology (working period from January 2015 to April 2019), Estonia.
Currently working at University of Lincoln, United Kingdom, as an Associate Professor

Dr. Mariliis Sihtmäe
Laboratory of Environmental Toxicology
National Institute of Chemical Physics and Biophysics
Estonia

Opponents: Prof. Jaak Truu
Institute of Molecular and Cell Biology
Faculty of Science and Technology
University of Tartu, Estonia

Dr. Andrew Hunt
Department of Chemistry
Khon Kaen University, Thailand

Defence of the thesis: 15/06/2021, Tallinn

Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, has been carried out in collaboration with the Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics. This thesis submitted for the doctoral degree at Tallinn University of Technology has not been submitted elsewhere for doctoral or equivalent academic degree.
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24 L-fenüülalaniini-põhise ioonvedeliku ökotoksikoloogiline ja antibakteriaalne iseloomustamine

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List of Publications

The list of author's publications, on the basis of which the thesis has been prepared:

I **Kusumahastuti, D. K. A.**, Sihtmäe, M., Kapitanov, I. V., Karpichev, Y., Gathergood, N., Kahru, A. (2019). Toxicity profiling of 24 L-phenylalanine derived ionic liquids based on pyridinium, imidazolium and cholinium cations and varying alkyl chains using rapid screening *Vibrio fischeri* bioassay. *Ecotoxicology and Environmental Safety*, *172*, 556–565. https://doi.org/10.1016/j.ecoenv.2018.12.076

II **Kusumahastuti, D. K. A.**, Sihtmäe, M., Gathergood, N., Kahru, A. (2019). Antibacterial activity of 24 L-phenylalanine derived surface-active ionic liquids (SAILs) towards two clinically relevant pathogens. *Ecology and Safety, 13,* 16–28. https://www.scientific-publications.net/en/article/1001856/

III **Kusumahastuti, D. K. A.**, Sihtmäe, M., Aruoja, V., Gathergood, N., Kahru, A. (2021). Ecotoxicity profiling of a library of 24-L-phenylalalnine derived surface-active ionic liquids (SAILs). *Sustainable Chemistry and Pharmacy*, *19*, 100369. https://doi.org/10.1016/j.scp.2020.100369.

Author's Contribution to the Publications

Contributions of the author to the publications in this thesis:

I The author participated in the study design and was responsible for collecting ecotoxicological information on 24 SAILs. She performed toxicity tests, data reduction and participated in the preparation of the manuscript.

II The author participated in the study design and performed the antimicrobial assays. She interpreted the data and participated in the preparation of manuscripts.

III The author performed crustacean toxicity tests and respective data reduction. She participated in the preparation of the original manuscript and visualisation.

Introduction

The combination of the global population's annual rise and the demand for a higher standard of living has the potential to lead to an increase in the release of hazardous materials into the environment. Different types of substances to meet consumer expectations have been manufactured using "green" technologies with the aim of reducing the adverse impact of hazardous materials on the environment. For instance, in the field of chemistry, there is a guidance framework for designing new chemical products and processes called the Twelve Principles of Green Chemistry.

One group of chemicals that have been studied extensively within the field of green chemistry are ionic liquids (ILs). They are considered "designer solvents" due to the facile manipulation of their anion and cation parts; the fine tuning of the functional groups present allows for the electronic and steric properties to be tailored for specific applications. Furthermore, ILs have been investigated in a broad range of different applications (e.g. electrolytes, agrochemicals, pharmaceuticals, and surfactants) where their ionic form is integral to their success.

In principle, ILs can be considered environmentally friendly due to several factors, such as low volatility, non-flammability, and high thermal stability. However, despite the many possible benefits achieved when using ILs in a process, they also can reach aquatic and terrestrial environments via various waste-flows.

In the current thesis, the ecotoxicities of a series of 24 L-phenylalanine derived surface-active ionic liquids (SAILs) with various cationic head groups (pyridinium, Py; imidazolium, Imid; and cholinium, Chol) and alkyl ester chains from C_2 to C_{16} were investigated. Additionally, the antibacterial potencies of these SAILs were determined.

As a result, the toxicological profiling of these SAILs towards algae and crustaceans was carried out. Two ranking scales were suggested based on mg/L and molarity concentration units. Quantitative structure analysis relationship (QSAR) studies could be determined using physicochemical and toxicity data. Recommendations for safer alternatives to "very toxic" commercial surfactants were made.

Finally, the thesis has been published as three peer-reviewed scientific articles. Furthermore, ecotoxicological data and antibacterial potency data have also been presented in some international science conferences.

Abbreviations

$[C_{10}mim][Br]$	1-decyl-3-methylimidazolium bromide
$[C_{12}mim][BF_4]$	1-dodecyl-3-methylimidazolium tetrafluoroborate
$[C_{12}mim][Br]$	1-dodecyl-3-methylimidazolium bromide
$[C_2Clmim][Cl]$	3-(2-cloroethyl)-1-methylimidazolium chloride
$[C_2Clmim][Tf_2N]$	3-(2-cloroethyl)-1-methylimidazolium bis-triflimide
$[C_2mim[[DMP]$	1-ethyl-3-methylimidazolium dimethyl phosphate
$[C_2mim[[MS]$	1-ethyl-3-methylimidazolium methyl sulphate
$[C_2mim[[NO_3]$	1-ethyl-3-methylimidazolium nitrate
$[C_2mim[[Ts]$	1-ethyl-3-methylmidazolium p-toluenesulfonate
$[C_2mim][Br]$	1-ethyl-3-methylimidazolium bromide
$[C_2OHmim][Tf_2N]$	1-(2-Hydroxyethyl)-3-methylimidazolium bis-triflimide
$[C_4mim][BF_4]$	1-butyl-3-methylimidazolium tetrafluoroborate
$[C_4mim][Br]$	1-butyl-3-methylimidazolium bromide
$[C_4mim][Cl]$	1-butyl-3-methylimidazolium chloride
$[C_4mim][DCA]$	1-butyl-3-methylimidazolium dicyanamide
$[C_4mim][DMP]$	1-butyl-3-methylimidazolium dimethyl phosphate
$[C_4mim][NTf_2]$	1-butyl-3-methylimidazolium bis-triflimide
$[C_4mim][PF_6]$	1-butyl-3-methylimidazolium hexafluorophosphate
$[C_4pyr][DCA]$	1-butylpyridinium dicyanamide
$[C_6mim][Br]$	1-hexyl-3-methylimidazolium bromide
$[C_6mim][Cl]$	1-hexyl-3-methylimidazolium chloride
$[C_6mim][SCN]$	1-hexyl-3-methylimidazolium thiocyanate
$[C_8mim][Br]$	1-octyl-3-methylimidazolium bromide
$[C_8mim][Cl]$	1-octyl-3-methylimidazolium chloride
$[EtNH_3][NO_3]$	ethylammonium nitrate
[Mor14][Br]	1-butylmorpholinium bromide
$[P_{6,6,6,14}][N(CN)_2]$	tetradecyl(trihexyl)phosphonium dicyanamide
$[P_{6,6,6,14}][NTf_2]$	tetradecyl(trihexyl)phosphonium bis[(trifluoromethane)sulfonyl]imide
$[P_{6,6,6,14}][Phosp]$	tetradecyl(trihexyl)phosphonium phosphate
[Pip14][Br].	1-butylpiperidinium bromide
[TBA][Arg]	tetrabutylammonium argininate
3,5-DCP	3,5-dichlorophenol
AE	atom economy
$AlCl_4$	tetrachloroaluminate
ANOVA	analysis of variance
API	active pharmaceutical ingredient
BAC	benzalkonium chloride
CBT	closed bottle test
CE	carbon economy
Cho[DBS]	cholinium dodecylbenzenesulfonate
CMC	critical micelle concentration
C_n	n number of carbons
CPB	hexadecylpyridinium bromide
CTAB	cetyltrimethylammonium bromide

D1222	didodecyldimethylammonium bromide
D1622	dihexadecyldimethylammonium bromide
DOC	dissolved organic carbon
DTAB	dodecyltrimethylammonium bromide
EC	European Commission
EC_{20}	median effective concentration of the studied chemicals, which produces adverse effect in 20% of the test organisms after a specified exposure time
EC_{50}	median effective concentration of the studied chemicals, which produces adverse effect in 50% of the test organisms after a specified exposure time
EDDAB	ethyldodecyldimethylammonium bromide
EDHAB	ethylhexadecyldimethylammonium bromide
E-factor	environmental impact factor
EMY	effective mass yield
ERA	European Research Area
Hyamine 1622	(diisobutylphenoxyethoxyethyl)dimethylbenzylammonium chloride
IC_{95}	median inhibitory concentration of the studied chemicals, which induces inhibition in 95% of the test organisms after a specified exposure time
ILs	ionic liquids
ISO	International Standard Organization
LC_{50}	median lethal concentration of the studied chemicals, which induces mortality in 50% of the test organisms after a specified exposure time
LCA	life cycle assessment
MBC	minimum bactericidal concentration
Mp	melting point
MI	mass intensity
MIC	minimum inhibitory concentration
MP	mass productivity
MRSA	methicillin-resistant *Staphylococcus aureus*
NOEC	no observed effect concentration
NOM	natural organic matter
OECD	Organization of Economic Co-Operation and Development
PMI	process mass intensity
PNEC	predicted no effect concentration
PO_2F_2	difluorophosphate
PO_3F	fluorophosphate
POF_3	phosphorus oxyfluoride
QACs	quaternary ammonium compounds
QSAR	quantitative structure–activity relationship
REACH	registration, evaluation, authorisation and restriction of chemicals
RME	reaction mass efficiency
SAILs	surface active ionic liquids
SALSILs	surface-active lauroyl sarcosinate ionic liquids

SARs	structure–activity relationships
SDGs	sustainable development goals
SI	solvent intensity
STAB	steartrimonium bromide
ThOD	theoretical oxygen demand
TSIL	task-specific ionic liquids
TTA	triterpene acids
TTAB	tetradecyltrimethylammonium bromide
UN	United Nations
VOCs	volatile organic solvents
WoS	web of science
WWI	waste water intensity

1. Literature review

Concerns about a sustainable world have been raised since the 1980s. The United Nations (UN) established a special commission to address the challenge of sustainable development. This commission is known as the Brundtland Commission. In 1987 this commission defined sustainable development as the "development that meets the present's needs without compromising future generations' ability to meet their own needs" (World Commission on Environment and Development, 1987). The increasing rate of deterioration of our planet influenced by social behaviour is not sustainable (Klotz et al., 2019). For instance, the global population's annual rise will trigger the release of hazardous materials into the environment in ever-increasing quantities (Kirchhoff, 2005).

1.1. Green Chemistry – a path to sustainability

An on-going and fundamental challenge of science is to support sustainable development. In 1991, a systematic approach to the design of chemistry aligned with sustainability was introduced as Green Chemistry (Anastas, 2017). This new perspective of chemistry was defined as "the design of chemical products and processes that reduce or eliminate the use and generation of hazardous substances". To achieve the goals of green chemistry, Anastas & Warner (1998) introduced a set of guidelines known as "The Twelve Principles of Green Chemistry". In recent decades, this approach has been implemented in academia and industry around the globe (Anastas & Zimmerman, 2018).

As the field of Green Chemistry continues to grow, there has been a common misperception of the terms "green" and "sustainable" within the chemistry community. Many publications reinforce perceptions that green chemistry and sustainable chemistry are synonymous (Dunn, 2012; Wilcox, 2020; Saha, M. et al., 2011; Jeon, 2018). The diagram (**Figure 1**) developed by Cannon, et al. in Zhang & Cue, (2012) illustrates their differences.

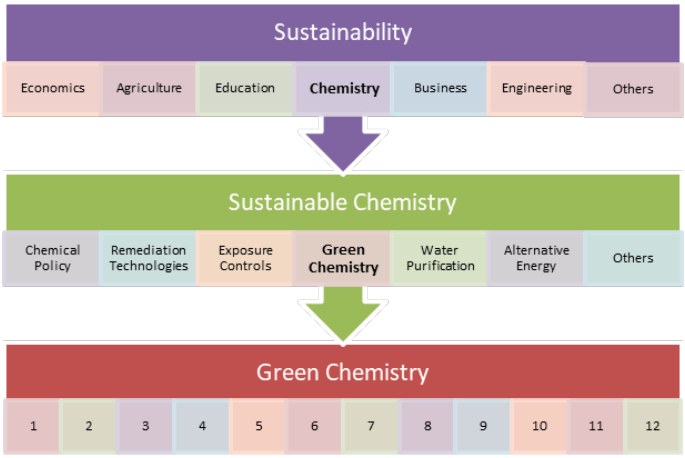


Figure 1*. The tiered relationship between sustainability, sustainable chemistry and the 12 principles of green chemistry (modified from Cannon et al. in* Zhang & Cue, 2012*).*

Sustainability can be achieved by considering numerous aspects, for example economics, agriculture, education, and engineering. Sustainable chemistry is also one subunit of sustainability and is the focus of this thesis. Sustainable chemistry can be defined as "the development of safer and more environmentally-friendly chemical methodologies and chemical products while equally integrating the priorities of economic competitiveness and societal concerns" (Marion et al., 2017). Many factors may be involved in achieving sustainable chemistry, including chemical policy, remediation technologies, exposure control, green chemistry, water purification, and alternative energy. Green chemistry is one essential subset of sustainable chemistry encoded by the 12 principles (Anastas & Warner, 1998) discussed above. Thus, progress in sustainable chemistry is a result of advances in green chemistry.

1.1.1. Twelve principles of Green Chemistry

As the high quality of life cannot be maintained without the chemical industry, the search for greener chemicals is a worthwhile goal. Careful consideration is required to produce greener chemicals. The Twelve Principles of Green Chemistry are a guiding framework for designing new chemical products and processes (**Figure 2**). They were composed by Paul Anastas and John Warner and are essentially a checklist of chemicals life-cycles, from raw materials to products' synthesis (Anastas & Warner, 1998). They were created to reduce the environmental impacts and the potential adverse health effects of chemicals and chemical processes.

1. Prevention
It is better to prevent waste than to treat or clean up waste after it is formed.
2. Atom Economy
Synthetic methods should be designed to maximise the incorporation of all materials used in the process into the final product.
3. Less Hazardous Chemical Synthesis
Whenever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
4. Designing Safer Chemicals
Chemical products should be designed to preserve the efficacy of the function while reducing toxicity.
5. Safer Solvents and Auxiliaries
The use of auxiliary substances (solvents, separation agents, etc.) should be made unnecessary whenever possible and, when used, innocuous.
6. Design for Energy Efficiency
Energy requirements should be recognised for their environmental and economic impacts and should be minimised. Synthetic methods should be conducted at ambient temperature and pressure.
7. Use of Renewable Feedstocks
A raw material or feedstock should be renewable rather than depleting whenever technically and economically practical.
8. Reduce Derivatives
Unnecessary derivatisation (blocking group, protection/deprotection, and temporary modification of physical/chemical processes) should be avoided whenever possible.
9. Catalysis
Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. Design for Degradation
Chemical products should be designed so that at the end of their function they do not persist in the environment and instead break down into innocuous degradation products.
11. Real-time Analysis for Pollution Prevention
Analytical methodologies need to be further developed to allow for real-time in-process monitoring and control before the formation of hazardous substances.
12. Inherently Safer Chemistry for Accident Prevention
Substance and the form of a substance used in a chemical process should be chosen to minimise the potential for chemical accidents, including releases, explosions, and fires

Figure 2. *The Twelve Principles of Green Chemistry (*Anastas & Warner, 1998*).*

1.1.2. Green Chemistry metrics

A fundamental aspect of the 12 principles (see **Figures 1** and **2**) is that new methods or chemical products must be demonstrated to be safer or greener than known examples. This is only possible by critical quantitative analysis of new and previously reported cases. In this section, the metrics created to evaluate Principle 1 and 2 are discussed. The relevance of Principle 4 as a green chemistry metrics (the design of chemicals of lower toxicity) is explained in a following section. Using a broad set of metrics is required to effectively answer the question "how green and sustainable is the chemical process?" The transition from pollution control and mitigation to pollution prevention began in the 1980s. This transition has had a positive impact on the environment and has strengthened economic competitiveness by cutting the costs of waste treatments (Sheldon, 2018).

The first principle of green chemistry is **waste prevention**. This principle is in good agreement with the Environmental Impact Factor (E-factor) metrics that determine the "environmental acceptability" of a manufacturing process. This E-factor can be applied to a multistep process and takes into account all substances that do not appear when viewing the reaction stoichiometry (for example, solvents and chemicals used in the workup step). This E-factor allows for a more detailed assessment of the entire process (compared to Atom Economy *vida infra*). As mentioned in the Twelve Principles of Green Chemistry, zero waste is the most desirable outcome; accordingly, the ideal E-factor is zero (meaning zero waste) (Sheldon, 2018). However, when a by-product is formed,

an alternative approach that leads to innovations in industrial ecology should be considered; e.g. the application of an aromatic aldehyde by-product of lignocellulosic biofuel production, which was utilised in the production of tertiary amine-based ILs (Socha et al., 2014). This has significant potential for the realisation of a "closed-loop" process for future lignocellulosic biorefineries.

The second principle is called the **atom economy** (AE). AE is often referred to as the Atom Efficiency concept introduced by Barry Trost in the 1990s (Trost, 1991). This metric is an assessment of reaction efficiency. Trost recommended incorporating the maximum number of atoms in reactants into final products. In principle, AE is defined as the ratio of the desired product's molecular weight over the molecular weights of all starting materials used in the reaction and is expressed as a percentage. The determination of AE is a useful tool for the rapid prediction of whether a small or large amount of waste will be generated from a reaction. It is worth noting that this metrics does not consider chemicals that do not appear in the stoichiometry calculation, and it is usually used to assess a single-step process (Sheldon, 2018).

These two metrics (AE and E-Factor) have inspired both industrial and academic chemists worldwide to address waste generation specifically. However, these metrics are not sufficiently comprehensive to measure the greenness and sustainability of the industrial process of pharmaceuticals and other fine chemicals (Sheldon, 2017). Some years later, additional mass-based metrics were proposed. The metrics analogous to AE include Reaction Mass Efficiency (RME), Mass Productivity (MP), Effective Mass Yield (EMY), and Carbon Economy (CE). Metrics analogous to the E-factor are Mass Intensity (MI), Process Mass Intensity (PMI), Waste Water Intensity (WWI), and Solvent Intensity (SI) (Sheldon, 2018). Another important metrics for supporting the mass-based metrics mentioned above is Life Cycle Assessment (LCA). LCA is a metric that measures the environmental impact of feedstock and waste (Sheldon, 2018). In the LCA method, the full process from raw material extraction, to the synthesis of ILs, to the process application of ILs, to the recovery of ILs for reuse should be assessed (Zhu et al., 2009). For instance, ISO 14040 and ISO 14044 are the standard guidelines available to evaluate LCA. The results found from the LCA approach are well-established and comprehensive, but the implementation of LCA is time-consuming and involves an extensive database (Ott et al., 2010).

1.1.3. How to improve sustainability from a Green Chemistry perspective

As the world population continues to grow, there are some issues that need to be addressed by the global community: e.g., demography, food, energy, and climate change. To address those concerns, the United Nations (UN) initiated the Sustainable Development Goals (SDGs) in September 2015. These SDGs include seventeen goals and 169 targets to be reached by 2030, including ending poverty, reducing inequality, and protecting the environment ("Transforming Our World: The 2030 Agenda for Sustainable Development", 2018). Chemicals are the gateway to achieving many of the SDGs from a molecular level perspective. The three dimensions of sustainability development (economy-social-environment) should be effectively integrated (Anastas & Zimmerman, 2018) and the cooperation of academia, industry, and government is essential (Kirchhoff, 2005) to reach the goals.

Academic research in green chemistry offers essential information about new chemical products and processes (i.e., the development of cleaner technologies). Moreover, those new processes and outcomes will have significant impacts on chemical industries. Academia also provides students with the education to promote green

chemistry and work towards a better future. Industries are encouraged to adopt cleaner technologies because economic factors, such as materials, safety and clean-up costs, are decreased. Lastly, the government can support research and education by increasing its funding and offering regulatory relief for adopting cleaner technologies (Kirchhoff, 2005).

1.2. Ionic liquids-based technologies

The history of ionic liquids (ILs) goes back to the mid-19th century when low-melting organic salts ("red oil") as a by-product of the aluminium chloride-catalysed alkylation reaction of benzene (Friedel-Crafts reaction) was described for the first time (Pernak et al., 2016). In 1888 the early reports on low-melting-point salts (e.g. ethanolammonium nitrate, melting point 52˚C) were published (Gabriel & Weiner, 1888). Walden discovered molten salts that were liquid at room temperature (e.g. $[EtNH_3][NO_3]$) in 1914 (Plechkova & Seddon, 2008). In 1908, organic salts (currently classified as ILs) with low Mp were discovered. However, these compounds were not highly soluble in water and interest waned ("Über organische und geschmolzene Salze," 1908). After years of silence, in 1934 a patent was issued on ILs that included the reaction between halide salts of nitrogen-containing bases (such as 1-benzylpyridinium chloride, 1-ethylpyridinium chloride, etc.) and cellulose at temperatures above 100 ˚C. These reactions generate cellulose in a very reactive form (Graenacher, 1934). Extensive research to develop electrolytes (e.g. for thermal batteries or capacitors) followed in later decades (Nardi et al., 1976; Nardiet al., 1977; Wilkes, , 2002a, 2002b, 2002c).

ILs remained a niche field of interest as non-volatile electrolytes until the year 2000. In the last two decades, ILs have been widely promoted to be "green" alternatives to volatile, flammable, and toxic volatile organic solvents (VOCs).

In general, ILs are composed of large organic cations (pyridinium, imidazolium, ammonium, etc.) with alkyl chain substituents and delocalised anions (e.g. hexafluorophosphate (PF_6^-), tetrafluoroborate (BF_4^-), or halides, chloride (Cl^-), bromide (Br^-) and iodide (I^-)) (Zhao et al., 2007). Usually, they are of high thermal stability and do not decompose over a large temperature range. The vast number of combinations of cations and anions make it possible to control the properties of ILs for specific tasks (Pernak et al., 2016). The adjustment of their cation and/or anion structures leads to different chemical and physical properties and can enhance yields, reaction rates, and selectivity (Sheldon, 1997).

The first advanced research workshop on ILs was in April 2000 organised by NATO and attended by leading ILs and green chemistry researchers (Shiflett, 2020). By 15 October 2020, there were 7015 ILs publications reported for the year 2020 mentioned in the Web of Science (WoS) compared to 710 publications in 2000 (**Figure 3**)**.** In total, the WoS indicates that more than 100 000 articles have been published (excluding patents) for the search term "ionic liquid".

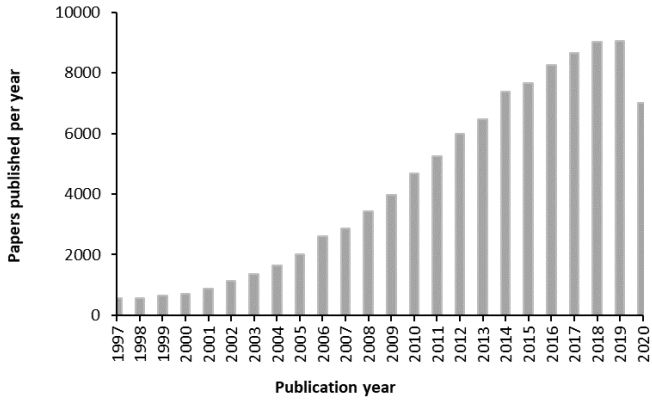


Figure 3. *The number of publications on ILs per year (time span: 1997-2020) retrieved from the Web of Science (WoS) on 15 October 2020 using the term "ionic liquid".*

The unique properties of ILs have attracted researchers' interest from a wide range of fields. **Figure 4** shows the top 25 research areas for ILs according to the WoS. From 1980 to 2020, the field of chemistry involved the highest number of publications on ILs (40.9%), followed by material science and engineering (about 9% each). Crucially for this thesis, publications of ILs in toxicology represent only about 0.2% (**Figure 4**).

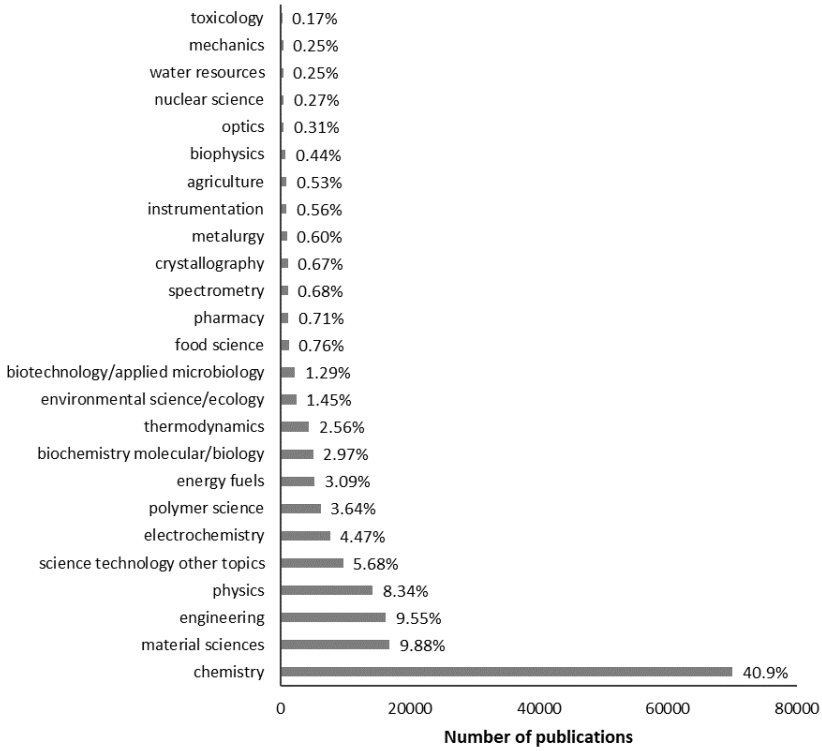


Figure 4. *The number of publications on ILs by different research areas (retrieved from WoS on 15 October 2020). The search was made using "ionic liquid" as the search term and 1980-2020 as the time frame.*

1.2.1. ILs and their relationship with Green Chemistry

In 1997, Sheldon determined that the E-factors for some industries (e.g. bulk chemicals, fine chemicals, and pharmaceuticals) were high (Sheldon, 1997) meaning their manufacturing processes were "dirty" with enormous amounts of waste generated that could harm the environment; this was partly because of the extensive use of VOCs in their production (Holbrey & Seddon, 1999). To reduce their pollution, manufacturers could work towards the targets stated in the Montreal Protocol (Holbrey & Seddon, 1999). Moreover, Holbrey & Rogers (2002) stated that ILs could be used as alternatives to VOCs because they had low vapour pressures and thus caused less air pollution than VOCs as solvents.

The introduction of green chemistry principles in 1998 was related to the remarkable growth of ILs as alternatives (safer) solvents to conventional solvents (Blanchard et al., 2002; Dai et al., 1999; Welton, 1999). However, in early 2000 the interest in studying the adverse effects of ILs (e.g. toxicity and biodegradability) was not significant. Researchers have recently acknowledged that reporting new ILs and their toxicity data is fundamental for designing greener ILs (Haiß et al., 2016). The sections below will address the toxicity, biodegradation, and green chemistry metrics used to show how the ILs community has developed greener ILs.

1.2.2. IL applications

The significant growth of IL production and use has driven experts to group ILs into three different "generations" or "evolutions". This classification is based on general assessments of their chemical structures, properties, and potential applications (Hough et al., 2007) (see **Figure 5**). The broad applicability of ILs is dependent on specific properties, such as non-volatility, good thermal stability, robustness (lack of chemical reactivity), non-flammability, stability to a wide electrochemical window, tenability, miscibility, and good extraction capability for various analytes (e.g. metal salts and natural biomolecules).

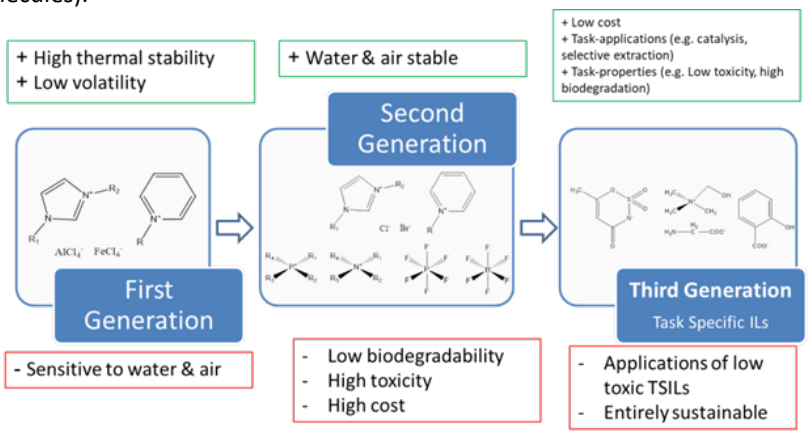


***Figure 5**. Evolution of ILs based on their properties: a scheme (modified from Caparica, 2017).*

Most dialkyl-imidazolium, alkyl-pyridinium cations, combined with metal halide anions are classified as the "first generation" of ILs. Briefly, **first-generation ILs** have specific physical properties controlling their cationic and anionic parts. One example is the selection of cationic and anionic structures for high conductivity in their applications

as electrolytes. Hough et al. in 2007 described how the first generation of ILs were aimed to achieve specific values of density, melting point, viscosity, hydrophobicity, refractive index, and thermal stability. The first generation of ILs have a large electrochemical window but requires water-free conditions. Due to the vigorous reaction of these ILs with water generating strong acids (e.g. HCl), their role as solvents beyond electrolytes was greatly restricted.

The **second generation ILs** with suitable physical parameters and chemical properties (Hough et al., 2007) have improved chemical stability compared to the first generation of ILs. For instance, they have air- and water-stable properties, high stability (chemical, thermal, and electrochemical) and a wide liquid range. Alkyl-substituted imidazolium, pyridinium, ammonium, and phosphonium cations were retained, and more stable (less reactive compared to first-generation ILs anion, e.g. $AlCl_4$) anions were selected (e.g. tetrafluoroborate [BF_4^-] and hexafluorophosphate [PF_6^-] and bis(trifluoromethylsulfonyl)imide [NTF_2^-]) in this generation. Egorova et al. (2017) reported that many ILs from this group have high toxicity and low biodegradability. Furthermore, despite the goal of water-stable ILs, the anions (BF_4 and PF_6) were subsequently reported to easily break down into toxic chemicals (HF, POF_3, PO_2F_2, and PO_3F_2) in aqueous conditions (Freire et al., 2010; Swatloski et al., 2003; Terborg et al., 2012). While NTF_2 has high stability to hydrolysis, considerable resources are required to manufacture this perfluorinated anion.

The third generation of ILs are often referred to as task-specific ILs (TSILs) and can be split into two classes:

(i) ILs tailored for specific applications (e.g. catalysis or selective extraction) and
(ii) ILs whose structures have been designed to reduce toxicity and improve biodegradability.

However, it is challenging to design a TSIL which is effective for a desired application and has a low toxicity to a wide range of organisms. Hough et al. (2007) have described ILs with specific biological activity (i.e. API = active pharmaceutical ingredients) as a third generation (evolution) of ILs. Another example is catalytic trans-esterification by [TBA][Arg] to produce biodiesel fuel (Li & Guo, 2017). Moreover, Shiflett (2020) proposed a fourth generation in which ILs will have to be entirely sustainable.

1.2.3. Worldwide production and commercial availability of ILs

The wide range of functionalities and applications of ILs has inevitably attracted the interest of industry. As a rule, second-generation ILs have been used in industrial processes and commercial applications (Plechkova & Seddon, 2008).

In 2008, Plechkova & Seddon reported four major European companies with IL portfolios, i.e. BASF SE, Merck KGsA, Degussa AG and Acros. Additionally, some small enterprises, such as Solvent Innovation GmbH, ECOENG™ 500 and Solvent Innovation GmbH have commercialised ILs. "Peg-5-cocomonium methosulphate" was the first IL produced on a ton scale and registered by the European Inventory of Existing Commercial Chemical Substances (EINECS) (Shiflett, 2020). Moreover, 22 ILs were available on a multi-kilogram scale, including substituted quaternary ammonium sulphate. A large number of ILs have also been used in industrial chemical processes, such as BASIL™ by BASF, where 1-methylimidazole was used as an acid scavenger and has been applied for eliminations, esterifications, deprotonations, phosphorylations acylations, sulfurylations, silylations, and acid removals in general in lab trials (Maase et al., 2004).

The Ionic Liquid Market-Growth, Trends, and Forecast (2020-2025) stated that the compound annual growth rate of the IL market is expected to increase more than 8%

globally during 2020-2025 (Research and Markets, 2020). The coronavirus's global outbreak has influenced the market's growth because of the aquatic toxicity of ILs and the halt in their industrial production. However, the application of ILs in pharmaceuticals and cosmetics has been predicted to grow over the next five years.

BASF is one of the key international players in IL production. BASF (BASF, n.d.) offers a broad range of ILs with a wide variety of properties, e.g. Basionics™. Basionics™ LQ 01 is an IL with low viscosity, and excellent thermal and mechanical stability; it is used as an additive for moulding substances and foams, e.g. as an antistatic agent for safety shoes. Basionics™ ST 80 is a precursor for the synthesis of other ILs or can be used as a reaction medium. Finally, Basionics™ VS 03 has been used as an antistatic additive for moulding polyurethane compounds and foams.

From Asia, the Koei Chemical Co., Ltd has IL products, e.g. KOELIC™. The KOELIC™ series can be used as antistatic agents for resins, dissolve cellulose, electrolytes, and solidifying agents in resins & inorganic materials (Koei Chemical, n.d.). A wide variety of ILs is available from TCI chemicals (TCI Chemicals, n.d.), e.g. ammonium, imidazolium, and morpholinium salts. In addition, Solvionic (a company specialising in IL chemistry) has developed ILs for catalysis, surface preparation, and energy storage (Solvionic, n.d.). The German company IoLiTec (Ionic Liquids Technologies) has an extensive IL portfolio on their website (Iolitec, n.d.), including ILs as antistatic additives, photopolymerisable ILs, supported ILs, ILs as lubricants, for CO_2 capture and for metal recovery.

1.3. Toxicity and ecotoxicity assessment of ILs

In principle, ILs can be considered environmentally friendly for several reasons: they are non-volatile (reduced air pollution), non-flammable (process safety), and relatively stable (potential for recycling and reuse) (Bubalo et al., 2014). However, as ILs are highly soluble in water, they can reach aquatic and terrestrial environments via various waste-flows and thus their potential toxicity and low biodegradability may become a problem (Bubalo et al., 2014). Therefore, the hazard assessment of ILs has become a crucial area of research. Moreover, the whole life cycle (from synthesis to disposal) of ILs should be considered to see how "green" ILs are based on green chemistry principles.

The bibliometric analysis of the scientific publications in the Web of Science (WoS) database revealed that studies of ILs have increased enormously during the past two decades. The awareness of adverse effects that may occur due to ILs or their by-products has also gradually increased. However, there is remarkably little data on the toxicity and especially on the ecotoxicity of ILs compared to the growing overall information on ILs (**Figure 6**), limiting the quantitative risk assessment of ILs.

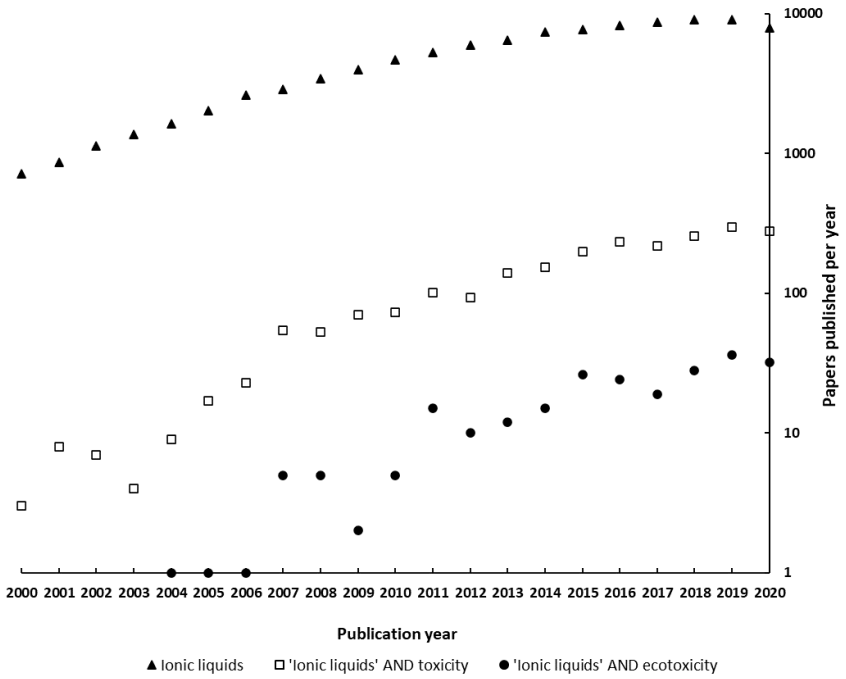


Figure 6. *The number of records in the Web of Science. A search was made on 25 November 2020, using 2000-2020 as a time-frame, with the keywords "ionic liquid", "ionic liquid" AND toxicity, and "ionic liquid" AND ecotoxicity (field: topic). "AND" indicates a combination of the respective keywords. Please note the logarithmic Y-axis.*

1.3.1. ILs release and the impact on the environment

Given the broad applications of ILs and the increasing industrial production (see sections 1.2.2 and 1.2.3), there is a growing possibility of the entry of ILs into various environmental compartments (water, soil and sediments) (Bubalo et al., 2014). As depicted in **Figure 7**, ILs can stay in the water column to interact with suspended particles and dissolved natural organic matter (NOM). They can also adsorb to the sediments/soil by different mechanisms, e.g. by ion-exchange and van der Waals interactions (Mrozik et al., 2008). ILs are relatively stable in the environment (Stepnowski & Zaleska, 2005) due to their resistance to photo-degradation (Gathergood & Scammells, 2002) and the limitation of specific ILs to biodegrading as mentioned above (Coleman & Gathergood, 2010). The degradation of ILs may occur aerobically when ILs stay in the water column or anaerobically when ILs reach sediments/soil (**Figure 7**).

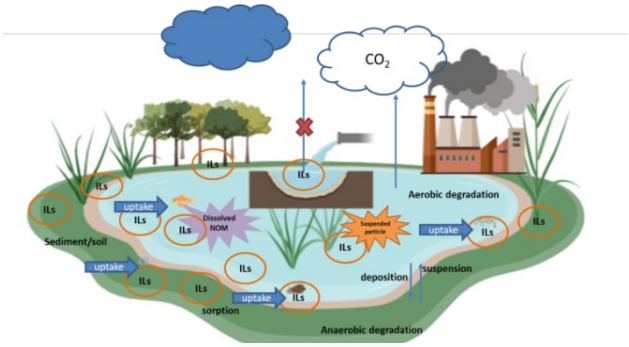


Figure 7. *A simplified scheme of the transport and transformation of ionic liquids (ILs) in the environment (modified from Amde et al., 2015 and created in Biorender.com).*

Ecotoxicity tests using species representing different food-chain levels from aquatic and terrestrial ecosystems can be used to evaluate the potential adverse effects of industrial chemicals (including ILs) on the environment (Amde et al., 2015). For that purpose, the dose (or concentration)-effect relationship must be created and analysed, and quantitative toxicity values (EC_{20}, EC_{50}, NOEC and PNEC) must be derived or predicted, depending on the parameter (see **Figure 8**).

In toxicology EC_n is the concentration of a chemical that yields an n% inhibitory effect on the tested organism. For instance, EC_{50} is the chemical concentration yielding a 50% inhibitory effect on the tested organism. NOEC (No Observed Effect Concentration) is the highest tested concentration for which there are no statistically significant differences in effect when compared to the control. PNEC (Predicted No-Effect Concentration) corresponds to a chemical concentration at which no adverse effect is expected to occur (below this concentration) for a specific organism. The value of PNEC is usually derived from NOEC values using specific safety coefficients.

When the given concentrations of chemicals in the environment are known (or predicted; PEC), the risk can be characterised as PEC/PNEC >1. There is no risk in PEC/PNEC <1; in the case of PEC/PNEC >1, there is a risk. Environmental risk assessment is vital to prevent future ecological deterioration (**Figure 8**).

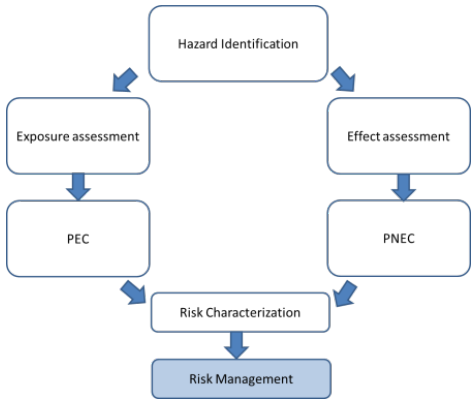


Figure 8*. The environmental risk assessment scheme (modified from* Vighi & Villa, 2013).

1.3.2. Ecotoxicological test battery for the evaluation of the environmental hazards of ILs

As mentioned above, the ecotoxicological effects of chemicals (including ILs) can be evaluated using different bioassays on environmentally relevant test species such as algae, plants, crustaceans, fish, rotifers, nematodes, earthworms, bacteria, and fungi. Some examples of these studies can be found in (Du et al., 2018; Jordan et al., 2016; Ventura et al., 2013a; Xia et al., 2018). A search in the WoS made on 23 November 2020 (see **Table 1** and **Figure 9**) showed that of algae, plants, crustaceans, fish, rotifers, nematodes, earthworms, bacteria and fungi, the most often used organisms for toxicity studies of ILs were bacteria (220 publications published), and toxicity data for rotifers were found only in one report published out of 633 published articles.

Table 1. *Share of scientific information on the ecotoxicity of ILs for different environmentally relevant organism groups. Data were retrieved from the WoS on 23 November 2020, using the search terms "ionic liquids" AND toxicity AND organism (e.g. algae/crustaceans/fish/rotifers/nematodes/earthworms/bacteria/fungi/plants), and the time period from 2000-2020. The total number of publications was 633. See also* ***Figure 9.***

Organism	Number of paper(s)
Bacteria	220
Plants	134
Algae	96
Crustaceans	61
Fish	56
Fungi	43
Earthworms	17
Nematodes	5
Rotifers	1

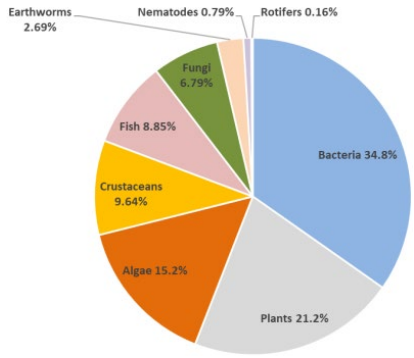


Figure 9. *Share of scientific information on the ecotoxicity of ILs for different environmentally relevant organism groups. Data are plotted from* ***Table 1****.*

Tests with algae, crustaceans, and fish – representatives of the simplified aquatic food web – are compulsory ecotoxicity tests in the REACH regulation (ECHA, 2017). According to REACH (ECHA, 2017), all chemicals produced in Europe or imported to Europe must be registered and characterised for their potentially hazardous effects using a test battery and the number of tests is based on the substances' quantity. Between 1 and 10 t annually produced or imported chemicals, the aquatic short-term tests on invertebrates

(preferably *Daphnia*) and aquatic plants (preferably algae) are mandatory. Chemicals produced/imported from 10 -100 t per year in/to Europe must be additionally tested with a fish acute toxicity test and an activated sludge test. Thirdly, chemicals produced/imported in amounts between 100-1000 t per year in/to Europe must be additionally tested with fish long-term toxicity tests (including various endpoints) and with invertebrates long-term toxicity test (preferably *Daphnia* species) and short-term tests with terrestrial invertebrates, plants, and soil organisms. For the chemicals produced/imported in amounts exceeding 1000 t per year in/to Europe, long-term tests with terrestrial invertebrates and plants, sediment organisms and birds must be performed.

Another group of organisms that plays an essential role in decomposing the organic matter in the food web are bacteria and fungi (Diaz et al., 2018; Hernández-Fernández et al., 2015; Samori et al., 2010). Bacteria are not only highly relevant test organisms but are also very suitable for the toxicity evaluation of chemicals because of the ease of handling bacteria in the laboratory and the cost efficiency of these tests. One of the bacterial species widely used for ecotoxicity studies (Kahru et al., 1996; Hernández-Fernández et al., 2015) and often used for QSARs (Aruoja et al., 2011; Cronin & Schultz, 1997) is *Vibrio fischeri* (previously known under the name of *Photobacterium phosphoreum* NRRL-B-11177 and currently known as *Aliivibrio fischeri;* Kurvet et al., 2011). *V. fischeri* has also often been used to evaluate ILs (Hernández-Fernández et al., 2015; Samori et al., 2010; Vieira et al., 2019). On this bacterial strain the first commercial ecotoxicity test –Microtox™ – is based (Bulich & Isenberg, 1981; Kaiser & Devillers, 1994; Wolska et al., 2007).

In general, ILs hazard potential ranges from low to high, depending on the chemical structure of the ILs and the test organism/toxicity test format (Amde et al., 2015). ***Table 2*** presents an example of the battery of aquatic tests that has been used to evaluate the toxicity of ILs.

Table 2. *Examples of the organisms, tests and toxicity endpoints that have been used for the evaluation of the aquatic toxicity of ILs.*

Trophic level of the test organism	Toxicity test	Toxicity endpoint	Exposure time, toxicity endpoint	References
Decomposers	Bacterial test *Vibrio fischeri* (Microtox™ test); ISO 21338	Inhibition of bacterial bioluminescence	5 min, 15 min, and 30 min -EC_{50}	(Ventura, et al., 2012)
Primary producers	Algal test (*Rapidocelis subcapitata*); OECD 201	Algal growth inhibition	72 h-EC_{50}	(Pretti et al., 2009)
Primary consumers	Microcrustacean *Thamnocephalus platyurus* (Thamno toxkit F™), ISO 14380	Mortality test	24 h-LC_{50}	(Tsarpali & Dailianis, 2015)
	Microcrustacean Daphnia magna (Daphtoxkit F™); OECD 202	Immobility/ mortality test	24 h and 48 h-LC_{50}	(Pretti et al., 2009)
Secondary consumers	Cnidarian test (*Hydra attenuata* assay); Trottier et al., (1997)	Acute sublethality and lethality indicated by morphological changes	24, 48, 72, and 96 h-LC_{50}	(Costa et al., 2015)
	Fish test (*Danio rerio*); OECD 203	Mortality test	92 h-LC_{50}	(Pretti et al., 2009)

1.3.3. Antibacterial activity of ILs

As was mentioned above, bacteria are very relevant test organisms for ecotoxicity analysis. However, bacteria/microbes inhabit not only various environmental compartments (soil, water and sediments) and play a dominant role in the biogeochemical cycling of nutrients (Briški & Domanovac, 2019), but are also important co-inhabitants in animal (Ezenwa & Williams, 2014) and human guts (Waters & Ley, 2019) and skin. Importantly, certain bacterial strains, e.g., *Staphylococcus aureus* and *Escherichia coli,* can cause various diseases in humans and animals (Bélanger et al., 2011; Vesterlund, et al., 2006).

To date, microbial infections in hospitalised patients are still challenging to treat, mostly due to drug resistant bacteria (Zheng et al., 2016). The research on new antibacterial agents that could overcome the resistance problem has rapidly developed (Gilmore, 2011; He et al., 2015; Wang et al., 2019; Zheng et al., 2016) and involves e.g., polymer hydrogels (Fan et al., 2014) metals (Wu et al., 2014), cationic compounds (Kubo et al., 2017; Zhao et al., 2016), and antimicrobial peptides (Yu et al., 2015).

The potential toxicity of ILs may be problematic for ”green” chemistry applications but can lead to the design of IL-based antimicrobials agents and pharmaceuticals, e.g. active

pharmaceutical ingredients (APIs). Indeed, ILs can be specifically tailored by varying the composition of their cations and anions resulting in a variety of ILs with a broad range of lipophilicity, acidity and viscosity (Rogers & Seddon, 2003; Stoimenovski et al., 2010). Usually, the antibacterial properties of ILs are tested against both gram-positive (for example *Staphylococcus aureus*) and gram-negative (for example *Escherichia coli*) bacteria as they have different types of cell walls and thus different tolerances to chemicals (including ILs). It has been shown that ILs have stronger inhibitory effects towards gram-positive bacteria than towards gram-negative ones, e.g. ILs derived from *N*-cinnamyl imidazole (Doria et al., 2018); $[P_{6,6,6,14}][Phosp]$, $[P_{6,6,6,14}][NTf_2]$, and $[P_{6,6,6,14}][N(CN)_2]$ (Ventura et al., 2012); 1-ethylpyridinium docusate and tributyl(2-hydroxyethyl)phosphonium docusate (Choi et al., 2011). However, it has also been shown that ILs have more potent inhibitory effects on gram-negative bacteria, for example $[C_4mim][DMP]$, $[C_4mim][PF_6]$, $[C_6mim][Cl]$ and $[C_8mim][Cl]$ (Ventura et al., 2012); $[C_6mim][SCN]$, $[C_4pyr][DCA]$ (Mester et al., 2015); cholinium amino acid based ILs (Hossain et al., 2013); 1-(2-hydroxethyl)-3-methylimidazlium based ILs (Hou et al., 2013), and choline and geranate (CAGE)-based ILs (Ibsen et al., 2018). Furthermore, Ferraz et al. (2014) showed that API-ILs based on ampicillin anions were potent inhibitors of Amp-resistant bacterial strains (namely *E. coli* TEM CTX M9, *E. coli* CTX M2 *and E. coli* AmpC MOX. Coleman et al. (2012) showed that most of the imidazolium salts (chiral and achiral) studied by them were non-toxic (IC_{95} > 2 mM) to eight bacterial strains and 12 fungal strains. However, one of the chiral imidazolium ILs showed the highest toxicity potency (125 µM after 24 h incubation and 500 µM after 48 h) to the methicillin-resistant *Staphylococcus aureus* (MRSA) and the presence of hydrophobic and aromatic phenylalanine units in ILs increased the toxicity of ILs (Coleman et al., 2012).

There is a range of methods available for the determination of microbial susceptibility to antimicrobial compounds. Furthermore, these tests allow for the determination of the relative potency of antimicrobial agents over a variety of microbial species and for the detection of antimicrobial compounds' synergies. The most widely used methods yield information on minimum inhibitory concentration (MIC) or the minimum bactericidal concentration (MBC) of the chemical of interest (Gilmore, 2011).

Like all chemicals with potential use as biocides, there are regulatory and commercial obstacles to their market entry. The regulatory instruments are essential to ensure public safety and ensure safely in taking ILs as biocides. In 1998, the Biocidal Products Directive (98/8/EC) was publicly introduced to harmonise the manufacture and use of biocides with the European biocidal product market. In addition, the purpose of the directive was to ensure a high degree of protection for humans, animals and the environment (Gilmore, 2011). Furthermore, medicines and cosmetics fell outside the scope of the directive, theoretically allowing ILs to be used as preservatives. In June 2009, on the basis of experience operating under the Biocidal Products Directive (98/8/EC), the European Commission adopted a proposal for a regulation on the marketing and use of biocidal products (COM (2009)267) intended as a complete revision of the current directive, which it would revoke and replace. The proposed new regulation was expected to come into effect on 1 January 2013. Finally, in 2013 the Biocidal Products Directive was replaced by the Biocidal Products Regulation (BPR, Regulation (EU) 528/2012)[1].

Based on data from the WoS (retrieved on 18 December 2020), there were 937 publications that contained the term "ionic liquid" AND "antimicrobial", from 1980 to

[1] https://echa.europa.eu/regulations/biocidal-products-regulation/understanding-bpr

2021. In general, the antimicrobial activity of Surface-Active Ionic Liquids (SAILs; see paragraph 1.6) the ILs studied in the current thesis is related to the length of the carbon chain, and membrane disruption has been proposed as the mechanism of antimicrobial action of ILs. Indeed, many ILs have similar structures to cationic surfactants whose primary mode of action is membrane-bound protein destruction (Bernot et al., 2005).

1.4. Toxicity mechanism of ILs

The properties of ILs are influenced by their composition: combinations of various cations and anions. According to Bubalo et al. (2017), there are approximately 10^{18} ILs with different chemical and physical properties such as melting, solubility, acidity, lipophilicity, density, viscosity and refractive index, which can be synthesised. Furthermore, the chemical structure of ILs, including cation, alkyl chain length and anion, may affect toxicity. Indeed, most of the toxicity studies of ILs using different organisms have shown that the chemical structures of ILs plays a significant role in their toxicity (see **Figure 10**).

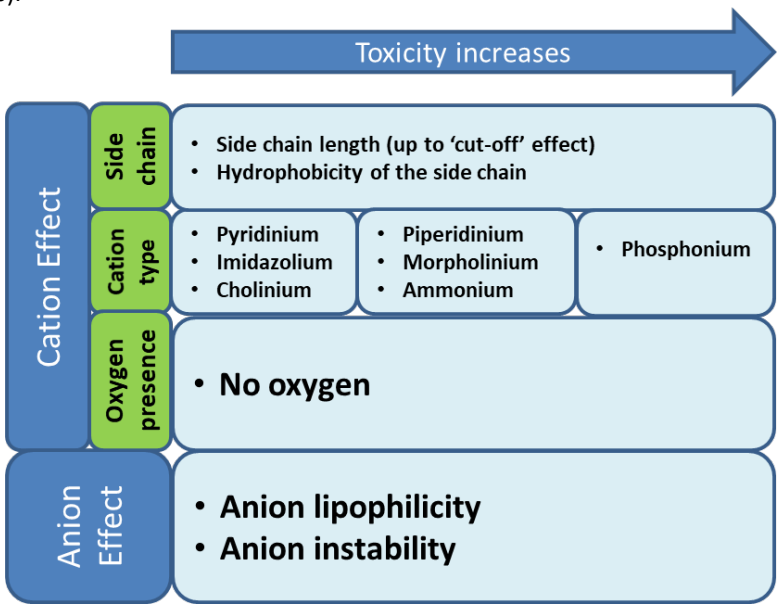


Figure 10. *General overview of the structural modifications of ILs which result in increased toxicity towards model test organisms (modified from Amde et al., 2015).*

The length of the alkyl chain has an effect on the toxicity of ILs: the longer the chain the higher the toxicity. For example, results of the toxicity studies of 29 imidazolium, pyridinium, and ammonium-based ILs towards bioluminescent bacteria *V. fischeri* revealed that the alkyl chain length had a more significant influence on the toxicity of ILs than the type of anion, cation core or functionalised side chain of the cation (Montalbán et al., 2016). Another relevant example is the decrease in the growth rate of algae *Scenedesmus quadricauda* in the presence of [C_4mim][Br], [C_6mim][Br], and [C_8mim][Br] (Kulacki & Lamberti, 2008). Elongation of the alkyl chain length of another set of ILs also increased the toxicity of ILs against *Artemia salina* crustaceans (Gouveia et al., 2014). Interestingly, there was also an irregularity in this continuum, called the "cut-off effect" (Montalbán et al., 2016) which was also observed with the elongation of the alkyl chain

length of the pyridinium, imidazolium and cholinium types of ILs in respect to their toxicity towards various bacteria and fungi (Kapitanov et al., 2019), towards *V. fischeri* bacteria, *Scenedemus vacuolatus* algae, and *Lemna minor* aquatic plants (Stolte et al., 2007) and earthworms (Du et al., 2018). Also, (Hou et al., 2013) showed that cholinium amino acid ILs had low toxicity against bacteria and good biodegradability.

The effect of the anion' type on the toxicity of ILs usually depends on the test organism. Frade & Afonso (2010) showed that toxicity to *V. fischeri* changed as follows: $[C_4mim][Br] < [C_4mim][DCA] < [C_4mim][Cl] < [C_4mim][BF_4] < [C_4mim][PF_6] < [C_4mim][NTf_2]$. In contrast, the phytotoxicity studies of $[C_2mim][Br]$, $[C_2mim][NO_3]$, $[C_2mim][Ts]$, $[C_2mim][dMP]$, and $[C_2mim][MS]$ using spring barley showed that there was no difference in toxicity between the five types of anions (Biczak et al., 2014). Toxicity studies of three ILs with different types of anions (Ma et al., 2020) using rice (monocotyledons representative) and capsicum (dicotyledon representative) as models showed that toxicity to rice increased in the following order $[TF_2N] > [PF_6] > [BF_4]$; the toxicity to capsicum increased as follows: $[BF_4] > [TF_2N] > [PF_6]$. Additionally, the toxicity studies of a set of 1-alkyl-3-methylimidazolium nitrate derived ILs with different anions towards *Danio rerio* fish showed that the LC_{50} values of these ILs were comparable (Zhang et al., 2017). Another study by Pinto et al. (2012) showed that the BF_4^- anion had a significant effect on *V. fischeri* compared to Cl^- of $[C_4mim]$ ILs.

The effect of cation type on ILs' toxicity also depends on the target organism. Reid et al. (2018) reported that cholinium derived aprotic IL showed different toxicities to the targeted bacteria. For instance, *Staphylococcus epidermidis* was more sensitive (MIC 1000 μM) than *S. aureus* with the MIC > 2000 μM against *N*,*N*,*N*-trimethylethanolammonium acetate. Stolte et al. (2007) showed that *V. fischeri* (EC_{50} > 20000 μM) was more tolerant than *Lemna minor* (EC_{50} 1280 μM) against [Mor14][Br]; but *Lemna minor* (EC_{50} 295 μM) was more tolerant than *V. fischeri* (EC_{50} 18600 μM) against [Pip14][Br].

There is an effect of the presence of oxygen atoms in the alkyl chain. A study by Morrissey et al. (2009) on oxygen-functionalised imidazolium ILs, i.e. containing ester and ether side chains demonstrated that these ILs reduced antimicrobial activity to three gram-positive (*Staphylococcus aureus*, *Enterococcus sp*., *Bacillus subtilis*) and four gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella sp*., *Salmonella sp*.) bacteria. Samorì et al. (2007) also found that the presence of an oxygenated side chain in the imidazolium cation can significantly reduce the toxicity of the ILs towards *D. magna* crustaceans and *V. fischeri* bacteria.

1.5. Biodegradation of ILs

Microorganisms (bacteria and fungi) play a vital role in Earth's biogeochemical cycles by biodegrading most of the natural products and industrial chemicals ending up in nature. Substances converted or degraded by microorganisms are used by other organisms as sources of energy, carbon, nitrogen, or other nutrients. "Biodegradation" involves the breakdown of organic compounds into biomass and less complex compounds, and eventually mineralising them into the water, carbon dioxide and oxides or mineral salts of other elements (Fetzner, 2002). The biodegradation potential of a compound determines and predicts the interaction between chemicals and the environment (including biodegrading microorganisms) (Jordan & Gathergood, 2015). The rates of chemicals biodegradation in the environment may vary from days or weeks to years or

decades (Fetzner, 2002). Jordan & Gathergood (2015) classified the biodegradation potential of compounds into five categories:

1) primarily biodegradable: loss of a particular structure moiety, e.g. the hydrolysis of the ester bond;
2) inherently biodegradable: given the chemical biodegrades by ~ 20%, the probability of further degradation is expected.
3) readily biodegradable: biodegradable by a particular percentage within a specified timeframe.
4) ultimately biodegradable: the chemical completely breaks down.
5) mineralisable: the breakdown of the chemical into molecules available to and taken up by microorganisms.

The Organization of Economic Co-Operation and Development (OECD) and the International Organization for Standardization (ISO) have developed guidelines for biodegradation tests. For example, ISO 10708 and OECD 301D are available for fresh-water biodegradation; both procedures are performed as "closed bottle tests" (CBT), with 60% ThOD (theoretical oxygen demand) after a 28-days test as a pass level; OECD 301A-D is available for inherent biodegradation, and OECD 306 is the testing guideline for biodegradability in seawater with > 70% DOC (dissolved organic carbon) after 28 days test as a pass level. Furthermore, Gore et al. (2013) have proposed a colour code (visible light metric system) for biodegradation level, with the following criteria: green (≥ 60% readily biodegradable), amber (20-59%) and red (0-19%).

From a green chemistry point of view, chemicals and their processes should be non-toxic, readily biodegradable, and satisfy technological and economic demands (Matzke et al., 2010). These parameters are useful for designing safer ILs and for reducing environmentally persistent molecules in ecosystems. For more information, there are several reviews on the toxicity, ecotoxicity, biodegradation, and bioaccumulation of ILs (Amde et al., 2015; Coleman & Gathergood, 2010; Jordan & Gathergood, 2015; D. Zhao et al., 2007).

Biodegradation rules of thumb for chemicals have been introduced by Boethling et al. (1994). As ILs have structure similar to conventional surfactants, the same "rules of a thumb" can be applied. These "rules of thumb" are several factors that can affect the percentage of the mineralisation of chemical compounds by microbial degradation. For example, beneficial for biodegradation is the presence of aromatic rings, long non-substituted alkyl chains, and hydrolysable groups, such as alcohols, aldehydes and carboxylic acids. On the other hand, the presence of halides, alkyl chain branching, quaternary carbon atoms, tertiary nitrogen atoms, heterocycles, and aliphatic ethers do not support compound's biodegradation. However, these "rules of thumb" do not guarantee that the presence of a single group correctly determines whether a compound will be biodegradable or persistent in the environment (Coleman & Gathergood, 2010).

Jordan & Gathergood (2015) examined the relevance of Boethling's "rules of thumb" for the biodegradation potential of ILs and concluded that the rules can be applied to the subset of chemicals known as ILs. For example, increases in alkyl chain length, due to the extra oxidisable carbons in the chain, lead to higher biodegradability. This was notably observed in both the anion and cation of the IL series studied. The use of an inorganic ion does not contribute directly as a carbon source for biodegradation determination, e.g. halides or pseudohalides. As many ILs contain inorganic anions, care must be taken when determining the percentage of biodegradation of an IL or a salt, where the fate of

both ions must be considered. Key features of ILs that increase biodegradability are long unbranched alkyl chains, the presence of esters (hydrolysable groups), alcohols (easily oxidised groups), and carboxylic acids.

1.6. The need for greener surface-active ionic liquids (SAILs)

According to Nandwani et al. (2020) SAILs are a special type of ILs of an amphiphilic character. In other words, SAILs are ILs with hydrophilic and lipophilic components. Usually, the lipophilic (tail) part consists of a long hydrocarbon (e.g. alkyl) chain and the hydrophilic group (head) can be either a cation or anion. SAILs undergo self-aggregation in solution. It has been observed that SAILs' properties include a low critical micelle concentration (CMC) comparable to conventional surfactants with similar chemical structures (Nandwani et al., 2020).

This class of ILs can be easily modified for task-specific functions by a simple change in their chemical structure, e.g. modifying the charge of the head or tail group. This particular "fine-tuning" property of all ILs, including SAILs, has drawn enormous attention among researchers in diverse fields, including chemistry, nanomaterials, and biotechnology (Pernak et al., 2016).

A literature search of "surface active ionic liquid" in WoS on 3 December 2020, showed 290 studies from 2000 to 2020 (see **Figure 11**): the search for SAILs began in 2007 with two studies and increased to 45 studies in both 2018 and 2019. In 2007, El Seoud and co-workers synthesised the first series of SAILs. Then, Dong et al. (2007) studied the surface tension and electrical conductivity of SAILs called 1-alkyl-3-methylimidazolium (e.g. $[C_{10}mim][Br]$, $[C_{12}mim][Br]$, and $[C_{12}mim][BF_4]$) and demonstrated that their surface activity (CMC values) was lower than that of commercial cationic surfactants (alkyltrimethylammonium bromides) and comparable to anionic surfactants (such as sodium alkyl sulphates). However, a major concern is the high microbial toxicity of these commercial cationic surfactants (including BAC salts). Therefore, the search to find safer (lower toxicity) surfactants (including SAILs) requires toxicological assessment.

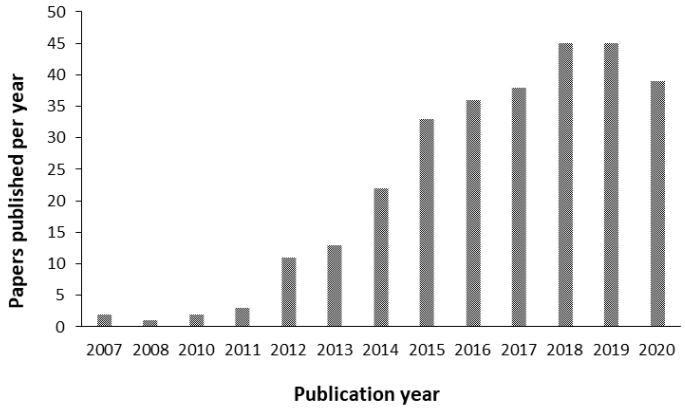


Figure 11. *The literature search in the Web of Science on the search term "surface active ionic liquid" on 3 December 2020, time-frame from 2000 to 2020.*

Given the self-aggregation properties of SAILs, they can be used as surfactants. Indeed, SAILs have exhibited superior surface activity, and are also capable of forming supramolecular nano- to giant aggregates *viz.* micelles (Blesic et al., 2007; Galgano &

El Seoud, 2011), vesicles (Liu et al., 2016; Wang et al., 2013) and wormlike micelles (Bi et al., 2015; Dong et al., 2008). As a result, SAILs can be useful in industrial, chemical (Cognigni et al., 2017) or pharmaceutical applications (Singla et al., 2018). The synthesis of an amphiphilic poly(ionic liquid) dendron from cashew nut shell oil was investigated by Atta et al. (2018) and applied as asphaltene dispersants and demulsifiers. An investigation by Bhat et al. (2019) showed that dodecyl-3-methylimidazolium chloride (DDMIMCl) was a safe, economical, eco-friendly, and efficient dehalogenation of halocarbons. Furthermore, De Faria et al. (2017) extracted triterpene acids (TTA) using a series of SAILs and as a result, higher yields of TTA were generated compared to chloroform and acetone under the same condition. In addition, cholinium dodecylbenzenesulfonate (Cho[DBS]) was confirmed to be a non-toxic SAIL against freshwater microalgae *Scenedesmus* sp, so the non-toxic nature of Cho[DBS] makes it useful in the laundry industry as a substitute for NaDBS (Gehlot et al., 2017).

However, Mustahil et al. (2019) found that there were problems with the toxicity of surface-active lauroyl sarcosinate ionic liquids (SALSILs). Three of the nine studied SALSILs were toxic against tested (gram-positive and gram-negative) bacteria and the six remaining SALSILs demonstrated moderate to low activity depending on the tested bacteria. Therefore, the low toxicity profile of the synthesized SALSILs and the higher biodegradability provides opportunities for the use of these surfactants in different industrial applications.

Additionally, efforts to prepare readily biodegradable and low-toxicity SAILs were reported by Kapitanov et al. (2019) and Suk et al. (2020) for a series of L-phenylalanine-derived SAILs. Suk et al. (2020) concluded that the biodegradibility of SAILs tested decreased according to the cation head group in the folowing order: PyC_nPhe > $ImidC_nPhe$ > $CholC_nPhe$. Petkovic et al. (2011) stated that SAILs toxicity and biodegradability depended upon their structure (e.g. the length of the alkyl chain and counter ion) and concentration in the environment.

As discussed in Sections 1.3 and 1.4, the commonly used ILs are not “benign”, fulfilling only some of the green chemistry principles, but not the majority. However, with the modular structure of ILs, ILs can be tuned to possess the unique properties needed (Zhao et al., 2007). Various studies have reported producing “greener” ILs from natural resources, e.g. from sugars (Chiappe et al., 2010) and amino acids (Ohno & Fukumoto, 2007). Kagimoto and Ohno in Plechkova & Seddon (2012) wrote that the properties of amino acid ILs that have attracted attention include being halogen-free, cheap, and easy to synthesise. Moreover, Gathergood and co-workers (Garcia et al., 2005; Gathergood et al., 2004; and Gathergood et al., 2006) have developed series of biodegradable imidazolium ILs by including ester or amide groups. ILs with ester functionality proved more biodegradable than others because of their susceptibility to enzyme hydrolysis. In general, molecules or ions derived from natural sources are not always considered non-toxic but their toxicity may depend on the side chain, the anion, the cation and their chemical modifications in a particular group (Plechkova & Seddon, 2012). Furthermore, Zhao et al. (2007) have suggested that the development of “greener” ILs should be done in cooperation with toxicologists. Therefore, considering the wide application and available data on SAILs, the study of environmentally favourable structural elements and the designing of inherently safer SAILs are essential.

Aims of the study

The overall objective of this study was to investigate the potential environmental hazard of a series of 24 L-phenylalanine SAILs (see **Figure 12**) to the aquatic ecosystem and identify the antibacterial potential of these SAILs.

Specific aims of the study were:

1. To determine the influence of different head groups and alkyl chain lengths of the above-described SAILs on their toxicity to three aquatic organisms of different food-chain levels (*Vibrio fischeri* bacteria, *Thamnocephalus platyurus* crustaceans, and *Raphidocelis subcapitata* algae) and to use these data:
    a. to predict the ecotoxicity ranking of these SAILs based on their EC_{50} values (in mg/L and μM);
    b. to suggest an initial screening ecotoxicity test battery for the SAILs;
    c. to examine the correlation between the (eco)toxicity and log K_{ow}, CMC, and $\Delta G°_{ad}/A_{min}$ of the SAILs and perform QSARs to assist the discovery of safer (lower ecotoxicity) SAILs.
2. To identify which of the 24 SAILs is preferred for future studies as green surfactants.
3. To compare the antibacterial potency of SAILs containing different head groups and having different lengths of the alkyl chain of the above-described SAILs using two medically relevant bacteria: gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus*.

2. Materials and methods

2.1. L-phenylalanine derived surface-active ionic liquids (SAILs)

Three families of L-phenylalanine-derived surface-active ionic liquids (SAILs) based on pyridinium, imidazolium, and cholinium cations, all paired with bromide-anion and varying alkyl chains from 2 to 16 carbon atoms were studied in this work. Altogether, a series of 24 L-phenylalanine SAILs (see **Figure 12**) were synthesised at Tallinn University of Technology in the laboratory of the ERA Chair of Green Chemistry. The full names, chemical structures, and abbreviations of the studied SAILs are given in **Table 3**.

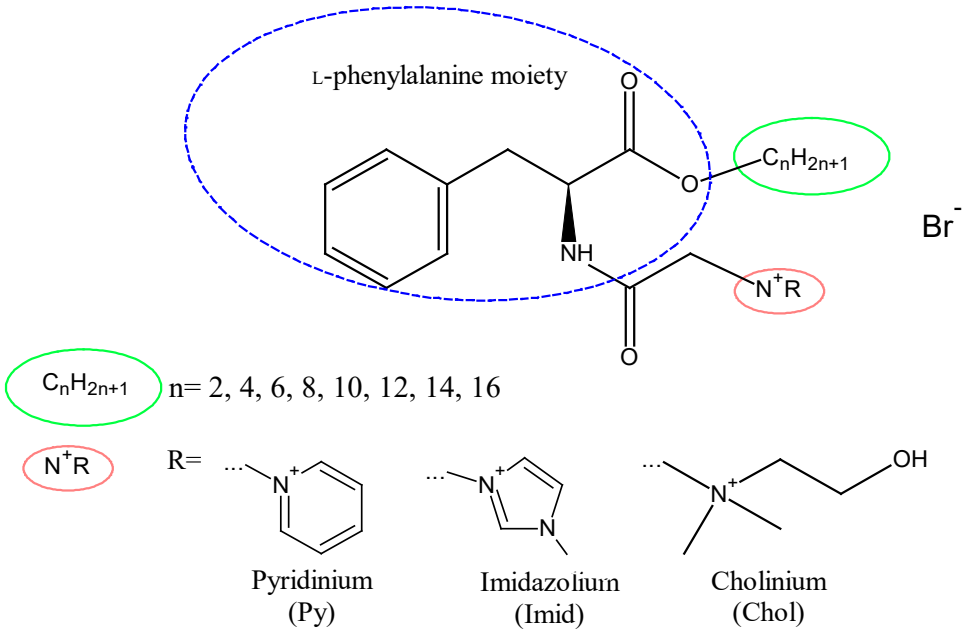
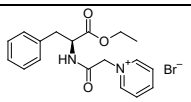
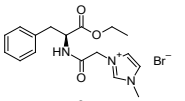
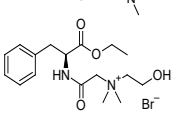
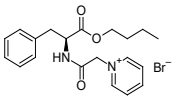
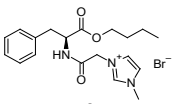
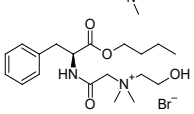
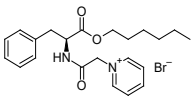
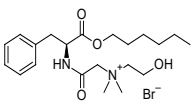
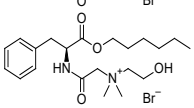
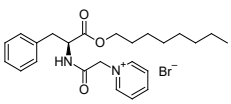
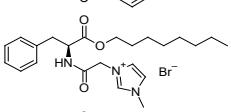
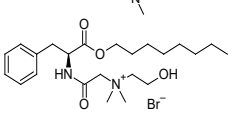
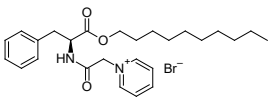
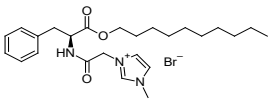
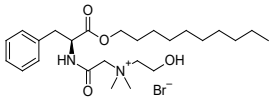
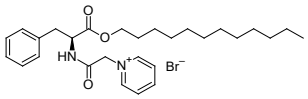
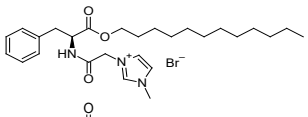
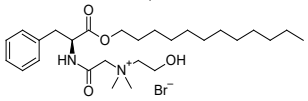
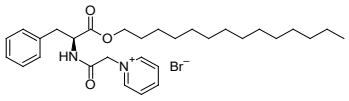
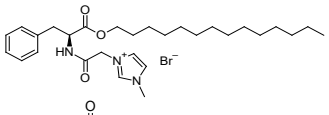
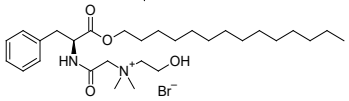
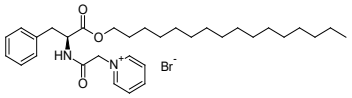
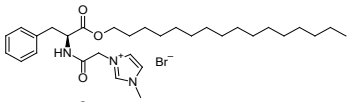
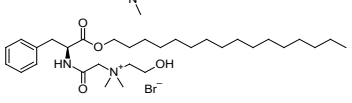


Figure 12. *A series of L-phenylalanine derived SAILs (PyC_nPhe, $ImidC_nPhe$, and $CholC_nPhe$) were studied in this work. All SAILs are Br^- salts. The figure has been modified from Figure 1 of* ***Publication I****.*

Table 3. *Chemical structures, names, and molecular weight (MW) of SAILs studied.*

Structure	Abbreviation and MW	Chemical Name
	PyC_2Phe 393.28	(*S*)-1-(2-((1-(Ethyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_2Phe$ 396.29	(*S*)-1-Methyl-3-(2-((1-(ethyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide
	$CholC_2Phe$ 403.32	(*S*)-2-((1-(Ethyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide
	PyC_4Phe 421.34	(*S*)-1-(2-((1-(Butyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_4Phe$ 424.34	(S)-1-Methyl-3-(2-((1-(butyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide
	$CholC_4Phe$ 431.37	(*S*)-2-((1-(Butyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide
	PyC_6Phe 449.39	(*S*)-1-(2-((1-(Hexyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_6Phe$ 452.39	(*S*)-1-Methyl-3-(2-((1-(hexyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide
	$CholC_6Phe$ 458.18	(*S*)-2-((1-(Hexyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide
	PyC_8Phe 477.44	(*S*)-1-(2-((1-(Octyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_8Phe$ 480.45	(*S*)-1-Methyl-3-(2-((1-(octyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide
	$CholC_8Phe$ 487.48	(*S*)-2-((1-(Octyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide
	$PyC_{10}Phe$ 505.50	(*S*)-1-(2-((1-(Decyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_{10}Phe$ 508.50	(*S*)-1-Methyl-3-(2-((1-(decyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide

	$CholC_{10}Phe$ 515.53	(*S*)-2-((1-(Decyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide
	$PyC_{12}Phe$ 533.55	(*S*)-1-(2-((1-(Dodecyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_{12}Phe$ 536.56	(*S*)-1-Methyl-3-(2-((1-(dodecyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide
	$CholC_{12}Phe$ 543.59	(*S*)-2-((1-(Dodecyloxy)-1-oxo-3-phenyl-propan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide
	$PyC_{14}Phe$ 561.60	(*S*)-1-(2-((1-(Tetradecyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_{14}Phe$ 564.70	(*S*)-1-Methyl-3-(2-((1-(tetradecyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide
	$CholC_{14}Phe$ 571.43	(*S*)-2-((1-(Tetradecyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide
	$PyC_{16}Phe$ 589.66	(*S*)-1-(2-((1-(Hexadecyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)pyridin-1-ium bromide
	$ImidC_{16}Phe$ 592.76	(*S*)-1-Methyl-3-(2-((1-(hexadecyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-2-oxoethyl)-1H-imidazol-3-ium bromide
	$CholC_{16}Phe$ 599.49	(*S*)-2-((1-(Hexadecyloxy)-1-oxo-3-phenylpropan-2-yl)amino)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2-oxoethan-1-aminium bromide

2.2. Toxicity tests

A library of 24 SAILs (**Table 3**) was toxicologically profiled using the naturally luminescent marine bacteria *Vibrio fischeri* (**Publication I**), two clinically relevant pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus* (**Publication II**) and the freshwater microalgae *Rapidhocelis subcapitata* (**Publication III**). In addition, a subset of the SAILs was tested for toxicity using the aquatic crustaceans *Thamnocephalus platyurus* (**Publication III**) (see **Table 4**).

***Table 4**. The series of SAILs evaluated for toxicity using different tests.*

	SAILs			
Test organisms	**Pyridinium**	**Imidazolium**	**Cholinium**	**Publication**
Bacteria				
Vibrio fischeri	C_2-C_{16}	C_2-C_{16}	C_2-C_{16}	I
Escherichia coli	C_2-C_{16}	C_2-C_{16}	C_2-C_{16}	II
Staphylococcus aureus	C_2-C_{16}	C_2-C_{16}	C_2-C_{16}	II
Algae				
Raphidocelis subcapitata	C_2-C_{16}	C_2-C_{16}	C_2-C_{16}	III
Crustaceans				
Thamnocephalus platyurus	C_2-C_{16}	C_6 & C_8	C_6 & C_8	III

All toxicity studies of this work were conducted at the National Institute of Chemical Physics and Biophysics at the Laboratory of Environmental Toxicology and performed according to the previously developed methods for which the exact details can be found in the publications included in this thesis (**Publication I** to **III**). The SAILs were analysed using the following multitrophic test battery:

- kinetic bioluminescence inhibition assay with *Vibrio fischeri* bacteria (ISO 21338:2010) – **Publication I**
- antimicrobial efficiency evaluation assay using *Escherichia coli* gram-negative bacteria and *Staphylococcus aureus* gram-positive bacteria (ISO 20776:1-2006) – **Publication II**
- growth inhibition assay with *Raphidocelis subcapitata* algae (OECD 201, 2011) – **Publication III**
- mortality test with *Thamnocephalus platyurus* aquatic crustaceans (ISO 14380:2015) – **Publication III**

The synthesis, characterisation and biodegradations studies of the 24 SAILs in this thesis were carried out in tandem with the work reported in this thesis (see **Figure 13**). Ecotoxicity studies are an integral part of the research and development of safer SAILs.

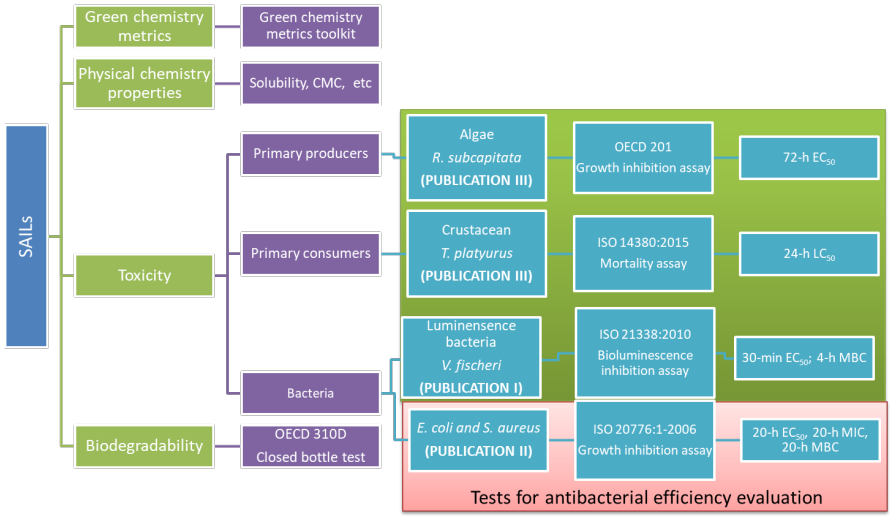


Figure 13. *Schematic representation of the overall work with the series of 24 L-phenylalanine-derived SAILs. Synthesis, green chemistry metrics, physical chemistry properties, and biodegradability studies were not performed in the framework of this thesis and were taken from the publications of the teams of Gathergood and Kümmerer* (Kapitanov et al., 2019; *Suk et al., 2020).*

3. Results and discussion

As described in section 1.3.2, according to the EU chemical regulation REACH, new industrial chemicals need to be assessed for their potential hazards using ecotoxicological tests the number of which is based on the annual production and/or import quantity of the respective chemicals. Previously, the team of Prof. Gathergood in TalTech used microbial toxicity test(s) to complement a biodegradation test (closed bottle test; CBT) to evaluate if ILs had undesirable inhibitory effects on bacteria and/ or fungi (Coleman et al., 2012; Gore et al., 2013; Jordan et al., 2016). The main drawback of the approach used by Gathergood and co-workers was that it was not possible to establish the quantitative numerical toxicity values (e.g. EC_{50}) necessary to conduct SARs for the ILs tested since the highest concentration tested 1-2 mM was below the EC_{50} value. In the current PhD thesis, we studied the concentration range of ILs that made it possible to quantify EC_{50} values (or MBC values) addressing both, ecotoxicity and the antibacterial potential of 24 L-phenylalanine SAILs.

3.1. Toxicity evaluation of SAILs

A library of 24 SAILs (see **Table 3**) was evaluated for their potential environmental hazards using organisms of different trophic levels in the aquatic food chain, e.g. *Rapidocelis subcapitata* (algae; representing primary producers), *Thamnocephalus platyurus* (crustaceans; representing consumers) and *Vibrio fischeri* (bacteria; representing decomposers). Although *V. fischeri* is not included in the ecotoxicity assessment of industrial chemicals for regulatory purposes, it is widely used in ecotoxicology and widely used to create toxicity libraries for QSARs (Aruoja et al., 2011; Cronin & Schultz, 1997). In addition, gram-positive and gram-negative medically relevant bacteria were used to study the potential antibacterial activity of these SAILs.
As **Publication III** also refers to the results of **Publication I** as part of the toxicity assessment of the SAIL, these publications will be discussed together first. Afterwards **Publication II** will be covered in Section 3.1.2.

The classification criteria of the hazard potential of the tested SAILs were applied based on EC_{50} values on a mg/L basis (adhering to Blaise et al., 2008; Sanderson et al., 2003) as well as molarity concentration units. Usually the REACH regulation operates with mg/L values (Bernot et al., 2005) and for QSARs molarity units are preferred to characterise the toxicity of compounds (Montalbán et al., 2018). For this reason, in all three publications we report toxicity data using both concentration units.

3.1.1. Ecotoxicity of SAILs towards *V. fischeri*, *R. subcapitata*, and *T. platyurus* (Publications I and III)

The toxicity studies of 24 L-phenylalanine SAILs towards algae, crustaceans, and bacteria were conducted to evaluate their potential hazard to environmentally relevant test organisms and to establish the relationship between chemical structures of SAILs and toxicity. First, the tests were conducted, and the concentration-response curves were created; then EC_{50} values were calculated for all tested SAILs towards the respective test organisms. The classification criteria are shown in **Table 6**. It should be noted that the hazard classification scale using molarity units is specific for this set of 24 SAILs as it is based on their average molecular weight. The overview of the toxicity effects for tested SAILs towards different aquatic organisms is presented in **Table 5**.

Table 5. *The overview of toxicity results (72-h EC_{50}, 24-h LC_{50}, and 30-min EC_{50} values) for pyridinium, imidazolium, and cholinium substituted phenylalanine derived bromide SAILs and used positive control (benzalkonium chloride; BAC) classified using a simplified hazard classification scheme. A full explanation for hazard classification can be found in Table 1 of* ***Publication III****. For colour codes see* ***Table 6****.*

SAILs	Algae *Rapidocelis subcapitata* (data from Publication III)		Bacteria *Vibrio fischeri* (data from Publication I)		Crustaceans *Thamnocephalus platyurus* (data from Publication III)	
	mg/L	µM	mg/L	µM	mg/L	µM
PyC_2Phe						
PyC_4Phe						
PyC_6Phe						
PyC_8Phe						
$PyC_{10}Phe$						
$PyC_{12}Phe$						
$PyC_{14}Phe$						
$PyC_{16}Phe$						
$ImidC_2Phe$					n.t	n.t
$ImidC_4Phe$					n.t	n.t
$ImidC_6Phe$						
$ImidC_8Phe$						
$ImidC_{10}Phe$					n.t	n.t
$ImidC_{12}Phe$					n.t	n.t
$ImidC_{14}Phe$					n.t	n.t
$ImidC_{16}Phe$					n.t	n.t
$CholC_2Phe$					n.t	n.t
$CholC_4Phe$					n.t	n.t
$CholC_6Phe$						
$CholC_8Phe$						
$CholC_{10}Phe$					n.t	n.t
$CholC_{12}Phe$					n.t	n.t
$CholC_{14}Phe$					n.t	n.t
$CholC_{16}Phe$					n.t	n.t
BAC						

n.t – not tested

Table 6. *The hazard classification criteria of chemicals for the aquatic environment applied in the current Thesis.*

$L(E)C_{50}$ (mg/L)	$L(E)C_{50}$ (µM)	Hazard classification
≤0.1	≤0.2	Extremely toxic
>0.1 and ≤ 1	>0.2 and ≤ 2	Very toxic
>1 and ≤ 10	>2 and ≤ 20	Toxic
>10 and ≤ 100	>20 and ≤ 200	Harmful
>100	>200	Non-toxic

According to the classification of hazard potential of the studied SAILs towards *V. fischeri* and *T. platyurus* (see **Table 5**) none of the studied SAILs was classified as *extremely toxic* or *very toxic*. In contrast (see **Table 5**), eight SAILs ($PyC_{12}Phe$, $PyC_{14}Phe$, $ImidC_8Phe$, $ImidC_{10}Phe$, $ImidC_{12}Phe$, $ImidC_{14}Phe$, $ImidC_{16}Phe$, and $CholC_{14}Phe$) were *extremely toxic* to *R. subcapitata* algae; eight SAILs were *very toxic* (PyC_8Phe, $PyC_{10}Phe$, $PyC_{16}Phe$, $ImidC_6Phe$, $CholC_8Phe$, $CholC_{10}Phe$, $CholC_{12}Phe$, and $CholC_{16}Phe$); three SAILs were *toxic* (PyC_6Phe, $ImidC_4Phe$, $CholC_6Phe$); three were *harmful* (PyC_4Phe, $ImidC_2Phe$ and $CholC_4Phe$); and two were *non-toxic* (**PyC_2Phe** and **$CholC_2Phe$**) when applying the mg/L scale classification. Several changes in the classification were observed using the molarity unit scale (**Table 6**), i.e. $PyC_{16}Phe$, $CholC_{12}Phe$ and $CholC_{16}Phe$ (*extremely toxic*), and $ImidC_2Phe$ (*non-toxic*). The results show that *R. subcapitata* green algae were more sensitive to the toxic effects of these SAILs than *V. fischeri* bacteria and *T. platyurus* crustaceans. The high sensitivity of algae to ILs has been observed before: [C_2Clmim][Cl], [C_2Clmim][Tf_2N] and [C_2OHmim][Tf_2N] where algae (*P. subcapitata*) were more sensitive than *Danio rerio* fish and *Daphnia magna* crustaceans (Pretti et al., 2009).

It is important to note that the toxicities of PyC_2Phe (EC_{50} 2203 μM), $ImidC_2Phe$ (EC_{50} 3096 μM) and $CholC_2Phe$ (EC_{50} 2858 μM) to *V. fischeri* determined in this study were all lower than the toxicities of benzene (EC_{50} 1148 μM) and toluene (EC_{50} 234 μM) to *V. fischeri* (Kaiser and DeVillers, 1994). These are promising results and are expected to aid in the design of low-toxicity ILs.

3.1.2. Antibacterial potency of 24 SAILs studied towards two clinically relevant bacteria (Publication II)

Publication II systematically analysed the antimicrobial potency of a library of 24 phenylalanine derived SAILs and reports the EC_{50}, MIC and MBC values for these compounds towards two clinically relevant pathogenic bacterial models: *Escherichia coli* and *Staphylococcus aureus*. **Table 7** provides an overview of the toxicity results (plotted from Table 1 of **Publication II**) using the hazard classification criteria and colour scheme presented in **Table 6**.

Table 7. *An overview of toxicity results (EC_{50}, MIC and MBC) for pyridinium, imidazolium, and cholinium substituted phenylalanine derived bromide SAILs and used positive control (benzalkonium chloride; BAC) classified using a simplified hazard classification scheme. For the hazard classification criteria and colour codes see* ***Table 6.***

SAILs	***Escherichia coli*** (20h of incubation at 37˚C in MHB)						***Staphylococcus aureus*** (20h of incubation at 37˚C in MHB)					
	EC_{50}		**MIC**		**MBC**		**EC_{50}**		**MIC**		**MBC**	
	mg/L	µM	mg/L	µM	mg/L	µM	mg/L	µM	mg/L	µM	mg/L	µM
PyC_2Phe												
$ImidC_2Phe$												
$CholC_2Phe$												
PyC_4Phe												
$ImidC_4Phe$												
$CholC_4Phe$												
PyC_6Phe												
$ImidC_6Phe$												
$CholC_6Phe$												
PyC_8Phe												
$ImidC_8Phe$												
$CholC_8Phe$												
$PyC_{10}Phe$												
$ImidC_{10}Phe$												
$CholC_{10}Phe$												
$PyC_{12}Phe$												
$ImidC_{12}Phe$												
$CholC_{12}Phe$												
$PyC_{14}Phe$												
$ImidC_{14}Phe$												
$CholC_{14}Phe$												
$PyC_{16}Phe$												
$ImidC_{16}Phe$												
$CholC_{16}Phe$												
BAC												

MIC – Minimum Inhibitory Concentration; MBC – Minimum Bactericidal Concentration; MHB – Muller Hinton Broth

All C_2 and C_4 SAILs were classified *non-toxic* to both bacterial strains (**Table 7**). C_6 derivatives were also *non-toxic* to *E. coli* but the pyridinium and imidazolium analogues were harmful to *S. aureus*. All C_8 to C_{16} SAILs were *harmful* to *E. coli*, while only $CholC_8Phe$ and the C_{16} derivatives were *harmful* to *S. aureus*. PyC_8Phe, $ImidC_8Phe$ and all C_{10}-C_{14} examples were *toxic* to *S. aureus*. This analysis is independent of the concentration unit scale used (**Table 6**) with the same classification resulting for all entries in **Table 7**.

3.2. Toxicity mechanism of SAILs

A positive correlation between the alkyl chain length and the toxicity of 24 studied L-phenylalanine SAILs was identified in this body of work. As shown in **Figure 14**, C_8-C_{14} SAILs have the most potent harmful effect on the tested organisms (depending on the tested organisms); this phenomenon is well supported by earlier studies evaluating the toxicity of long-chain imidazolium cationic surfactants (Pernak et al., 2003) and 4,5-dialkylimidazolium based ILs (Wang et al., 2018).

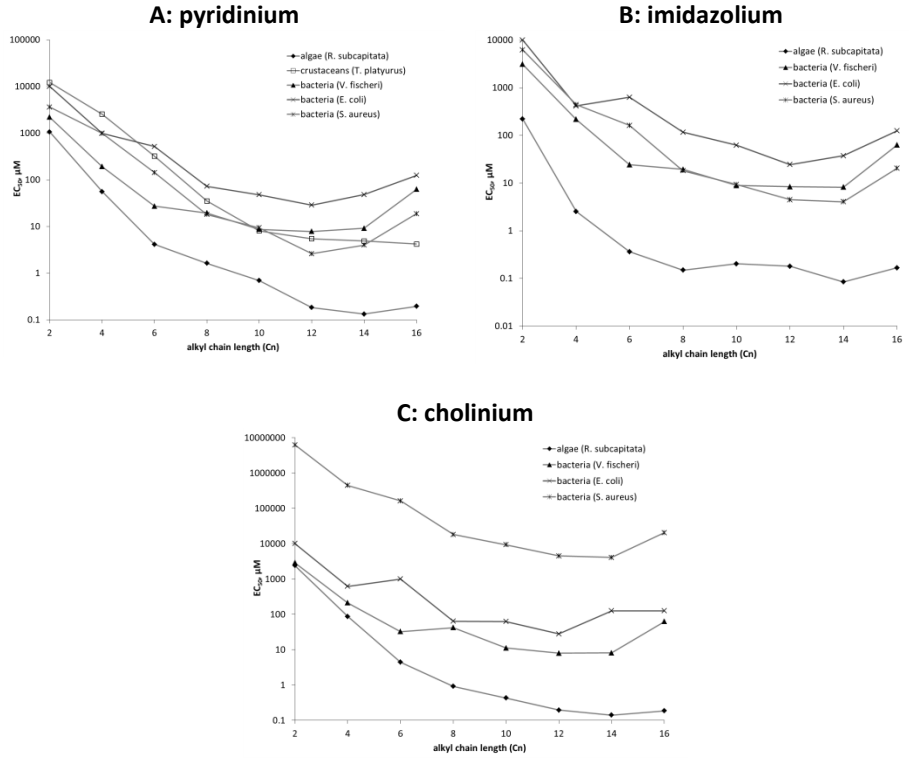


Figure 14. *Effects of alkyl chain length and head group on the toxicity of tested SAILs: pyridinium (A), imidazolium (B), and cholinium (C) of L-phenylalanine SAILs on tested organisms. Data plotted from Table 1 of* ***Publication I*** *for V. fischeri, Table 1 of* ***Publication II*** *for S. aureus and E. coli, and Table 1 of* ***Publication III*** *for R. subcapitata and T. platyurus. Note that the Y-axis is on a logarithmic scale.*

The shape of the curves in **Figure 14 A-C** for all bacteria (i.e. *V. fischeri*, *E. coli*, and *S. aureus*) were similar for all of the studied SAILs. This indicates that toxicity was independent of the head group (pyridinium, imidazolium, and cholinium). However, in the case of algae, the curves were similar only for pyridinium and cholinium and distinctly different for imidazolium SAILs (see also **Figure 15A**). Notably, ImidC_8Phe and ImidC_{12}Phe had similar toxicities (EC_{50} values) against the algae, while the trend for pyridinium and cholinium SAILs was C_{12} derivatives being more toxic than C_8 analogues to the algae (**Figure 14**).

Thus, the length of the alkyl chain is the dominant factor for the toxicity of studied SAILs. From C_2 to C_{12} the toxicity increased as predicted regardless of the organisms

tested. The toxic effect was consistent up to C_{16} on crustaceans. However, there was no enhancement of the toxicity in the bacteria case, beginning from C_{12} and the toxicity for C_{16} on algae was lower than for C_{14}. Some different explanations to account for this "cut-off effect" have been proposed (Matzke et al., 2010; Stolte et al., 2007; Ventura et al., 2012). 1) based on insufficient solubility (nominal concentration differs from real test concentration), 2) kinetic aspects (uptake deceleration because of steric effects for compounds with large molecular sizes), and 3) is the formation of IL aggregates.

The three longest alkyl chain (C_{16}) SAILs were less toxic to the *R. subcapitata* and *V. fischeri* used in this study than were the C_{14} analogues. An example of the "cut-off effect" can be seen in **Figure 15**; which shows the effect of the alkyl chain length of pyridinium SAILs on toxicity to *R. subcapitata* algae. The highest toxicity was observed for the $PyC_{14}Phe$ SAIL and the same overall trend regarding chain length and toxicity to algae was demonstrated using either mg/L or molarity-based concentration units.

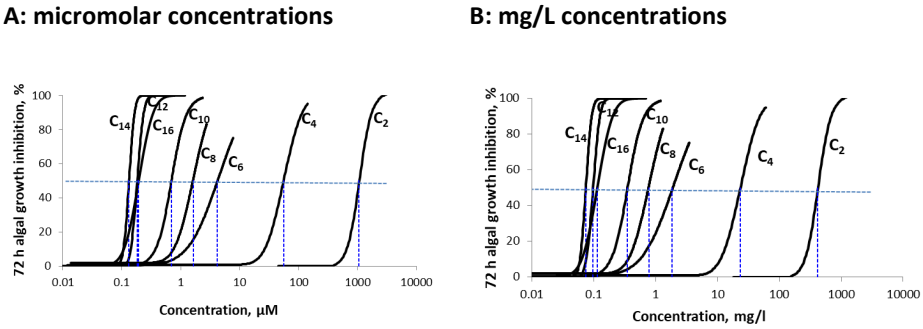


Figure 15. *Concentration-response curves of pyridinium L-phenylalanine SAILs towards algae R. subcapitata (A) in micromolar (µM), and (B) in mg/L concentrations. The least toxic SAILs (C_2) are located on the right. The EC_{50} values data are taken from Table 1 of* ***Publication III****. Note that the X-axis has a logarithmic scale.*

As mentioned above, the effect of different **head groups** on toxicity showed different trends to the various tested organisms. **Figure 16** illustrates the toxicity for SAILs with different head groups towards test organisms used in the current thesis. The toxicity evaluation of SAILs using algae showed a steeper slope of the concentration-response curve of $ImidC_8Phe$ than that of the $CholC_8Phe$ and PyC_8Phe (**Figure 16A**), indicating imidazolium ILs are more toxic and there is a different toxicity mechanism towards *R. subcapitata* algae compared to SAILs with other head groups. These results were analogous to the study of Xia et al. (2018), which showed that imidazolium ILs were more harmful than pyridinium ILs towards *Scenedesmus obliquus* green algae.

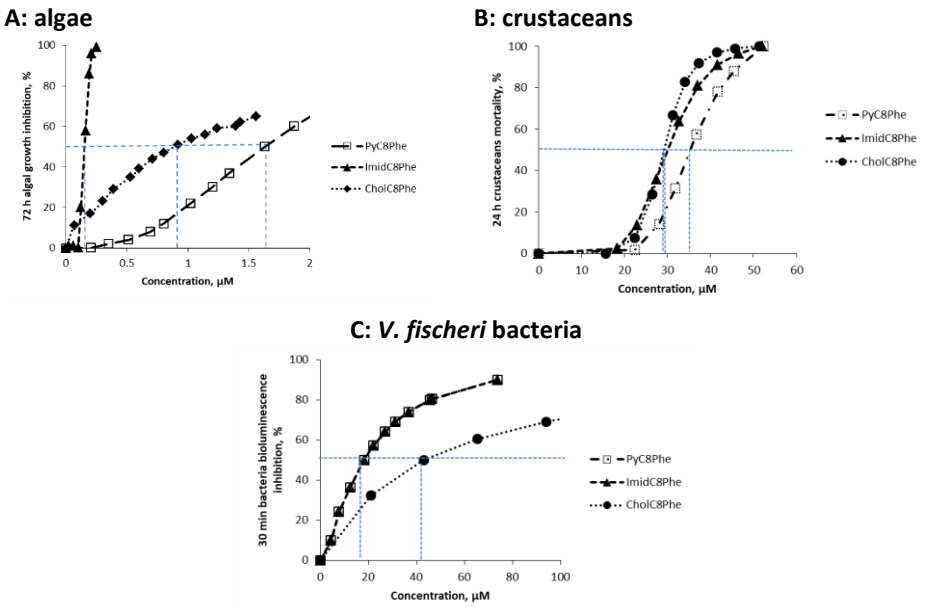


Figure 16. *Effect of the SAILs head groups with the same alkyl chain length (C_8) to (A) R. subcapitata, (B) T. platyurus, and (C) V. fischeri: concentration-response curves. Data are plotted from Table 1 of* ***Publication III.***

Concentration-effects curves for SAILs (using PyC_6Phe as a representative example, **Figure 17)** clearly show that the studied SAILs follow the toxicity trend: algae (*R. subcapitata*) > bacteria (*V. fischeri*) > crustaceans (*T. platyurus*). These results were in agreement with Costa et al. (2017) and Pretti et al. (2009), which stated that a chemical's toxic effect depends on the test organism.

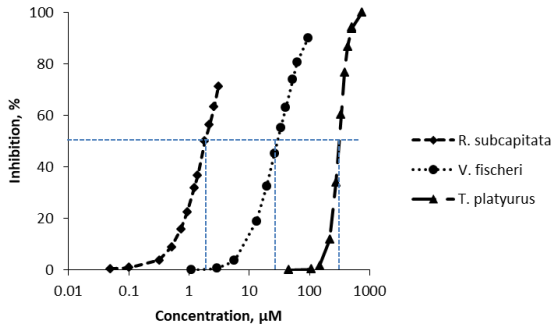


Figure 17. *Concentration-effect curve of PyC_6Phe SAILs towards R. subcapitata, V. fischeri, and T. platyurus. Data are plotted from Table 1 of* ***Publication III****. Note that the X-axis is on a logarithmic scale.*

The studied long alkyl chain length SAILs have an increased lipophilicity compared to the SAILs with shorter chains. The link between lipophilicity and toxicity is a common phenomenon, known as the basic toxicity of chemicals to aquatic organisms (Megaw et al., 2013; Bubalo et al., 2017). According to Stolte et al. (2007), the acute toxicity attributed to the alkyl chain length lipophilicity of ILs results in membrane interactions similar to polar narcosis. In addition, toxic action mechanisms of ILs are dependent on

surface activity (Rosen et al., 2001), accumulation in membranes and membrane disruption (Ranke et al., 2006; Łuczak et al., 2010). The increased lipophilicity of SAILs with longer alkyl chains could contribute to an increase in surfactant-like alterations in cell-membranes (Bernot et al., 2005) which then could lead to an increase in membrane permeability that can accelerate the death of the cell (Roberts & Costello, 2003). Another possible mode of action could be enzyme inhibition (Agrawal & Bagchi, 2001) or structural DNA damage (Shugart, 1996).

Furthermore, the lipids-containing cell membrane is the first entry point for all chemical compounds including toxicants, such as surfactants. Scammells et al. (2005) have noted that SAILs have modes of chemical action similar to surfactants. Indeed, Sena et al. (2010) reported the critical role of the cell wall in transportation of chemicals, including toxicants, into and out of algal cells. Additionally, Couling et al. (2006) proposed that the presence of ILs in the phospholipid bilayer in chloroplasts of algae can inhibit their photosynthesis.

The complete understanding of the toxicity action mechanism for ILs to bacteria remains unclear. Nevertheless, ILs have structural similarities with cationic biocides and surfactants, especially quaternary ammonium compounds (QACs) (Blesic et al., 2007). A charged hydrophilic head group with one or more hydrophobic tails is a common chemical structure of ILs that is common to QACs, and Gilmore's group have stated that they have a common mode of action (Pendleton & Gilmore, 2015). For example, ILs have a tendency to form amphiphilic micelles (aggregation in solution) (Blesic et al., 2007) and many classes of ILs exhibit significant surface activity (Cornellas et al., 2011; Garcia et al., 2013; Łuczak et al., 2008), in some cases by increasing hydrophobicity (Jungnickel et al., 2008). Cationic biocides and surfactants disrupt cell membrane integrity. Some ILs have been observed to display surface-active properties which interact adversely with cell walls and membranes (Borkowski et al., 2016).

The phenyl ring's effect on the surface activity of the C_6 SAILs (pyridinium, imidazolium, and cholinium) used in this study was determined by Kapitanov et al. (2019). The same group also studied the surface activity properties (e.g. Critical Micelle Concentration; CMC in water) of the 24 L-phenylalanine SAILs used in this study. CMC is one of the parameters that can explain the toxicity of SAILs towards organisms. Notably, a significant decrease in surface activity (CMC value) was observed when phenylalanine residue was exchanged with alanine. The correlation between CMC values and the toxicity of the tested SAILs in this study is presented in the next section. According to the surface activity results presented by the Gathergood group in Kapitanov et al. (2019), the CMC values were not significantly dependent on the SAILs head group (imidazolium, pyrdinium and cholinium) in this study. The lipophilicity of the studied SAILs is predominantly dependent on the two hydrocarbon groups (i.e. the alkyl chain of the ester group and the benzyl substituent of the phenylalanine subunit). The length of the alkyl chain in the cationic part of SAILs contributes to SAILs' molecules' lipophilicity, which means that increases in the alkyl chain's length will increase the lipophilic character of SAILs. These results agree with previous studies' reported results (Latała et al., 2009; Pham et al., 2008; Ranke et al., 2004; Docherty & Kulpa, 2005). Furthermore, the ILs studies by Montalbán et al. (2018) showed that ILs with longer alkyl chains attach more strongly to the surface of lipid bilayers and induce strong micellisation effect. This in turn, triggers the membrane lipid bilayer to lose its integrity.

3.3. Correlations between toxicity and CMC, $\Delta G^{\circ}_{ad}/A_{min}$ and Log K_{ow}

3.3.1. Correlation between toxic effect and the CMC of the studied SAILs

The determination of the correlation of algae toxicity and CMC for all 24 SAILs resulted in low R^2 values. However, it was apparent that by refining the dataset according to headgroup type, higher R^2 values would be attained. This was indeed the case for pyridinium and cholinium SAILs (**Figure 18A** and **18C**, respectively), but a low R^2 value was still observed for the imidazolium series. It is clear from the three plots in **Figure 18**, that higher R^2 values would be achieved if the C_4 data point were also omitted. However, for complete transparency we have included below the correlations calculated using all data points.

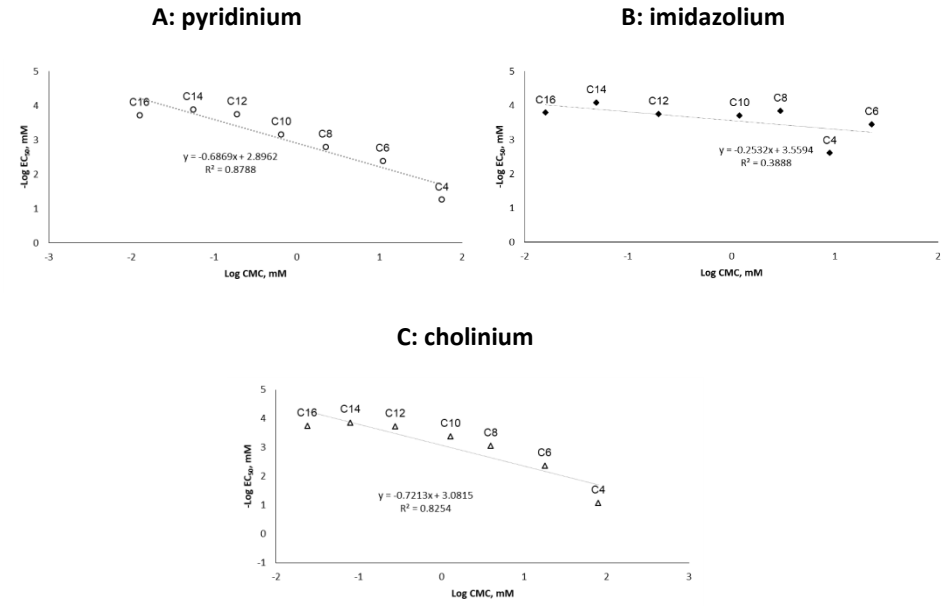


Figure 18. *Correlation between algal toxicity –Log EC_{50} and Log CMC for the SAIL series tested: pyridinium (A), imidazolium (B) and cholinium (C). The EC_{50} values were taken from Table 1 of* ***Publication III*** *and the log CMC data were taken from Kapitanov et al. (2019).*

3.3.2. Correlation between the toxicity of SAILs to algae and $\Delta G^{\circ}_{ad}/A_{min}$ of the studied SAILs

Additionally, Kapitanov et al. (2019) reported that the $\Delta G^{\circ}_{ad}/A_{min}$ that was introduced by (Rosen et al., 2001) could also be used to correlate the toxicity values of SAILs and their hydrophobicity. $\Delta G^{\circ}_{ad}/A_{min}$ is a physicochemical parameter for the interfacial activity of surfactants, where ΔG°_{ad} is the standard free energy of adsorption of surfactants at the air/solution interface, and A_{min} is the minimum cross-sectional area of the surfactants. It was also apparent on first inspection of the dataset that an analysis should be performed on IL headgroups separately for optimal fitting. The correlation between $\Delta G^{\circ}_{ad}/A_{min}$ is depicted in **Figure 19**. Similar results were observed when compared to **Figure 18**. The highest R^2 values were calculated for the pyridinium and cholinium SAILs, while low values were found for the imidazolium examples. However, we predict that in

Figure 19 the only plot that would result in a significantly high R^2 value if the C_4 data were omitted is **Figure 19B**.

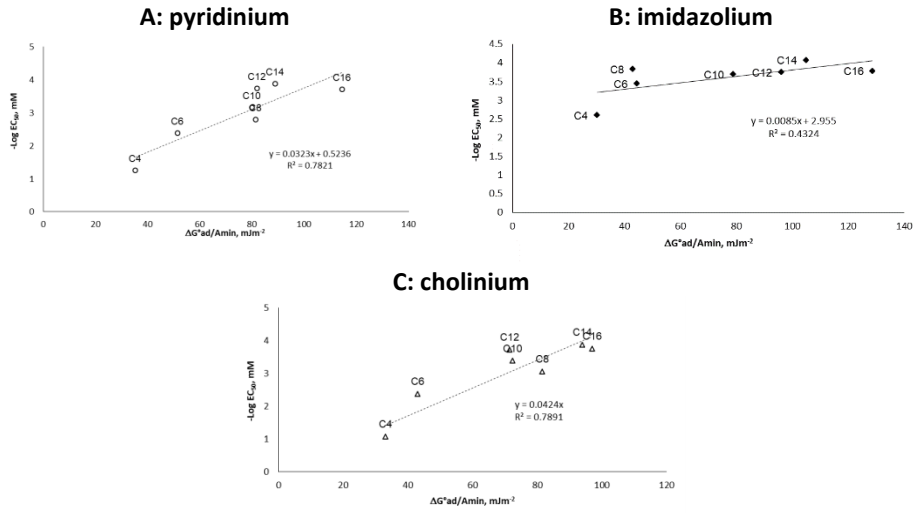


Figure 19*. Correlation between –Log EC_{50} and $\Delta G^{\circ}_{ad}/A_{min}$ for the series tested SAILs: pyridinium (A), imidazolium (B) and cholinium (C). The EC_{50} values were taken from Table 1 of* ***Publication III*** *and* $\Delta G^{\circ}_{ad}/A_{min}$ were taken from Kapitanov et al. (2019)*.*

3.3.3. Correlation between toxic effect and the log K_{ow} of the studied SAILs

Log K_{ow} is another chemical property related to the toxicity effects of chemicals towards organisms. This parameter describes the hydrophobicity of the chemicals, their ability to enter the membrane's lipid phase and to cross the biological membrane (Stock et al., 2004; Ventura et al., 2012). The higher the log K_{ow} number, the larger the chemical compound interaction and the greater the binding to the membrane and toxicity (Montalbán et al., 2018). Chemicals with log $K_{ow} \geq 4.0$ are considered to significantly impact the environment (EC, 2008). The correlation between the log K_{ow} of the tested chemicals and the most sensitive organism (*R. subcapitata* algae) in this study is presented in **Figure 20.** The order for higher correlation between log K_{ow} and -log EC_{50} was pyridinium > cholinium > imidazolium.

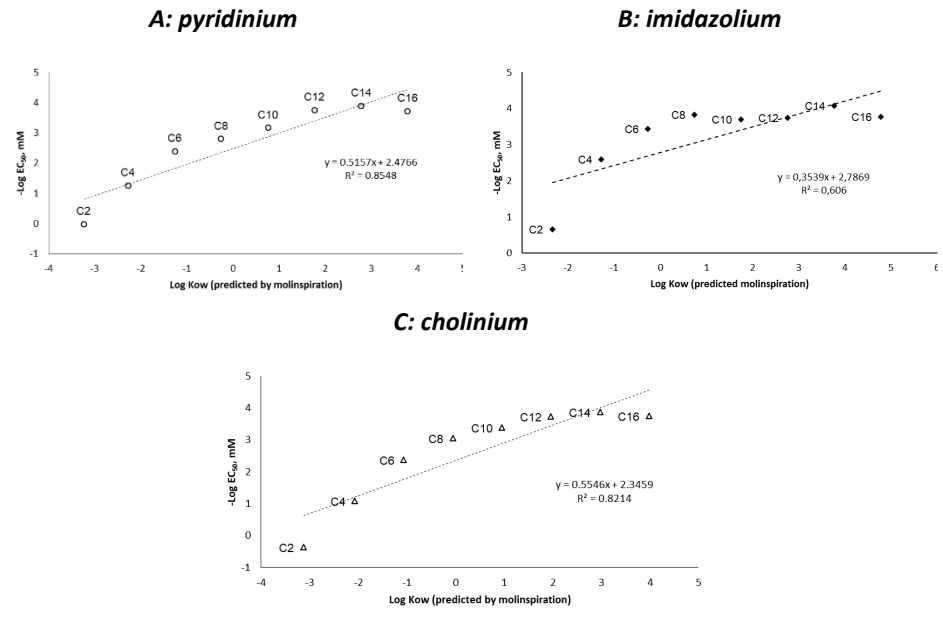


Figure 20. *Correlation between –Log EC_{50} and Log K_{ow} for the series tested SAILs: pyridinium (A), imidazolium (B) and cholinium (C). The EC_{50} and log K_{ow} values were taken from Table 1 of* ***Publication III****. log K_{ow} values were predicted using the free software of molinspiration.com.*

An estimation of the EC_{50} values for SAILs can be made from **Figure 20** as the log K_{ow} used in this thesis was predicted by the free software from molinspiration.com. For instance, if one is investigating the effect of alkyl chain modification on an ester, the fitted line of **Figure 20** can be used to predict the EC_{50} value of an analogous structurally-related derivative. While one must exercise caution when selectively removing data points to refine a dataset, it is apparent from the three graphs in **Figure 20** that R^2 values are improved if only the C_6-C_{14} data are considered. For instance, for pyridinium the equation would be y = 0.3891x + 2.8929 and the R^2 is 0.9777; for imidazolium the graph equation would be y = 0.117x + 3.5506 and the R^2 is 0.6688, and for cholinium the graph equation would be y = 0.625x + 2.1086 and the R^2 is 0.9079. However, this reduces the log K_{ow} range of the chemicals within which the full data set can predict EC_{50} values for C_6 to C_{14} instead of C_2-C_{16}.

3.3.4. QSAR studies to support the design of safer SAILs

Sections 3.3.1 to 3.3.3 show the correlations (including R^2 values) between the toxicity of SAILS to algae and CMC, $\Delta G^{\circ}_{ad}/A_{min}$ and log K_{ow} respectively. Toxicity to algae was selected as algae were the most sensitive test species in this study (see **Table 5**). R^2 is a statistic that gives some information about the "goodness of fit" of a model (Draper & Smith, 1998). While this is an important basis for the statistical analysis of the data, applying modelling results to support future investigations is a fundamental reason for performing QSAR studies.

A key distinction between the datasets used to plot **Figures 18**-**20** is that in the **Figure 20** case the computer software predicted one set of values (log K_{ow}). Whereas, in **Figures**

18 and **19**, all the datapoints were determined by experiments in the laboratory. These results in two approaches one can employ to predict the toxicity of SAILs.

The first is to calculate the log K_{ow} of the novel SAIL using the software from molsimulation.com and use **Figure 20** to estimate the EC_{50} (algae) value. The advantage is that a large number of SAILs can be processed expediently. The judicious selection of the most appropriate fitting plot (i.e. the head-group and alkyl chain length range) has the potential to yield an accurate result based on a high R^2 value. SAILs with a close similarity in structure to the 24 studied herein are considerably more suitable candidates to screen this way than other ILs. A disadvantage is that inaccurate predictions of log K_{ow} are difficult to identify and therefore errors in toxicity assessment due to this error are possible outcomes. It is acknowledged that log K_{ow} can be determined experimentally, however, **Figure 20** is based solely on the predicted values from the molsimulation.com software.

The second approach is more labour intensive and time consuming, due to the experimental laboratory work required to determine CMC and $\Delta G^{\circ}_{ad}/A_{min}$ values. This is less suitable for screening large numbers of SAILs. However, an advantage of this approach is that one can determine if the novel SAIL has an expected CMC value or not. For instance, the SAIL $PyC_{11}Phe$ would be expected to have a CMC value between that of $PyC_{10}Phe$ and $PyC_{12}Phe$. If this is not found to be the case experimentally, then the researcher has to consider how suitable **Figure 18A**is to estimate the toxicity. Conversely, if an expected CMC value were experimentally obtained, then one would be more confident of the accuracy of the predicted toxicity.

We propose that using both approaches in tandem to predict the toxicity of SAILs leads to more accurate results. QSAR studies using **Figure 20** enables a large library of SAILs to be screened, and a representative sub-set can be evaluated using **Figure 18** and **19**.

Examination of the R^2 values in **Figure 18**-**20** informs us that pyridinium and cholinium SAILs have higher R^2 values than imidazolium has in all cases. The highest R^2 value for imidazolium SAILs was calculated in **Figure 20B**, but this is significantly lower than the R^2 values for the other two headgroup types. It is more likely that larger errors will occur when predicting the toxicity of imidazolium SAILs using **Figures 18B**, **19B** and **20B** (c.f. pyridinium **Figures 18 A**, **C** and cholinium **Figures 20 A**, **C**).

Ultimately, QSARs are best applied in an iterative manner supported by accompanying experimental results within a well-defined chemical space that allows accurate predictions of properties. This project provides tools that can be tested in future studies to support the development of lower toxicity SAILs.

3.4. SAILs as safer alternatives to commercial cationic surfactants

As mentioned in the literature review, the aim of green chemistry is to encourage the use of safer chemicals. One goal of this thesis is to establish which SAILs in our series of 24 compounds are safer than commercially available cationic surfactants (**Publication III**). Based on the toxicity effects on the tested organisms herein, algae proved to be the most susceptible. A comparison of the toxicities of the studied SAILs towards algae was provided in this thesis and the toxicities of eleven selected commercially available cationic surfactants are shown in **Figure 21**.

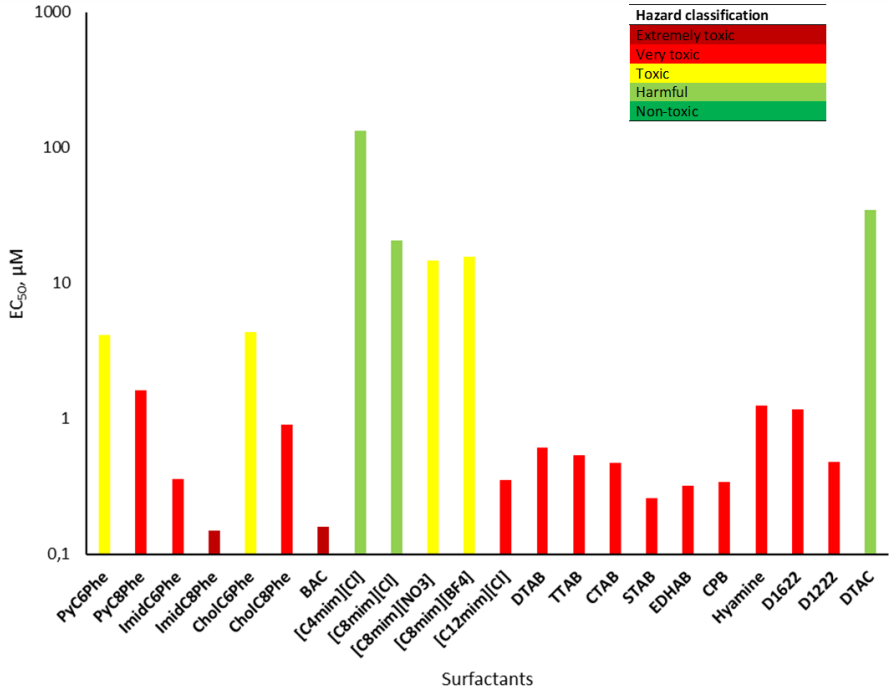


***Figure 21**. Toxicity effect to algae of studied SAILs and 10 commercially available surfactants. [C_4mim][Cl] and [C_8mim][Cl] ILs are included as references. The graph (including colour ranking scale) is plotted according to Table A1 of **Publication III**.*

As can be seen from **Figure 21** two SAILs, PyC_6Phe and CholC_6Phe are rated as "*toxic*" (yellow bars) and are recommended for investigation as alternatives of "*very toxic*" (red bars) surfactants, such as DTAB, TTAB, STAB, EDHAB, CPB, hyamine, D1622 and D1222, as well as BAC (*extremely toxic*; dark red bar) (**Publication III**). Although, PyC_8Phe, ImidC_6Phe, and CholC_8Phe, are "*very toxic*" they are still suggested as suitable substitution for BAC and STAB. ImidC_8Phe is classified as "*extremely toxic*" (dark red bar), so this is the same rating as BAC and is not recommended as a substitute for any of the commercial surfactants in the study.

It is important to note that the studies above are based on the reported data available and some variabilities could lead to errors in interpreting the results. These differences in test conditions are explicitly highlighted in **Publication III,** including a citation to the primary literature sources. For instance, the literature EC_{50} values reported are for different algae (i.e. *C. pyrenoidosa*, *C. vulgaris*, *S. capricornutum*, *N. gaditana* and *C. pyrenoidosa*) and different time periods of exposure such as 96h or 72h. Therefore, we propose that the recommendations (while encouraging for promoting the use of PyC_6Phe and CholC_6Phe) be used as a guide to stimulate and direct a more detailed toxicology assessment.

Conclusions

The lack of toxicity data, relative to the large number of novel ILs synthesised, hampers the design of greener and safer ILs. In order to fill this gap, this thesis evaluated 24 SAILs derived from L-phenylalanine that represent a particularly challenging subset of ILs due to the widely reported high toxicity of cationic surfactants, which are close analogues to the SAILs of this study. The testing focus was set across different level of food-web aquatic organisms (i.e. algae, crustaceans, and bacteria) as well as clinically relevant test bacteria, to provide general view of the antibacterial properties of these SAILs.

The results obtained showed that:

(i) Only two out of the 24 studied SAILs could be considered green SAILs (EC_{50} >100 mg/L). More specifically, the ecotoxicological test battery (*R. subcapitata* algae *T. platyurus*, crustaceans, *V. fischeri* bacteria) classified three SAILs as "*harmful*" (10< EC_{50} ≤100 mg/L), three SAILs as "*toxic*" (1< EC_{50} ≤10 mg/L, eight SAILs as "*very toxic*" (0.1< EC_{50} ≤1 mg/L) and eight SAILs as "*extremely toxic*" (EC_{50} ≤0.1 mg/L).

(ii) Alternatively, a classification scale dependent on the average MW of the compound dataset (based on molar and not mg/L concentrations) ("*non-toxic*" – EC_{50} >200 μM; "*harmful*" – 20< EC_{50} ≤200 μM; "*toxic*" – 2< EC_{50} ≤20 μM; "*very toxic*" – 0.2< EC_{50} ≤2 μM and "*extremely toxic*" – EC_{50} ≤0.2 μM) was obtained to rank the SAILs as a more accurate appraisal for suggesting greener alternatives to certain commercial SAILs/surfactants.

(iii) The toxicity results proved that the *R. subcapitata* algae growth inhibition assay was the most sensitive test used and could be used for the ecotoxicity classification of SAILs (algae representing the weakest link in the aquatic food chain).

(iv) The *V. fischeri* bioluminescence inhibition test as rapid and cost-efficient assay can be recommended for initial (eco)toxicity screening as its data correlated well with the data obtained using an algal test (R^2 = 0.79).

(v) PyC_6Phe and $CholC_6Phe$, ranked as "*toxic*" on the ecotoxicological test battery are recommended for further investigation as alternatives to the "*very toxic*" commercial surfactants DTAB, TTAB, STAB, EDHAB, CPB, hyamine, D1622 and D1222, and especially to the "*extremely toxic*" BAC.

(vi) SAILs, especially from C_{10}, proved to be efficient antibacterial. The most potent antibacterial were the three C_{12} SAILs (MIC and/or MBC values from 4.2 to 34 mg/L); Gram-positive bacteria (represented by *S. aureus*) were more sensitive (about eight times) to the tested SAILs than were Gram-negative bacteria (represented by *E. coli*).

(vii) The antimicrobial potency of the tested SAILs was dependent on alkyl C-chain length but not on head group.

(viii) QSAR studies were refined by head-group type to improve the fitting of the data. High R^2 values were obtained for the pyridinium and cholinium SAIL QSAR plots. These models are expected to yield good predictions of SAIL toxicity. The highest R^2 value (0.98) was determined for the –log EC_{50} and log K_{ow} plot of the pyridinium SAIL subset C_6-C_{14}.

(ix) Future QSAR analysis of SAIL libraries is recommended by first calculating the log K_{ow} value (**Figure 20**), and then screening a subset in the laboratory to determine surface activity properties (**Figure 18** and **Figure 19**).

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Abstract
Ecotoxicological Profiling and Antibacterial Potency of a Series of 24 L-Phenylalanine Based SAILs

Ionic liquids (ILs) are salts that are composed by the structural arrangement of a wide spectrum of cations and anions, often also known as "designer solvents". This is due to the very large number of possible combinations of the cations and anions which allows us to control the properties of ILs for a specific task. IL research has developed rapidly, from the synthesis of the first generation of ILs in the laboratory to their current industrial applications. However, the growth of research in the synthesis and application of ILs has not been followed by sufficient research to obtain the toxicity data of ILs. Most ILs are soluble in water and they can reach aquatic and terrestrial environments via various waste-flows. Therefore, the hazard assessment of ILs is a crucial area of research. Moreover, the whole life cycle (from synthesis to disposal) of ILs should be considered to determine how "green" ILs are based on green chemistry principles.

One of the IL types that has been growing rapidly are surfactants, commonly known as surface-active ionic liquids (SAILs). SAILs are a special type of ILs of an amphiphilic character, with hydrophilic and lipophilic components. This thesis provides the toxicological profiling of 24 SAILs derived from L-phenylalanine which have many structural features similar to those of common cationic surfactants. The key feature of our SAIL series is the incorporation of two lipophilic groups: the alkyl ester and the benzyl group of the L-phenylalanine. This is in contrast to many cationic surfactants where alkyl groups are the sole contributor to the compound's lipophilicity.

The main aim of this thesis was to investigate the potential environmental hazards of a series of 24 L-phenylalanine derived SAILs with pyridinium, imidazolium and cholinium cations paired with bromide–anions and varying alkyl chains, from 2 to 16 carbon atoms (C_2 to C_{16}) to the aquatic ecosystem use of different trophic levels in the aquatic food chain (e.g. algae – primary producers, crustaceans – consumers and bacteria – decomposers). In addition, the antibacterial potential of these SAILs was evaluated.

The first tier of testing was performed using the naturally luminescent marine bacteria *Vibrio fischeri* and bioluminescence inhibition as a toxicity endpoint (ISO 21338:2010). Additionally, the antibacterial potency was tested towards the medically important bacterial strains *Staphylococcus aureus* Gram-positive bacteria, and *Escherichia coli* Gram-negative bacteria using a standard broth microdilution method (ISO 20776-1:2006) for antimicrobial susceptibility testing. The second tier of assays used a 72-h algal growth inhibition assay (OECD 201) with *Raphidocelis subcapitata* and the third tier used a 24-h mortality test with *Thamnocephalus platyurus* aquatic crustaceans (ISO 14380:2011).

The results showed that the toxicity of SAILs was mainly dependent on the length of the side chain, with only a minor head group effect observed. According to the used ecotoxicological test battery (*R. subcapitata* algae, *T. platyurus* crustaceans, *V. fischeri* bacteria) only two SAILs (PyC_2Phe and $Chol_2Phe$) out of the 24 studied were considered to be green SAILs (EC_{50} >100 mg/L). The C_6 SAILs ($ImidC_6Phe$ and $CholC_6Phe$) were classified as "*toxic*" and are recommended for investigation as alternatives to "*very toxic*" surfactants (e.g. DTAB, TTAB, STAB, EDHAB, CPB, hyamine, D1622 and D1222) as well as "*extremely toxic*" benzalkonium chloride. Of the test battery used, the algal (*Raphidocelis subcapitata*) growth inhibition assay was the most sensitive and this could be used for the ecotoxicity classification of SAILs. The *V. fischeri* bioluminescence inhibition test proved to be as rapid and cost-efficient assay that can be recommended for initial

ecotoxicity screening as its data correlated well with the data obtained using an algal test (R^2 = 0.79). In terms of antibacterial potential, the most efficient antibacterials proved to be SAILs starting from C_{10}. The most potent antibacterial were the three C_{12} SAILs (MIC and/or MBC values from 4.2 to 34 mg/L); Gram-positive bacteria (represented by *S. aureus*) were more sensitive (about eight times) to the tested SAILs than were Gram-negative bacteria (represented by *E. coli*).

QSAR studies were refined by head-group type to improve the fitting of the data. High R^2 values were obtained for the pyridinium and cholinium SAIL QSAR plots. These models are expected to yield good predictions of SAIL toxicity. The highest R^2 value (0.98) was determined for the –log EC_{50} and log K_{ow} plot of the pyridinium SAIL subset C_6-C_{14}. However, future QSAR analyses of SAIL libraries are recommended to determine toxicity and surface activity properties.

Lühikokkuvõte
24 L-fenüülalaniini-põhise ioonvedeliku ökotoksikoloogiline ja antibakteriaalne iseloomustamine

Ioonsed vedelikud (IL) on soolad, mis koosnevad anioonide ja katioonide teatud kombinatsioonidest ja mis on toatemperatuuril vedelas olekus. Anioone ja katioone, mida saab omavahel kombineerida, on lõpmatult palju ning see annab võimaluse luua uusi ioonvedelikke vastavalt (tööstusele) soovitud omadustele. Kuna tänapäeval on suund kestlikele tehnoloogiatele, siis eelistatakse järjest enam taaskasutatavaid, keskkonnasõbralikke, funktsionaalseid ja odavaid materjale. Ka ioonseid vedelikke võib nende unikaalsete omadusete tõttu (madal lenduvus, mittesüttivus, kõrge keemiline stabiilsus) pidada sobilikeks 'rohelise keemia' kemikaalideks. IL on palju erinevaid rakendusi, neid saab kasutada näiteks nii lahustitena, elektrolüütidena kui ka pindaktiivsete ainetena. Pindaktiivsed IL (ingl.k SAIL) on sellised IL, millel on nii hüdrofoobne kui ka hüdrofiilne osa.

Käesolevas doktoritöös uuriti L-fenüülalaniini-põhiseid SAIL-e, millel on palju sarnaseid struktuurseid omadusi tavaliste katioonsete pindaktiivsete ainetega, ent millel erinevalt traditsioonilistest katioonsetest pindaktiivsetest ainetest (näiteks tuntud desinfektant bensalkooniumkloriid, mille lipofiilsuse tagavad alküülrühmad) on kaks lipofiilsust tagavat rühma - L-fenüülalaniini alküülester ja bensüülrühm. Antud doktoritöös uuritud kemikaali-raamatukogu hõlmas 24 erinevat L-fenüülalaniini-põhist SAIL-i (koosnesid püridiinium-, imidasoolium- ja koliinium-katioonidest, mis olid ühendatud bromiidi-aniooniga ning alküülahelaga pikkuses C_2 - C_{16}).

Töö peaeesmärk oli nende 24 SAIL-i 'rohelisuse' hindamine ökotoksikoloogilises plaanis, ent ka nende SAILide antibakteriaalsete omaduste hindamine. Ökotoksikoloogiliseks analüüsiks kasutati selgrootutest organismidest koosnevat multitroofset biotestide patareid: i) OECD 201 test vetikatega *Raphidocelis subcapitata* (esmased tootjad - CO_2 sidujad), ii) ISO 14380:2011 test kirpvähkidega *Thamnocephalus platyurus* (tarbijad) ja iii) ISO 20776-1:2006 test looduslike bioluminestseeruvate bakteritega *Vibrio fischeri* (orgaanilise aine lagundajad). SAILide mürgisust hinnati, mõõtes kas rakkude kasvu pidurdumist (testid vetikate ja bakteritega), bioluminestsentsi vähenemist (*V. fischeri* test) või siis hinnates organismide surevust (kirpvähilised) nende ainetega kokku puutudes.

Testiti erinevaid SAIL-de kontsentratsioone ja arvutati toksilisuse väärtused (EC_{50} või LC_{50}; mg/l ja µM), mille alusel jagati SAILid erinevatesse toksilisuse klassidesse. Praktiliselt kõigi testide tulemused näitasid, et SAILide toksilisus ei sõltunud katiooni-tüübist, ent sõltus SAILi alküülahela pikkusest. Ökotoksikoloogiline analüüs näitas, et 24 uuritavast ühendist osutusid mitte-toksilisteks (EC_{50}> 100 mg/L või > 200 µM) ehk siis nö 'roheliseks' vaid kaks SAILi (PyC_2Phe ja $Chol_2Phe$). Ehkki SAIL-id alküülahela pikkusega C_6 klassifitseerusid 'toksilisteks' (EC_{50} >1-10 mg/l või >2-20 µM), võib neid siiski soovitada alternatiividena 'väga toksiliste' (EC_{50} >0,1-1 mg/L või >0,2-2 µM) (nt DTAB, TTAB, STAB, EDHAB, CPB, hüamiin, D1622 ja D1222) ning 'äärmiselt toksiliste' (EC_{50} ≤0,1 mg/l või ≤0,1 µM) pindaktiivsetele ainetele (bensalkooniumkloriid).

Töös kasutatud multitroofse testpatarei põhjal osutus SAILide ökotoksilisuse määramisel kõige tundlikumaks vetika (*R. subcapitata*) kasvu inhibeerimise test. Näitasime, et *V. fischeri* bioluminestsentsi inhibeerimise testi saab edukalt kasutada SAIL-ide ökotoksikoloogilise toime esmaseks hindamiseks, kuna selle testi tulemused korreleerusid hästi vetikatestide tulemustega (R^2 = 0,79). Lisaks hinnati ka SAILide

antibakteriaalset mõju, kasutades meditsiinile huvi pakkuvaid (haigustekitajad!) gram-positiivseid baktereid *Staphylococcus aureus* ja gram-negatiivseid baktereid *Escherichia coli.* Antibakteriaalse mõju osas osutusid testitud ühendite puhul kõige tõhusamaks SAIL-id alates alküülahela pikkusega C_{10}, eriti aga SAIL-d alküülahela pikkusega C_{12} (MIC ja/või MBC väärtused 4,2 kuni 34 mg/l). Ühtlasi selgus, et gram-positiivsed bakterid *S. aureus* olid testitud SAILide suhtes ligi 8 korda tundlikumad kui gram-negatiivsed bakterid *E. coli*. Lisaks eeltoodud tulemustele võimaldavad saadud toksilisuse andmed (homogeenne andmekogum!) edasist QSAR analüüsi, et saaks arvutuslikult ennustada 'rohelisemaid' ioonvedelikke.

Appendix

Publication I

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Toxicity profiling of 24 L-phenylalanine derived ionic liquids based on pyridinium, imidazolium and cholinium cations and varying alkyl chains using rapid screening *Vibrio fischeri* bioassay

Dewi K.A. Kusumahastuti[a,b,c], Mariliis Sihtmäe[b], Illia V. Kapitanov[a], Yevgen Karpichev[a], Nicholas Gathergood[a,*], Anne Kahru[b,d,**]

[a] ERA Chair of Green Chemistry, Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn 12618, Estonia
[b] Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Tallinn 12618, Estonia
[c] Department of Chemistry, Satya Wacana Christian University, Salatiga 50711, Indonesia
[d] Estonian Academy of Sciences, Kohtu 6, Tallinn 10130, Estonia

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ABSTRACT

A library of 24 pyridinium-, imidazolium-, and cholinium-based ionic liquids (ILs) with varying alkyl chain from C_2 to C_{16} was toxicologically profiled using naturally luminescent marine bacteria *Vibrio fischeri*. The toxicity (30-min EC_{50}) of studied ILs to *Vibrio fischeri* ranged from 7.82 μM (4.2 mg/L) (PyC_{12}Phe) to 3096 μM (1227 mg/L) ($ImidC_2$Phe), i.e. from "toxic" (EC_{50} 1–10 mg/L) to "not harmful" (EC_{50} > 100 mg/L). Inhibition of the bacterial luminescence upon 30-min exposure to ILs correlated well with bacterial viability (exposure for 4 h). The toxicity of studied ILs was largely driven by the length of the alkyl chain (hydrophobicity) and not the type of cationic part of the IL: starting from C_{10} all the ILs irrespective of the cationic part proved "toxic". The toxicity of the studied ILs was increasing in parallel to their hydrophobicity up to log K_{ow} = 1 (C_8–C_{10}) and then levelling up, being consistent with the previously obtained analogous data sets. The "cut-off" effect reported in this study for longer chain length members of the ILs series leads to the "limit" toxicity level for this type of ILs to be ca. 8 mM. Two open-access online tools (www.molinspiration.com and www.vcclab.org) have been applied for the calculation of the K_{ow} values for the 24 ILs reported in this study and 21 ILs reported in the literature. This lead to plotting two nonlinear monotonic correlations between the values of experimental log ($1/EC_{50}$) and calculated log K_{ow}. The limitation of the online tools and an effect of the ILs structure on the "cut-off" effect have been discussed. The challenge of developing low microbial toxicity surface active ILs remains a significant task to overcome. Our results shed light on the new approaches for designing environmentally benign ILs and functional surfactants. As the hydrophobicity of the ILs significantly correlated with the toxicity, the *Vibrio fischeri* assay could be considered a powerful tool in providing toxicity data for building and evaluating the QSAR toxicity models for ILs.

1. Introduction

During the past two decades the number of scientific papers on ionic liquids (ILs) has increased almost exponentially (Fig. A1). The rapid developments in the field of ILs have led to a remarkable number of publications (> 57,000; Scopus search: "ionic liquid*", title-abs-key, as of July 10, 2018 and > 85,000 publications in WoS) and related patents (> 750,000; Google Patents as of July 10, 2018). The increasing interest and expanding number of publications in ILs is mainly due to the unique physicochemical properties of certain ILs, e.g. low vapor pressure, non-flammability, electrochemical and thermal stability, high ionic conductivity. This makes them an often screened desirable component for different fields of research and applications, e.g., as alternative solvents. ILs have been extensively studied within the field of green chemistry and have been indicated as "green chemicals" (due to their synthesis from sustainable compounds) and thus as promising alternatives for various chemicals (Jordan et al., 2016). Recent efforts have been directed towards better understanding the persistency, bioaccumulation and toxicity (PBT; REACH Regulation, Annex XIII; EC, 2006) assessment of ILs in the context of the "safe-by-design" approach

* Corresponding author at: ERA Chair of Green Chemistry, Department of Chemistry and Biotechnology, Tallinn University of Technology, Tallinn 12618, Estonia.
** Corresponding author at: Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Tallinn 12618, Estonia.
E-mail addresses: nicholas.gathergood@ttu.ee (N. Gathergood), anne.kahru@kbfi.ee (A. Kahru).

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(Docherty et al., 2007; Samorì et al., 2007; Ventura et al., 2012; Jordan and Gathergood, 2015; Haiß et al., 2016; Tang et al., 2017).

The (eco)toxicological effects of ILs have been investigated in a series of interdisciplinary studies (Ranke et al., 2004; Docherty and Kulpa, 2005; Matzke et al., 2010; Jordan et al., 2016). Typically, (eco)toxicological hazard industrial chemicals are evaluated using bioassays of different trophic levels using e.g., bacteria, algae, daphnids and fish. Among these the three latter assays - forming a proxy for a simplified aquatic food web - are compulsory ecotoxicity tests for REACH Regulation (EC, 2006). However, bacteria as destructors are not only the important members of the natural food-webs but also the most suitable test organisms for rapid screening of chemicals' toxic potency, due to the ease of handling in the laboratory and cost-efficiency (Ventura et al., 2012; Suppi et al., 2015). Gram-negative naturally luminescent marine bacteria *Vibrio fischeri* (also known under the name of *Photobacterium phosphoreum* NRRL-B-11177 and *Aliivibrio fischeri)* (Kurvet et al., 2011) are probably the most widely used bacteria for (eco)toxicological studies of so called "regular chemicals" (Kaiser and Devillers, 1994) but also in case of ILs (Garcia et al., 2005; Abbas et al., 2018). In addition, *V. fischeri* data are remarkably often used for the development of QSARs (Cronin and Schultz, 1997; Aruoja et al., 2011). *V. fischeri* responds rapidly to bioavailable toxicants with decrease in its natural bioluminescence in the time-scale of seconds-to-minutes, depending on the toxicant and its concentration. This is due to the disturbance of the integrity of cellular membrane, which functionality is essential for the central energy metabolism of the bacteria (Hastings et al., 1987). The *V. fischeri* test (e.g., Microtox™) toxicity data obtained on various chemicals (mostly organic industrial chemicals) have demonstrated significant correlations with toxicity data obtained with fish, crustaceans and algae (Kaiser, 1998; Aruoja et al., 2011), and also with animal cells *in vitro* (Kahru and Borchardt, 1994). Although the ecotoxicity of ILs is not so extensively studied yet, the same principle appears to be valid also for ILs. Indeed, Das et al. (2015) have conducted interspecies analysis of toxicities of comparative ILs toxicity data sets of 33–40 data points. They reported that due to the similar mechanism of toxicity of ILs to *V. fischeri*, *D. magna* and green algae *S. vacuolatus*, the *V. fischeri* could be used as a surrogate species to daphnia and algae as *V. fischeri* test is the most cost-efficient of these assays and also has the most abundant toxicity data. It was also shown that the computed lipophilicity parameter for the cations (*log* k_o) was an important predictor of ecotoxicity of ILs.

The inclusion of functional groups (including heteroatoms O and N) into the structure of ILs to reduce microbial toxicity and improve biodegradability includes ethers (Morrissey et al., 2009; Gathergood et al., 2006) and esters (Gore et al., 2013; Harjani et al., 2009). Examples including both ether and an ester bond in the side chain of the IL have lower lipophilicity than hydrocarbon analogues and low toxicity to seven different bacterial strains has been demonstrated (Morrissey et al., 2009). Substitution of the imidazolium ring with esters and amide functional groups also gave ILs which were not highly toxic towards bacteria and fungi (Gore et al., 2013). This study also investigated tandem ester and amide bond modification in the side chain of the imidazolium ILs, with the same trend identified. Research groups with the goal of designing green chemicals often evaluate toxicity and biodegradation together as an overall strategy. Recent advances have led to the development of an ultimately biodegradable ionic liquid, which includes an ester and amide bond (PyC_2Phe, see Fig. 1) (Haiß et al., 2016; Jordan et al., 2016). The key structural motif required was an amino acid ethyl ester subunit beta to the pyridinium cation headgroup. While imidazolium and cholinium analogues passed primary biodegradation criteria within 28 or 42 days, these compounds did not pass the Closed Bottle Test (CBT), and persistent transformation products were identified (Haiß et al., 2016; Jordan et al., 2016). Our group has stressed the importance of preliminary microbial toxicity SAR studies before undertaking a biodegradation evaluation.

In this study, a series of 24 L-phenylalanine derived ILs was studied

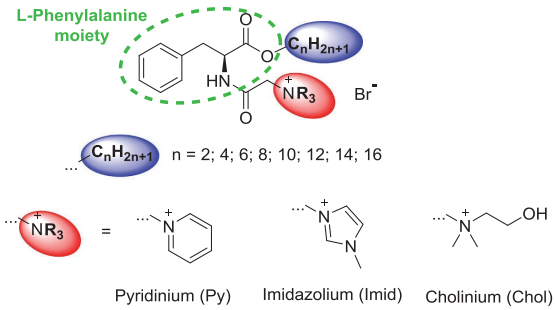

Fig. 1. A series of L-phenylalanine derived ILs studied in this work: PyC_nPhe, $ImidC_nPhe$ and $CholC_nPhe$. All ILs are Br^- salts.

with a high-throughput *V. fischeri* toxicity screening assay using two different endpoints: (i) inhibition of bacterial natural bioluminescence after 30 min of contact with the chemical and (ii) ability to yield visible colonies on agar plate after 4 h of contact with the chemical (see below). Three families of L-phenylalanine ILs based on pyridinium, imidazolium and cholinium cations and varying alkyl chain from 2 to 16 carbon atoms (C_2–C_{16}) were investigated (see Fig. 1).

The aim of this study was to shed light on the effect of the IL side chain length and headgroup structure on the antibacterial activity towards *V. fischeri*. Empirical correlations between the toxicities of the surface active ILs and their partitioning coefficients predicted by free access online QSAR tool were examined. We have analyzed the relationships to estimate a "cut-off" effect and a "limit" of the toxicity one would expect for the ILs of the similar structures. Analysis of the predictability and suitability of this approach for preliminary modelling was also performed.

2. Materials and methods

2.1. Test chemicals

A series of 24 of L-phenylalanine ILs was synthesized in the Green Chemistry laboratories of Tallinn University of Technology. Full description of synthesis and characterization data is described elsewhere (Jordan et al., 2016; Kapitanov et al., 2019).

For the experiments, stock solutions of ILs were prepared in MilliQ water, stored in the dark at room temperature and tested for toxicity within 2 weeks. Initial concentration for ILs was 20,000 μM for C_2, 5000 μM for C_4 to C_8 and 500 μM for C_{10} to C_{16} (Table A1). The initial pH values of the stock solutions were 4.6–6.5. All studied salts were soluble at the concentrations used under the experimental conditions.

2.2. Vibrio fischeri kinetic bioluminescence inhibition assay (a "Flash-assay")

Acute bioluminescence inhibition assay (exposure time 30-min) with bacteria *V. fischeri* was performed at room temperature (~20 °C) on white sterile 96-well polypropylene microplates (Greiner Bio-One GmbH) following the Flash-assay protocol (ISO, 2010). To 100 μL of test suspension in the microplate well 100 μL of bacterial suspension was added by automatic dispensing in the luminometer testing chamber. The bioluminescence was recorded during the first 30 s after dispensing of the bacterial suspension in each well without additional mixing of the sample and once after 30 min incubation. The Microplate Luminometer Orion II (Berthold Detection Systems, Pforzheim, Germany), controlled by Simplicity Version 4.2 Software was used. Reconstituted *V. fischeri* Reagent (Aboatox, Turku, Finland) was used as the test bacteria suspension and all chemicals and their dilutions were prepared in 2% NaCl. Each test was performed in 5–7 dilutions, each in

Table 1
Toxicity results (30-min EC_{50} and 4-h MBC values) and a simplified hazard classification scheme for pyridinium, imidazolium and cholinium substituted L-phenylalanine derived bromide ILs and used positive controls (BAC and 3,5-DCP) according to the toxicity data obtained using *Vibrio fischeri*.

Compound	30-min EC_{50}±SD	4-h MBC	30-min EC_{50}±SD	4-h MBC
	μM		mg/L	
PyC_2Phe^{b}	2203±281	> 10000	866±110	>3933
PyC_4Phe^{c}	192±31.8	2000	81.0±13.4	843
PyC_6Phe^{ef}	27.3±1.62	1000	12.3±0.73	449
PyC_8Phe^{g}	19.5±2.53	250	9.32±1.21	119
$PyC_{10}Phe^{hi}$	8.71±1.23	31.3	4.40±0.62	15.8
$PyC_{12}Phe^{i}$	7.82±0.82	15.6	4.17±0.44	8.32
$PyC_{14}Phe^{hi}$	9.16±2.17	15.6	5.14±1.22	8.76
$PyC_{16}Phe$	> 62.5	> 62.5	>36.9	>36.9
$ImidC_2Phe^{a}$	3096±282	> 10000	1227±112	>3963
$ImidC_4Phe^{c}$	216±25.2	2000	91.7±10.7	849
$ImidC_6Phe^{fg}$	24.3±3.50	1000	11.0±1.58	452
$ImidC_8Phe^{g}$	19.4±3.95	250	9.31±1.90	120
$ImidC_{10}Phe^{h}$	8.89±0.83	62.5	4.52±0.42	31.8
$ImidC_{12}Phe^{i}$	8.36±0.62	15.6	4.49±0.33	8.37
$ImidC_{14}Phe^{i}$	8.22±0.83	15.6	4.64±0.47	8.81
$ImidC_{16}Phe$	> 62.5	> 62.5	>37.0	>37.0
$CholC_2Phe^{ab}$	2858±271	> 10000	1153±109	>4033
$CholC_4Phe^{c}$	209±34.2	2000	90.1±14.8	863
$CholC_6Phe^{e}$	32.3±4.40	1000	14.8±2.02	458
$CholC_8Phe^{d}$	42.0±2.51	500	20.5±1.23	244
$CholC_{10}Phe^{h}$	11.1±1.49	62.5	5.72±0.77	32.2
$CholC_{12}Phe^{i}$	7.90±0.81	15.6	4.29±0.44	8.48
$CholC_{14}Phe^{hi}$	8.03±0.73	15.6	4.59±0.42	8.91
$CholC_{16}Phe$	> 62.5	> 62.5	>37.5	>37.5
BAC^{j}	3.31±0.62	31.25	0.94±0.18	8.87
$3,5\text{-}DCP^{g}$	29.0±2.92	400	4.72±0.48	65.20

[a–j] indicate statistical significance. Means with the same letter are not significantly different at $p = 0.05$.
Color code: ≤1 mg/L ( ) = very toxic; >1–10 mg/L ( ) = toxic; >10–100 mg/L ( ) = harmful; >100 mg/L ( ) = "not classified/not harmful"

two replicates. Controls, both negative (2% NaCl) and positive (3,5-dichlorophenol; 3,5-DCP and benzalkonium chloride; BAC), were included in each measurement series. The inhibition of bacterial luminescence (INH%) by the analyzed compounds was calculated as follows:

$$INH\% = 100 - \frac{IT_{30}}{KF*IT_0}*100 \quad \text{with } KF = \frac{IC_{30}}{IC_0};$$

KF (correction factor) characterizes the natural loss of luminescence of the control (i.e. bacterial suspension in 2% NaCl). IC_0 and IT_0 are the maximum values of luminescence during first 5-sec after dispensing of 100 μL of test bacteria to 100 μL of control or test sample, respectively. IC_{30} and IT_{30} are the respective luminescence values after 30-min. EC_{50} is the concentration of a compound reducing the bioluminescence by 50%. EC_{50} were calculated from the concentration versus INH% curves based on nominal exposure concentrations and using the log-normal model of MS Excel macro Regtox (Vindimian, 2016). The concentration-effect curves were based on at least five concentrations, and at least five concentrations were used for the regression.

2.3. *Vibrio fischeri* viability assay (a "Spot-test")

V. fischeri viability assay (a "Spot-test") was performed as described in detail in Kurvet et al. (2017) to evaluate the ability of the toxicant-exposed bacteria to form colonies on toxicant-free nutrient agar after exposure to the tested chemicals in 2% NaCl.

Briefly, at the end of the bioluminescence inhibition assay (see

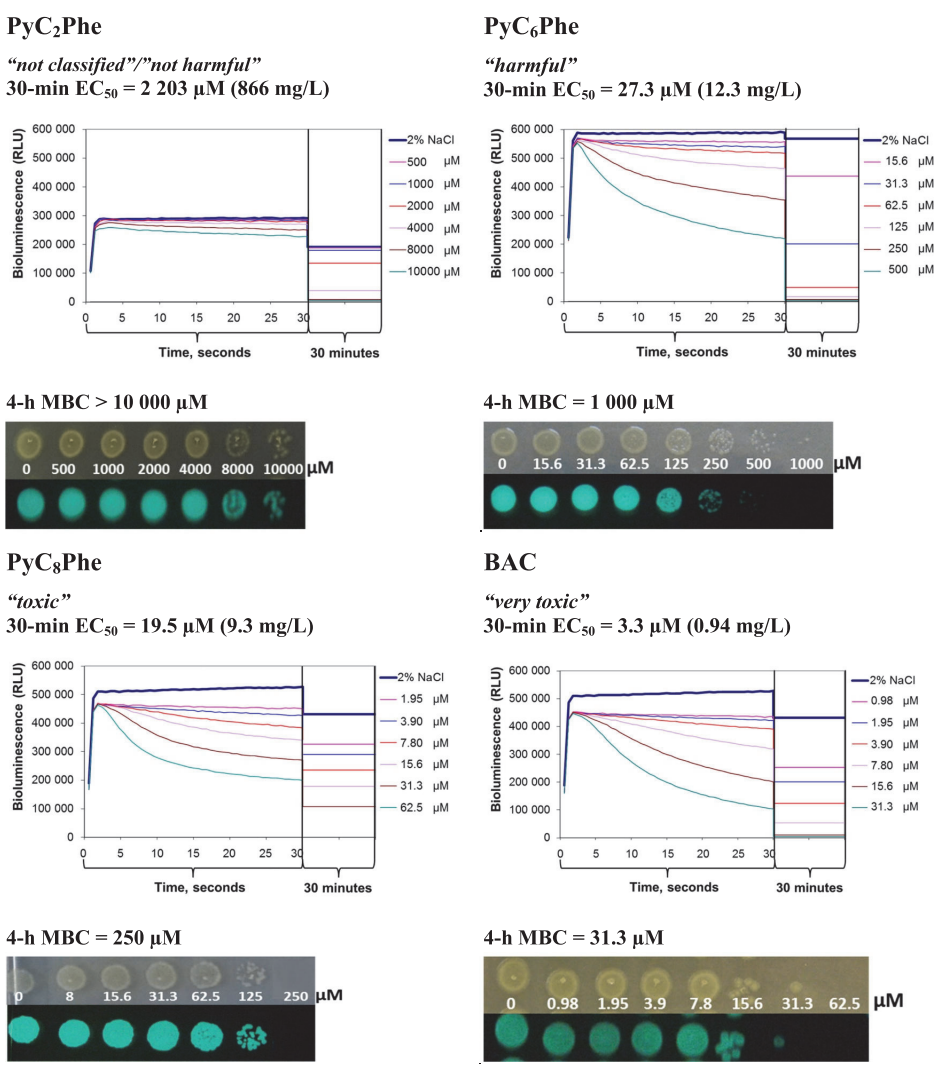

Fig. 2. Inhibition of bioluminescence (concentration-effect curves; Flash-assay) and colony-forming ability (visualization of bacterial growth on nutrient agar plates; viability assay) of bacteria *Vibrio fischeri* exposed to different concentrations of PyC_2Phe, PyC_6Phe, PyC_8Phe ILs and benzalkonium chloride (BAC). 30-min EC_{50} values calculated from concentration-effect curves, 4-h minimal bactericidal concentration (MBC) values and respective toxicity rankings are taken from Table 1. RLU – relative light units; 2% NaCl – negative control. See also Fig. A2.

Section 2.2.) microplates were incubated for 4-h at room temperature and 3 µL of each sample was removed and pipetted as a spot onto an agarized Beneckea Harvey (BH) growth medium containing (per liter): yeast extract 3 g, tryptone 5 g, glycerol (99%) 2 mL, NaCl 30 g, $Na_2HPO_4{\cdot}12H_2O$ 9.45 g, KH_2PO_4 1 g, $(NH_4)_2HPO_4$ 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.3 g, agar 15 g. The inoculated agar plates were incubated at room temperature in the dark for 72-h and the minimal bactericidal concentration (MBC) was determined as the lowest tested nominal concentration of a chemical which totally inhibited the ability of the cells to yield visible (bioluminescent) colonies on toxicant-free nutrient agar-plates.

2.4. Statistical analysis

All tests were performed in at least three independent experiments and duplicates in every single experiment. Statistical analysis was performed using SPSS 21.0 Edition for Windows. Results were expressed as mean ± standard deviation (SD). Data were analyzed by one-way analysis of variance, and Tukey's test was used to compare differences among all samples. A p value < 0.05 was considered statistically significant.

3. Results and discussion

In our previous work, microbial toxicity tests supporting the biodegradation studies (CBTs) were to establish whether ILs have undesirable high toxicity to bacteria and/or fungi (Gore et al., 2013; Jordan et al., 2016). Our group first used this approach to develop green imidazolium ionic liquids including amino acid esters in the side chain (e.g. Phe, Val, Ala or Ile) (Coleman et al., 2012). These ILs did not

exhibit high toxicity to 8 gram-negative and gram-positive bacterial strains. The microbial toxicity screen was expanded to 8 bacterial and 11 fungal strains in the following studies (Jordan et al., 2016; Gore et al., 2013), where these ILs also did not exhibit high toxicity (e.g. bacteria IC_{95} > 500 μM and fungi IC_{50} > 500 μM). The main limitation to this approach of microbial toxicity screening is that SARs of desirable ILs with low toxicity cannot be performed. This is due to the maximum concentration (1–2 mM) limit used in the reported studies and therefore exact quantitative toxicity values (e.g. IC_{50}) are not known. While the ultimately biodegradable pyridinium compound (PyC_2Phe) we reported can be classified as not highly toxic to the microbial strains screened (e.g. bacteria IC_{95} > 500 μM and fungi IC_{50} > 500 μM), based on current data we cannot claim it has low microbial toxicity. In addition the microbial toxicity studies performed were on drug susceptible and drug resistant strains of bacteria (e.g. SA and MRSA) and fungi (e.g. *Candida* strains) to assess whether PyC_2Phe has potential as an antimicrobial pharmaceutical (Jordan et al., 2016). Although high toxicity (e.g. bacteria IC_{95} > 10 μM and fungi IC_{50} > 10 μM) was not observed for all strains screened, this is only considered as a preliminary microbial toxicity study before more relevant (from the viewpoint of designing safer ionic liquids) environmental toxicity data can be obtained (Jordan et al., 2016; Gore et al., 2013; Coleman et al., 2012).

3.1. *Vibrio fischeri* kinetic bioluminescence inhibition assay

This study provides new toxicological data for a series of pyridinium, imidazolium and cholinium substituted phenylalanine derived ILs (Table 1) as these compounds have not been analyzed for the toxicity using *V. fischeri* bioluminescence inhibition assay. Bacterium *V. fischeri* (also known under the name of *Photobacterium phosphoreum* NRRL-B-11177 and *Aliivibrio fischeri)* was chosen to study the toxic effects of ILs due to the extensive prior use of *V. fischeri* toxicity data for remarkable amount of chemicals in QSAR modelling (Cronin and Schultz, 1997; Aruoja et al., 2011). Moreover, as *V. fischeri* is naturally luminescent bacterium isolated from the sea water, it is of environmental relevance. The Microtox test that uses *V. fischeri* was the first widely used ecotoxicological assay due to its environmental relevance, cost-efficiency and availability as a commercial test kit (Bulich and Isenberg, 1981; Kaiser and Devillers, 1994; Wolska et al., 2007). There are various luminescence inhibition tests of *V. fischeri*, most of them are developed for the analysis of aqueous samples (Microtox, LUMIStox, BioTox, ToxAlert), whereas one of the test formats – a Flash-assay – is also applicable for the analysis of turbid suspensions (including solid/colored environmental samples, e.g. sediments, soil suspensions, wastewater, sludge extracts, etc.) and is also an ISO standardized acute toxicity test. The decrease in bacterial luminescence occurs already after brief contact of bacteria with toxicants (on the scale of seconds to minutes, depending on the compounds) (Mortimer et al., 2008) as shown in Fig. 2.

The decrease in bioluminescence reflects the inhibition of bacterial metabolic activity and is proportional to the toxicity of test sample (Bulich and Isenberg, 1981). To connect the toxicity data obtained using 30-min bioluminescence inhibition with viability of the bacteria after more prolonged incubation with ILs (up to 4 h), we also conducted a viability assay (see Section 2.3). Results of the ILs from the 30-min bacterial bioluminescence inhibition assay and viability test (4-h) are presented in Table 1. Experimentally determined mean 30-min EC_{50} values for the studied ILs ranged from 8 μM ($PyC_{12}Phe$; $ImidC_{12}Phe$; $ImidC_{14}Phe$; $CholC_{12}Phe$; $CholC_{14}Phe$) to more than 2000 μM (PyC_2Phe; $ImidC_2Phe$; $CholC_2Phe$). The minimal bactericidal concentration (4-h MBC) values were between 16 μM to > 10,000 μM, and the general tendency was in agreement with the obtained EC_{50} values (Fig. 3). The most toxic ILs against *V. fischeri* were C_{12} (Py, Imid, Chol) and their toxicity was comparable to benzalkonium chloride (BAC), a well-known antimicrobial compound.

For environmental risk assessment, the toxicity of chemicals is usually calculated on mg/L basis and not on molar-basis (useful for QSARs). According to EU Chemicals Regulation REACH, all substances that are manufactured in the EU or imported to the EU more than 1 t/y have to be registered and characterized for their potential hazardous effects (EC, 2006). To estimate the toxicity to the aquatic environment, usually one species of algae, crustaceans, and fish are used, in addition substance degradation rate (if absent, the log K_{ow}) and its bioconcentration factor are considered (EC, 2008). The toxicity of chemicals to bacteria is not taken into account in ecotoxicity assessment for regulatory purposes. However, it has been shown that *V. fischeri* bioluminescence inhibition assay is a suitable test for rapid screening of chemicals' toxic potency (Kaiser, 1998; Aruoja et al., 2011; Kahru and Borchardt, 1994). Based on the simplified hazard classification scheme described in (Aruoja et al., 2011) the studied ILs were categorized according to the experimentally obtained EC_{50} or MBC values as: ≤ 1 mg/L = very toxic; > 1–10 mg/L = toxic; > 10–100 mg/L = harmful; > 100 mg/L = "not classified/not harmful". The 30-min EC_{50} and 4-h MBC values (mg/L) for *V. fischeri* ranged from 4 ($PyC_{10}Phe$; $PyC_{12}Phe$; $ImidC_{12}Phe$; $CholC_{12}Phe$) to > 800 (PyC_2Phe; $ImidC_2Phe$; $CholC_2Phe$), hence the classification ranged from toxic to "not harmful" substances (Table 1).

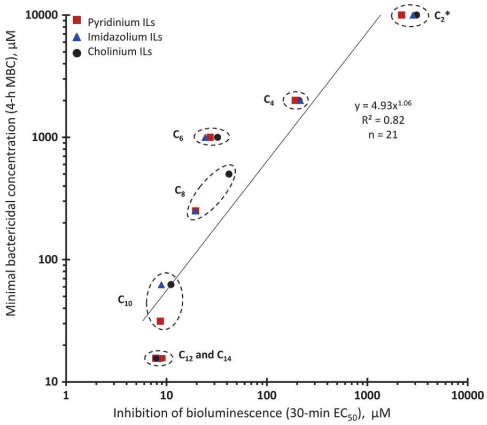

Fig. 3. Correlation of the results of two toxicity assays applied for the evaluation of the toxicity of 21 ILs: 30-min bioluminescence inhibition assay versus 4-h viability assay (a "Spot-test"). A log-log plot; R^2 value 0.82 ($p < 0.05$). Data are plotted from Table 1. *4-h MBC > 10,000 μM.

The luminescence inhibition of *V. fischeri* caused by toxicants is a sub-lethal response and does not fully reflect the viability of cells. The minimal bactericidal concentration (MBC), however, is based on the viability/mortality endpoint, i.e., the inability of the cells to grow after the exposure to toxic concentration of the chemical. Despite the above described toxicity endpoints are different, the 30-min EC_{50} and 4-h MBC values for studied ILs correlated reasonably well (log-log R^2 = 0.82), the MBC values were on average up to 10 fold higher compared to 30-min EC_{50} values (see Fig. 3).

Analogous results were obtained in the study of MEIC chemicals: the reduction of light output of luminescent bacteria was about 10-fold more sensitive endpoint than the minimal inhibitory concentration, MIC, for *Escherichia coli* and *Bacillus subtilis* (Kahru and Borchardt, 1994). Also, Kurvet et al. (2017) in case of lanthanides showed that the *V. fischeri* 24-h MBC values were on average 2-fold higher compared to the 30-min EC_{50} values. Interestingly, C_6 ILs were outliers as compared to the rest of the toxicity data, the luminescence inhibition assay "presented" these ILs more toxic than confirmed in the viability assay, as shown on Fig. 3.

Viboud et al. (2012) has studied the effect of different components of ILs (e.g. cation headgroup, alkyl chain and anion) using *V. fischeri*

Table 2
Comparison of the toxicity of imidazolium and pyridinium-based bromide ILs and selected volatile organic compounds (VOCs) to *Vibrio fischeri*. Data from the current work were compared with literature data.

V. fischeri data from this work		*V. fischeri* data from literature		
Ionic liquids*	Log EC_{50} (30-min, ~20 °C) μM	Ionic liquids and volatile organic compounds	Log EC_{50} (15-min, 15 °C) μM	Log EC_{50} (30-min, 15 °C) μM
		[bmim] Br	3.66[a]	
($ImidC_2Phe$) Br	3.49			
($CholC_2Phe$) Br	3.46	[bmim] Cl	3.46[b]	
		[bpyr] Br	3.39[c]	
(PyC_2Phe) Br	3.34			
		Benzene	3.24[d]	3.06[d]
		Phenol	3.24[a,e]	3.35[e]
		[hmim] Br	3.13[a]	
		[hpyr] Br	2.85[a]	
		Toluene	2.38[d]	2.37[d]
($CholC_4Phe$) Br	2.34	$C_8(MeIm)_2Br_2$	2.34[f]	
($ImidC_4Phe$) Br	2.33			
(PyC_4Phe) Br	2.28			
		$C_{12}(Pyr)_2Br_2$	2.07[f]	
		[hmim] Cl	1.94[g]	
		[opyr] Br	1.89[a]	
($ImidC_{16}Phe$) Br	> 1.80			
($PyC_{16}Phe$) Br	> 1.80			
($CholC_{16}Phe$) Br	> 1.80			
($CholC_8Phe$) Br	1.62			
($CholC_6Phe$) Br	1.51			
(PyC_6Phe) Br	1.40			
($ImidC_6Phe$) Br	1.38			
(PyC_8Phe) Br	1.29			
($ImidC_8Phe$) Br	1.29			
($CholC_{10}Phe$) Br	1.04			
($PyC_{14}Phe$) Br	0.96			
($ImidC_{10}Phe$) Br	0.95			
($PyC_{10}Phe$) Br	0.94			
($ImidC_{12}Phe$) Br	0.92			
($ImidC_{14}Phe$) Br	0.92			
($CholC_{12}Phe$) Br	0.90			
($CholC_{14}Phe$) Br	0.90			
($PyC_{12}Phe$) Br	0.89			
		[omim] Cl	0.69[b]	
		[omim] Br	0.63[c]	

Notes:
* Structures of the ILs are collected in the Table A2.
[a] Viboud et al. (2012).
[b] Montalbán et al. (2016).
[c] Docherty and Kulpa (2005).
[d] Kaiser and Devillers (1994).
[e] Aruoja et al. (2011) (test was performed at ~20 °C).
[f] Montalbán et al. (2018a).
[g] Luis et al. (2007).

inhibition test (15-min EC_{50}). *V. fischeri* data (30-min EC_{50}) on selected ILs obtained in the current study were compared to *V. fischeri* data (15-min EC_{50}) of the suitable imidazolium and pyridinium-based ILs as well as data of some well-known volatile organic compounds (VOCs) by Viboud et al. (2012). The highest toxicity was observed for phenol and is included in Table 2, while other VOCs screened (such as acetonitrile, ethanol, ethyl acetate, and acetone) by Viboud have lower toxicity than $ImidC_2Phe$. As can be seen from Table 2, ILs from this work were more toxic compared to the VOCs reported by Viboud et al. (2012), with the exception of the C_2 series which was less toxic than phenol. Kaiser and Devillers (1994) report a similar value for benzene as phenol, however toluene was more toxic to *V. fischeri.* From our study the C_4 series has similar log EC_{50} *V. fischeri* toxicity values to toluene in the range 2.28–2.34. In general, we consider the short chain examples (C_2 and C_4 series) as possible replacements for VOCs, however only the C_2 series was shown to be less toxic than toluene in the test performed.

3.1.1. Influence of IL's cationic part on its toxicity

The data available in literature, either experimental studies (Ranke et al., 2004; Ruokonen et al., 2017) or QSAR models for predicting toxicity of ILs against *V. fischeri* (Grzonkowska et al., 2016; Ben Ghanem et al., 2017) have pointed out that toxicity of IL is determined by its structure and depends mostly on the cation's structure – its size and chain length – and the influence of the anion on the toxicity level is not substantial. Imidazolium and pyridinium are among the most widely synthesized ILs (Heckenbach et al., 2016). In this study, we have compared the effects of different cationic head groups (pyridinium, imidazolium and cholinium) of ILs on the toxicity towards *V. fischeri*. Several studies have indicated that ILs with aromatic cations such as pyridinium and imidazolium, are in general more toxic than the non-aromatic cation ring of ILs, such as cholinium (Nockemann et al., 2007; Matzke et al., 2010; Stolte et al., 2007). In the current study no statistical differences were observed for pyridinium. imidazolium and cholinium based ILs with similar number of carbon in the alkyl chain (Fig. A3). The only exception was $CholC_8Phe$ which was less toxic than PyC_8Phe and $ImidC_8Phe$. Montalbán et al. (2018b) have explained as both pyridinium and imidazolium are planar heteroaromatic groups, this could promote the interaction with the membrane lipid bilayer and consequently increase their toxicity. Furthermore, increase of membrane activity might be directly connected to an increase in the size of cationic side chain length. Gal et al. (2012) mentioned that the cation core and their orientation are the key factors that establish membrane interactions and concurrent toxicity. Polarity in the cationic ring could contribute for the toxicity of imidazolium and pyridinium based ionic liquids (Viboud et al., 2012). These results are in agreement with the data from Costa et al. (2015) and Nockemann et al. (2007) reports. However, Silva et al. (2014) described that cholinium salts, either mono- or dicationic, exhibit a different toxicity mechanism as compared to imidazolium. The cholinium group contains a non-aromatic quaternary nitrogen and a hydroxyl group. This charge headgroup of the molecule is distinctly different to the imidazolium and pyridinium ionic liquids and uptake by the bacteria and membrane activity may be different. Especially, 30-min EC_{50} values for $CholC_8Phe$ do not follow the general trend observed for imidazolium and pyridinium analogues. Explicitly that C_8 analogues are more toxic than C_6, C_4 and C_2 and less toxic than C_{10}, C_{12} and C_{14}. $CholC_8Phe$ is less toxic than $CholC_6Phe$ and this also suggests a different toxicity mechanism for $CholC_8Phe$ compared to other cholinium ILs studied.

3.1.2. Influence of alkyl chain length on toxicity of ILs

The effect of IL alkyl chain length (and hence lipophilicity) on toxicity towards bacteria has been discussed by several researchers (Ranke et al., 2004; Matzke et al., 2010; Montalbán et al., 2016; Silva et al., 2014) demonstrating that the elongation of the alkyl chain results in enhanced toxicity. Analogously, Kubo et al. (2017) recently reported that polyoxometalate ionic liquids (POM-ILs) having longer-chain alkylammonium cations showed higher antimicrobial activity. It has been observed that the tendency of increasing toxicity for all head groups (pyridinium, imidazolium and cholinium) follows this side chain length effect up to certain alkyl chain length as mentioned in Matzke et al. (2010). The results of the current study showed that irrespectively of the cationic head group structure, toxicity increased with the tail length from C_2 to C_{10} (see Fig. A3, the only exception was for $CholC_8Phe$). Latter is consistent with the literature findings (Ranke et al., 2004; Luczak et al., 2010; Shao et al., 2017). Similarly to our data, it has been reported that the toxicity to *V. fischeri* (Couling et al., 2006; Viboud et al., 2012; Ventura et al., 2012), to Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana* (Latala et al., 2005) and to *D. magna* (Couling et al., 2006; Das et al., 2015), correlated positively with the length of the alkyl chain.

However, the toxicity *vs* chain length profile undergoes levelling-off for the chain length from C_{10} to C_{14} (almost no changes in toxicity), and even lower toxicity against *V. fischeri* when the alkyl group is extended

to C_{16} (Fig. A3). Therefore, the toxicity increased monotonously with the alkyl chain elongation but at a certain point levels up or even decreases. This phenomenon has been labeled as a "cut-off" effect is widely discussed in the studies of the biological activities of long-chain amphiphilic molecules, including ILs (Stolte et al., 2007; Matzke et al., 2010; Luczak et al., 2010; Ventura et al., 2012). There are several explanations proposed for this effect (Luczak et al., 2010): (i) the elongation of the side chain may result in the "limit in solubility" caused by the solubility in the lipid phase to increase faster than the partitioning of the organic salt occurs; this caused the concentration to become insufficient to disrupt a biological membrane; (ii) the long-chain ILs may act as the mimicking molecules for the lipid bilayer, which have a lower impact on the membrane; (iii) the tendency of the ILs to form micelles (the longer the chain length the lower the critical micelle concentration) becomes more significant rather than the tendency to interact with a membrane, it may cause decreasing IL monomer concentration at the site of action.

3.2. Relationship between octanol/water partition coefficient (K_{ow}) of ILs and their toxicity to Vibrio fischeri

The basic structural unit of any biological membrane is a lipid bilayer. For unicellular organisms this lipid bilayer is also part of the barrier between the cell and the environment and therefore ability of a chemical to dissolve in lipids is an important characteristic whether it is able to cross the membrane and enter the cell or not. Entering the cell, in turn, is a pre-requisite for the chemical to act as a toxicant. Among the most important and fundamental properties of chemical compounds is octanol/water partition coefficient (K_{ow}) – a parameter which describes the hydrophobicity and the tendency of a chemical to mimic behavior in a membrane's lipid phase and ability to cross the biological membrane (Ventura et al., 2012; Ranke et al., 2004). Correlations between toxicity and K_{ow} of the ILs have been reported to determine various properties such as environmental hazard profile of ILs (Lee and Lee, 2008; Montalbán et al., 2016). ILs with longer alkyl chain show stronger attachment of the molecule to the lipid bilayer surface, most likely contributing to a substantial interference of the membrane bilayers, suitable with a strong micellization effect (Montalbán et al., 2018b). This relation can be defined by accumulation of the substances in the membrane lipid bilayer that may cause loss of the integrity of membrane lipid bilayer. Heipieper et al. (1994) reported about two decades ago that chemicals with log K_{ow} values (between 1 and 5) effect the membrane lipid bilayer. Recently, Heipieper and co-workers (Piotrowska et al., 2017; Atashgahi et al., 2018) developed this idea further in the area of prokaryotes' response in natural environments to high concentrations of chemicals and physical stress by membrane "repair" to reestablish membrane fluidity and rigidity.

For the structurally similar ILs, the greater is log K_{ow}, the larger its membrane accumulation and toxicity (Montalbán et al., 2018a). The experimental log K_{ow} values for ILs with C_{10} to C_{14} were found to be around 1 (Montalbán et al., 2018b), which means these substances might have effect to the membrane lipid bilayer. Along with various experimental methods developed for the experimental measurements of K_{ow} for different classes of ILs (Montalbán et al., 2018c) or relationships proposed for the certain IL series (Montalbán et al., 2018a), several QSAR approaches are available as open access chemoinformatics tools, e.g. *molinspiration.com*, *vcclab.org*, *chemicalize.org*, etc. An empirical relationship was reported by Montalbán et al. (2016) for the experimental data on the toxic effect of the wide series of ILs, mainly imidazolium salts with short to medium chain length and variable anions, 24 compounds in total, towards *V. fischeri* as a function of the log K_{ow} of ILs, Eq. (1):

$$\log(1/EC_{50}) = -1.436 + 0.859 \log K_{ow} - 0.119(\log K_{ow})^2 \quad (1)$$

This relationship was reported for moderately hydrophobic monocationic salts, most of them with log K_{ow} in the range from ca. −2 to ca. −1. Later, these authors extended their study with a series of more hydrophilic dicationic (bolaform) ILs with remarkably lower toxicity towards *V. fischeri* along with a series of the more hydrophobic octyl side chain ILs with various counterions (Montalbán et al., 2018a). The bolaform salts data with log K_{ow} values calculated by means of online tool www.molinspiration.com was reported by Montalbán et al. (2018a) to demonstrate positive deviation from the Eq. (1). On the other hand, the octyl ILs were calculated to have the log K_{ow} in a wide range from −1 to 3 with relatively similar toxicities within a series and EC_{50} values, which are lower than expected from the polynomial correlation (1). Although Montalbán et al. (2016) have demonstrated a correlation between the log EC_{50} estimated by online tools with the experimental data for some of the ILs tested, the applicability of different online tools is an outstanding issue. In addition, Eq. (1) can hardly be applied for the ILs with log $K_{ow} > 1$ since the predicted toxicity values are even higher for BAC, one of the most efficient antibacterial of quaternary ammonium compounds. Therefore, studying ILs with medium (C_6 - C_8) to longer ($\geq C_{10}$) side chain length (corresponding to moderate to high hydrophobicities in terms of log K_{ow}) may elucidate either the question of the toxicity limit for ILs or applicability of different online tools predicting physical properties, e.g. lipophilicity and solubility in water.

In this work, the K_{ow} values were estimated with the two open access tools (http://www.molinspiration.com/services/logp.html and http://www.vcclab.org/lab/alogps/) (Tetko and Tanchuk, 2002) using SMILES (Simplified Molecular Input Line Entry System) notation. The predicted log K_{ow} values along with experimental log ($1/EC_{50}$) data are collected in the Table A3 and plotted on the Fig. 4 together with the data reported by Montalbán et al. (2016, 2018a). Since the K_{ow} is also one of the key indicators in the assessment of environmental risk and the fate in the natural environment, chemicals with high log K_{ow} values (≥ 4.0) are considered to have greater concern to the environment (EC, 2008). In this work, all log K_{ow} values of the studied ILs were estimated

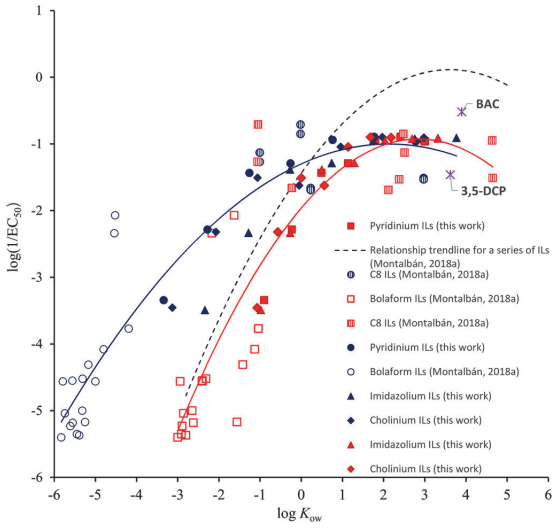

Fig. 4. Correlation between log ($1/EC_{50}$) and log K_{ow} for the series of studied ILs and monocationic and dicationic ILs reported by Montalbán et al., (2016, 2018a). The K_{ow} values were estimated by means of www.molinspiration.com (blue markers) and www.vcclab.org (red markers). The values of K_{ow} for imidazolium ILs (dashed line) have been reported by Montalbán et al. (2016) as experimentally determined. Data for 3,5-DCP and BAC were taken from the work of Hansch and Hoekman (1995) and US EPA EPI Suite™ (2012), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to be < 4, see Table A3, with one exception $ImidC_{16}Phe$ (4.78). At the same time, the different online tools give different predicted log K_{ow} values. For less hydrophobic bolaform salts with methyl groups in side chain (Montalbán et al., 2018a) molinspiration.com predicts log K_{ow} in the range between −6 and −4, and the vcclab.org values represents a more hydrophobic range from −3 to −1. It is worth noting that in the latter case the points corresponding to pyridinium and imidazolium dicationic salts (Montalbán et al., 2018a) are reasonably close to the Eq. (1) proposed by these authors earlier (dashed line in Fig. 4), and only pyrrolidinium salts are shifted to the higher K_{ow} region (-2 < log K_{ow} < −1). There are also some differences in the predicted K_{ow} for the ILs presented in this work by means of the two online resources. The vcclab.org provides the values with no remarkable differences for various cation head groups, only chain length plays a role in the predicted values. In the case of molinspiration.com, this calculation gives some difference to distinguish pyridinium (the lowest K_{ow}), cholinium (slightly higher K_{ow}), and, in essence, imidazolium (the highest K_{ow}) salts with the same chain length, see Fig. 4.

The correlations including the ILs reported in this paper along with dicationic bolaform ILs and octyl ILs (Montalbán et al., 2018a) were obtained for the log K_{ow} estimated from www.molinspiration.com, Eq. (2) and www.vcclab.org, Eq. (3):

$$\log(1/EC_{50}) = -1.300 + 0.280\log K_{ow} - 0.066(\log K_{ow})^2); r^2 = 0.904 \quad (2)$$

$$\log(1/EC_{50}) = -1.963 + 0.729\log K_{ow} - 0.128(\log K_{ow})^2; r^2 = 0.825 \quad (3)$$

The two correlations (for 45 ILs each) shown on the Fig. 4 gave satisfactory correlation and demonstrate that different online tools have different deviations from the experimental correlation (1), represented as a dashed line on the Fig. 4. The overall series has an increase in toxicity when transfer from dicationic salts to short- and medium chain length ILs (C_2 to C_6 -C_8), and further to the long chain derivatives (between C_8–C_{10} and C_{14}). When the log K_{ow} values for the less hydrophobic ILs, including dicationic (bolaform) salts (Montalbán et al., 2018a) and Phe ILs with short chain length (C_2 to C_6) are obtained from molinspiration.com (blue color signs on Fig. 4), the corresponding points experience positive deviations from the dependence described by Eq. (1). At the same time, the deviations for imidazolium ILs (namely, $ImidC_2Phe$, $ImidC_4Phe$, $ImidC_6Phe$) are less remarkable, when compared to other salts. Alternatively, vcclab.org predicts the log K_{ow} values to have negative deviations, which is less remarkable as the negative deviations observed for the molinpiration.com results, particularly, for the bolaform ILs. With elongation of the side chain, the predicted values taken from the two tools become less significant, and in both cases the points undergo negative deviation from the Eq. (1). The highest toxicity values experimentally found for the C_{10} - C_{14} ILs are remarkably lower than that for BAC, see Fig. 4 (log K_{ow} for BAC was illustratively taken as estimated value for C_{14} BAC using EPA tool https://www.epa.gov/tsca-screening-tools; 3,5-DCP data reported by Hansch and Hoekman, 1995). The Eq. (1) built for short-chain ILs should be used judiciously for predicting behavior of the hydrophobic (e.g. long-chain) ILs. In particular, limit of toxicity (predicted values higher that experimentally found for number of the ILs) and the "cut-off" effect (predicted by Eq. (1) for ILs with higher K_{ow} than actually occurs) should be taken into account. The "cut-off" effect we report here for longer chain length members of the ILs series leads to the "limit" toxicity level for this type of ILs and is similar to the toxicity values previously reported by Montalbán et al. (2018a) for octyl ILs, the longest chain length reported in that study.

Therefore, our studied series of 24 ILs can be represented as three categories of compounds with three different toxicity mechanisms, dependent on their structure. The ILs with the shortest chain C_2 are non-toxic with PyC_2Phe reported to be biomineralisable (Haiß et al., 2016; Jordan et al., 2016). According to the work of Ruokonen et al. (2017), they may not rupture cell membrane but could affect the cell metabolism at toxic concentration. The main group of the ILs, from C_2 to C_{14} (18 compounds in total) form a monotonic dependence for their short and medium chain length members (C_2–C_{10}) with partially cell membrane rupturing effect followed by levelling-off for the longer chain (C_{12} and C_{14}) salts with the highest toxicity values within the series. This "cut-off" effect turns to the reverse dependence of toxicity on the hydrophobicity observed for the longest chain length ILs (C_{16}), which may be connected either to the aggregation of the C_{16} salts (preferred micellization occurs) or their insufficient solubility (Krafft temperature constraint).

To summarize, it can be noted that for the ILs with log $K_{ow} \geq 1$ the toxicity is expected to undergo the levelling-off with log $1/EC_{50}$ ca. 1.0. None of the ILs reported in this work have higher toxicity against *V.fischeri* than BAC.

4. Conclusions

A library of 24 pyridinium-, imidazolium-, and cholinium-based ionic liquids (ILs) with varying alkyl chain from C_2 to C_{16} was toxicologically profiled using naturally luminescent marine bacteria *Vibrio fischeri*. The toxicity (30-min EC_{50}) of studied ILs to *Vibrio fischeri* ranged from 7.82 μM (4.2 mg/L) ($PyC_{12}Phe$) to 3096 μM (1227 mg/L) ($ImidC_2Phe$), i.e. from "toxic" (EC_{50} 1–10 mg/L) to "not harmful" (EC_{50} > 100 mg/L.) The inhibition of the bacterial luminescence upon exposure to ILs correlated well with bacterial viability. The toxicity of studied ILs was mostly dependent on the length of the alkyl chain (i.e. hydrophobicity) and not the type of the cationic part of the IL: starting from C_{10} all the ILs irrespective of the cationic part proved "toxic". The elongation of the chain length result in the "cut-off" effect to demonstrate similar EC_{50} values for the ILs for the chain length longer than C_{10}. All twenty-four ILs reported in this study were comparably or less toxic than [omim]Br. Noteworthy, the highest toxicity against *V.fischeri* reported in this study was remarkably lower than that for BAC, one of the most potent antimicrobial agents. The values of EC_{50} ca. 8.0 μM can be considered as the "limit" (the least small value) of the toxicity towards *V. fischeri* one could expect for the ILs of the similar chemical structure.

Two open-access online tools (www.molinspiration.com and www.vcclab.org) applied for calculation of the K_{ow} values for the 24 ILs reported in this study, along with 21 ILs reported in the literature, result in plotting nonlinear monotonic correlations between the experimental log ($1/EC_{50}$) values and calculated log K_{ow}. The plots built up by means of two different tools depict similar shape and limit log ($1/EC_{50}$) values at the "levelling-off" region but demonstrate remarkable differences in the predicted K_{ow} values. The discrepancies are significant for less hydrophobic ILs to have the data predicted by vcclab.org more hydrophobic as compared to those calculated by means of molinspiration.com, and almost consistent for the long chain length derivatives. The experimentally determined empirical correlation reported recently by Montalbán et al. (2016) depicts the tendency similarly to the vcclab.org in the range of log K_{ow} < 0 (except the pyrrolidinium salts and octyl imidazolium ILs with variable counter ions) but results in the remarkable positive deviation in the extrapolation onto more hydrophobic ILs with of log K_{ow} > 0. Studying ILs with medium (C_6–C_8) to longer ($\geq C_{10}$) side chain length reported in this work elucidates applicability of two online tools predicting physical properties and provide the toxicity data complementary to the less hydrophobic ILs reported previously by Montalbán et al. (2016, 2018a). This study allows us to provide a broader vision on the effect of lipophilicity on toxicity towards *V. fischeri*. The online tools' are shown to have limitation in the predictability and should be used judiciously. However, when supported by sufficient experimental data, they can be considered as a suitable supplementary means for preliminary modelling empirical correlation.

The design of green ILs depends on many factors which includes desirable low toxicity and favourable biodegradation properties. While

progress towards mineralisable ILs has been demonstrated in recent years (Jordan et al., 2016; Haiß et al., 2016), the challenge of developing low microbial toxicity surface active ILs remains significant. The results of the present study shed light on the new approaches for designing environmentally benign ILs and functional surfactants. As the hydrophobicity of the ILs is significantly correlated with the toxicity, the *Vibrio fischeri* assay could be considered a powerful tool providing toxicity data for building and evaluating the QSAR toxicity models for ILs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2018.12.076.

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Publication II

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ANTIBACTERIAL ACTIVITY OF 24 L-PHENYLALANINE DERIVED SURFACE-ACTIVE IONIC LIQUIDS (SAILS) TOWARDS TWO CLINICALLY RELEVANT PATHOGENS

Dewi K. A. Kusumahastuti[1,2], Mariliis Sihtmäe[2]*, Nicholas Gathergood[1], Anne Kahru[2,3]

[1]Tallinn University of Technology, Akadeemia tee 15, Tallinn 12618, Estonia

[2]National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn 12618, Estonia

[3]Estonian Academy of Sciences, Kohtu 6, Tallinn 10130, Estonia

Abstract

Ionic liquids - low melting point salts - are attractive for a wide range of applications, from material science to medicinal chemistry. The vast number of possible combinations of different cations and anions enables researchers to fine-tune the physico-chemical and/or biological properties (e.g., hydrophobicity or toxicity). This paper systematically analyses the antimicrobial potency of a library of 24 L-phenylalanine derived surface-active ionic liquids (SAILs; C_2-C_{16}) and provides EC_{50}, MIC and MBC values for these compounds towards two clinically relevant pathogenic bacterial models – Escherichia coli and Staphylococcus aureus using standard broth microdilution method ISO 20776-1:2006. We demonstrated that the antimicrobial potency of SAILs containing different cationic headgroups (pyridinium, imidazolium, and cholinium) increased with the length of the alkyl ester chain from C_2 to C_{12} and then similar values obtained for C_{14} followed by a decreased toxicity for C_{16}. This trend was not dependent on the type of headgroup. The minimum inhibitory concentration (MIC) ranged from 8000 mg/L (C_2 SAILs) down to 4 mg/L (C_{12} SAILs). Most potent were C_{12} SAILs that inhibited the growth of S. aureus and E. coli at concentration of few milligrams per liter: EC_{50} values ~2 and ~15 mg/L (or ~4 and ~27 μM), respectively. These data are comparable to the antimicrobial efficiency of benzalkonium chloride, which is widely used antimicrobial compound. As a rule, gram-positive S. aureus was 7-fold more susceptible to the SAILs than gram-negative E. coli. In addition, the results obtained in this study on medically relevant bacteria were in agreement with our previous data on Vibrio fischeri – a naturally luminescent marine bacterium. We hypothesize that the toxic effects of studied SAILs was manifested via disturbing the bacterial membranes. To summarise, the SAILs are promising antimicrobials which toxicity towards bacteria can be tuned by modifying the alkyl ester chain properties.

Keywords: *Surface active ionic liquids, alkyl ester chain length, Escherichia coli, Staphylococcus aureus,* ISO 20776-1:2006

1. INTRODUCTION

Due to the continuously increasing occurrences of antibiotic resistant bacteria and hospital-acquired infections [1], scientists have examined alternative approaches to solve this problem, e.g. developing new antimicrobial chemicals. Within the vast library of chemical compounds that can be envisaged, numerous ionic liquids (ILs), including surface active ionic liquids (SAILs), with intrinsic antimicrobial properties and highly tuneable nature, have been developed to meet this need [2, 3].

SAILs can be considered analogues of native lipopeptides as they are cationic amphiphiles consisting of one or two amino acids connected to a hydrophobic part. In consequence, they show the toxic properties towards microorganisms and are of low potency to induce resistance [4]. At least two functional groups, carboxylic groups, and amino groups are inherently present in amino acids. These substances can be transformed into single chain surfactants with reactive sites in their hydrophobic chain, for example, fatty acids, fatty esters, fatty amines, and fatty alcohols. The hydrophobic chain can be linked to the structure of amino acid through alkyl ester or amide linkages [5].

Various studies on the biological activity of ILs on bacteria have been carried out, including gram-negative and gram-positive bacteria [6-10]. In addition, several test methods for measuring toxicity

towards bacteria have been introduced. One of the most recognized antibacterial activity tests is a standard broth microdilution method described in ISO 20776-1:2006 "Reference method for testing the *in vitro* activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases" [11]. The method is based on the serial dilution of tested chemical and incubating the dilutions with test bacteria for determination of minimum inhibitory concentration (MIC). In addition, half-effective concentration (EC_{50}) values of test chemicals can be calculated from the test data. Moreover, as a prolongation of the test, the incubation mixtures can be plated out on agarized nutrient medium to determine minimal bactericidal concentrations of the tested chemicals (MBC).

Escherichia coli (gram-negative bacterium) and *Staphylococcus aureus* (gram-positive bacterium) were used in this study as model organisms that may cause skin and soft tissue infections (SSTIs) [12]. Moreover, within these bacteria there are strains that have developed resistance towards common antibiotics. The latter motivates the studies for discovery of novel compounds (including ionic liquids) with antimicrobial properties [13].

In general, research on the antibacterial activity of ILs has been limited to determination of MIC [14] and comprehensive data on bacterial growth inhibition, MIC and MBC, are still rare. The current study aimed to explore the antibacterial activity of 24 L-phenylalanine derived SAILs, including cyclic cation (pyridinium and imidazolium) and acyclic cation (cholinium), all as bromide salts, containing the same linker (L-phenylalanine), and different alkyl ester chain lengths (C_2 to C_{16}) (see Fig. 1) against *E. coli* and *S. aureus*.

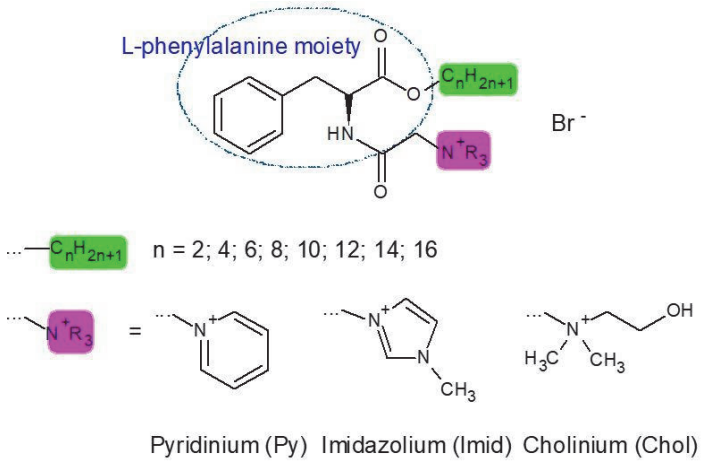

Fig. 1. A library of 24 L-phenylalanine derived SAILs with three different cations (pyridinium, imidazolium and pyridinium), all paired with bromide–anion and varying alkyl chain (C_2 to C_{16}) studied in this work: PyC_nPhe, $ImidC_nPhe$ and $CholC_nPhe$. Figure modified from Kusumahastuti et al. [15].

2. MATERIALS AND METHODS

2.1. Materials

A series of 24 L-phenylalanine derived SAILs was synthesised in the Green chemistry laboratory of Tallinn University of Technology, Estonia. The description of synthesis and characterization data is described in Kapitanov et al. [3]. The stock solutions of SAILs (1 to 8000 mg/L; soluble concentrations) were prepared in sterile MilliQ water, stored at ambient temperature in the dark and tested for toxicity in nominal concentrations within 1-2 weeks.

2.2. Methods

2.2.1. Antimicrobial activity evaluation of SAILs

Antibacterial activity evaluation was adapted from the standard broth microdilution method ISO 20776-1:2006 for antimicrobial susceptibility testing. Altogether 24 L-phenylalanine derived SAILs were evaluated using 2 bacterial strains: *Staphylococcus aureus* 6538 and *Escherichia coli* MG1655. Prior to testing, bacteria were first cultivated overnight in 3 ml of Mueller-Hinton Broth (MHB, Oxoid CM 405, Oxoid Microbiology Products; containing per 1 litre: dehydrated infusion from beef 300 g, casein hydrolysate 17.5 g, starch 1.5 g, Ca^{2+} 4.19 mg and Mg^{2+} 5.6 mg) at 30 °C with shaking at 200 rpm, and then diluted with fresh medium to a bacterial density of ~$5x10^5$ CFU/mL in the test (verified by viable plate counts on agarized medium). For toxicity testing, bacteria were incubated with different concentrations of SAILs by combining the bacterial suspension (50 µL) and test compound (SAILs) solution (50 µL) in 96 well transparent microtiter plates (BD Falcon) at 37 °C for 20 hours in the dark in aerobic conditions with minor shaking every 15 min. Wells containing 50 µL of control broth and 50 µL of bacterial culture in MHB were included for each tested strain and served as non-treated controls. The growth of bacteria was evaluated by the increase of the absorbance at 600 nm in 15 min intervals using microplate Spectramax Paradigm spectrophotometer (Molecular Devices, USA). Based on the bacterial growth data two parameters were determined: 1) minimum inhibitory concentration (MIC) as the lowest tested concentration of SAILs where no visible growth of bacteria was observed and 2) half effective concentration (EC_{50}) of the SAILs that reduced bacterial growth by 50%, calculated from the dose-effect curves of bacterial log-phase growth (between 3 h and 10 h of incubation, depending on the bacterial strain, i.e. inhibition of the growth rates of chemical exposed bacteria versus bacterial growth in the control broth) using MS Excel macro Regtox [16]. In addition, minimum bactericidal concentration (MBC) was determined in the end of the experiment (20 hours). For that 3 µL of each sample was removed from the microplate and pipetted as a spot onto Muller Hinton Agar (MHA) growth medium. The inoculated agar plates were incubated at 30 °C in the dark for 24 hours and MBC was designated as the lowest tested concentration of SAILs which totally inhibited the ability of the test bacteria to yield visible colonies on toxicant-free MHB plates.

2.2.2. Statistical analysis

All tests were performed in at least three independent experiments and duplicates in every single experiment. The data are expressed as mean ± standard deviation. To define statistically significant differences between samples, the data were analyzed by SPSS 21.0 Edition. Data were analyzed by one-way analysis of variance and Tukey's test was used to compare differences among all samples. A p value < 0.05 was considered statistically significant difference.

3. RESULTS

In this study the toxicity of 24 SAILs with cationic headgroups (pyridinium, imidazolium, and cholinium) and varying alkyl ester chain (C_2-C_{16}) described in Kapitanov et al. (2019) were analysed against 2 different bacteria *S. aureus* and *E. coli* that are models for clinical pathogens. The heat-map of colour-coded toxicity data (mg/L) is presented in Table 1: the SAILs that according to their EC_{50}, MIC or MBC values are presumably non-hazardous (> 100 mg/L; green); harmful (>10-100 mg/L; yellow), toxic (>1-10 mg/L; pink) or very toxic (≤1 mg/L; red). This simplified hazard ranking system has been initially used to heat-map the ecotoxicological results by Aruoja et al. [17]. The same toxicity ranking criteria were recently used by us for the same library of SAILs towards naturally luminescent marine bacterium *Vibrio fischeri* (also known under the name of *Photobacterium phosphoreum* NRRL-B-11177 and *Aliivibrio fischeri*) which is a well-known model organism for ecotoxicity studies [15]. For the comparison *V. fischeri* data are also presented in Table 1.

Table 1. Toxicity data (EC_{50}, MIC, and MBC) of studied 24 SAILs towards *E. coli* and *S. aureus*. Benzalkonium chloride (BAC) was used as a positive control. For the comparison, 30-min EC_{50} values for the same SAILs towards bacteria *Vibrio fischeri* [15] are presented. All toxicity values are expressed in both, mg/L and μM (in brackets).

Compound	***E. coli*** (20 h of incubation at 37°C in MHB)			***S. aureus*** (20 h of incubation at 37°C in MHB)			***V. fischeri*** (30 min of incubation at ~20°C in 2%NaCl)
	EC_{50} mg/L (μM)	**MIC** mg/L (μM)	**MBC** mg/L (μM)	**EC_{50}** mg/L (μM)	**MIC** mg/L (μM)	**MBC** mg/L (μM)	**EC_{50}** mg/L (μM)
PyC_2Phe	>3933 (>10000)	>3933 (>10000)	>3933 (>10000)	1426±21.6 (3625±54.8	7866 (20000)	7866 (20000)	866±110 (2203±281)
$ImidC_2Phe$	>3969 (>10000)	>3969 (>10000)	>3969 (>10000)	2474±8.67 (6234±21.9)	7926 (20000)	7926 (20000)	1227±112 (3096±282)
$CholC_2Phe$	>4033 (>10000)	>4033 (>10000)	>4033 (>10000)	1580±10.7 (3917±10.7)	8066 (20000)	8066 (20000)	1153±109 (2858±271)
PyC_4Phe	418±1.15 (993±2.73)	843 (2000)	843 (2000)	418±19.6 (991±43.7)	527 (2000)	527 (2000)	81.0±13.4 (192±31.8)
$ImidC_4Phe$	176±44.2 (415±95.1)	849 (2000)	849 (2000)	187±49.7 (442±117)	424 (1000)	424 (1000)	91.7±10.7 (216±25.2)
$CholC_4Phe$	265±17.8 (613±41.2)	863 (2000)	863 (2000)	201±54.2 (466±126)	431 (1000)	431 (1000)	90.1±14.8 (209±34.2)
PyC_6Phe	233±14.6 (519±32.6)	449 (1000)	899 (2000)	64.8±2.98 (144±6.63)	112 (250)	112 (250)	12.2±0.73 (27.3±1.62)
$ImidC_6Phe$	285±27.6 (629±61.0)	905 (2000)	905 (2000)	73.0±14.7 (161±32.4)	113 (250)	113 (250)	11.0±1.58 (24.3±3.50)
$CholC_6Phe$	456±35.1 (996±76.5)	916 (2000)	916 (2000)	113±5.03 (246±11.0)	229 (500)	229 (250)	14.8±2.02 (32.3±4.40)
PyC_8Phe	34.8±2.93 (72.9±6.14)	59.7 (125)	59.7 (125)	8.64±0.48 (18.1±1.02)	14.9 (31.3)	14.9 (31.3)	9.32±1.21 (19.5±2.53)
$ImidC_8Phe$	56.1±0.08 (117±0.16)	60.1 (125)	60.1 (125)	8.72±0.38 (18.2±0.79)	15.0 (31.3)	15.0 (31.3)	9.30±1.90 (19.4±3.95)
$CholC_8Phe$	30.9±1.48 (63.4±3.03)	60.9 (125)	60.9 (125)	12.2±1.15 (25.1±2.35)	15.2 (31.3)	15.2 (31.3)	20.5±1.23 (41.9±2.51)
$PyC_{10}Phe$	24.2±6.48 (47.9±12.8)	63.2 (125)	63.2 (125)	4.74±0.24 (9.37±0.47)	7.89 (15.6)	7.89 (15.6)	4.40±0.62 (8.71±1.23)

$ImidC_{10}Phe$	31.7±1.50 (62.3±2.9)	63.6 (125)	63.6 (125)	4.72±0.10 (9.28±0.21)	7.93 (15.6)	7.93 (15.6)	4.52±0.42 (8.89±0.83)
$CholC_{10}Phe$	32.2±3.25 (62.5±6.31)	64.4 (125)	32.2 (62.5)	5.00±0.08 (9.69±0.15)	8.04 (15.6)	8.04 (15.6)	5.72±0.77 (11.1±1.49)
$PyC_{12}Phe$	15.4±2.14 (28.8±4.00)	33.3 (62.5)	33.4 (62.5)	1.39±0.20 (2.61±0.37)	4.16 (7.80)	4.16 (7.80)	4.17±0.44 (7.82±0.82)
$ImidC_{12}Phe$	13.1±2.42 (24.4±4.50)	33.5 (62.5)	33.5 (62.5)	2.41±0.09 (4.49±0.17)	4.19 (7.80)	4.19 (7.80)	4.48±0.33 (8.36±0.62)
$CholC_{12}Phe$	15.1±1.20 (27.8±2.22)	34.0 (62.5)	34.0 (62.5)	2.87±0.58 (5.28±1.07)	4.24 (7.80)	4.24 (7.80)	4.29±0.44 (7.90±0.81)
$PyC_{14}Phe$	27.2±1.29 (48.4±2.29)	>70.2 (>125)	>70.2 (>125)	2.25±0.49 (4.01±0.87)	4.38 (7.80)	4.38 (7.80)	5.14±1.22 (9.16±2.17)
$ImidC_{14}Phe$	21.1±6.28 (37.3±11.12)	>70.6 (>125)	>70.6 (>125)	2.28±0.31 (4.05±0.55)	4.40 (7.80)	4.40 (7.80)	4.64±0.47 (8.22±0.83)
$CholC_{14}Phe$	>71.4 (>125)	>71.4 (>125)	>71.4 (>125)	2.62±0.08 (4.58±0.14)	4.46 (7.80)	4.46 (7.80)	4.59±0.41 (8.03±0.73)
$PyC_{16}Phe$	>73.7 (>125)	>73.7 (>125)	>73.7 (>125)	11.1±1.45 (18.8±2.46)	18.4 (31.3)	18.4 (31.3)	>36.9 (>62.5)
$ImidC_{16}Phe$	>74.1 (>125)	>74.1 (>125)	>74.1 (>125)	12.1±1.59 (20.4±2.69)	18.5 (31.3)	18.5 (31.3)	>37.05 (>62.5)
$CholC_{16}Phe$	>74.9 (>125)	>74.9 (>125)	>74.9 (>125)	12.1±2.99 (20.3±4.98)	18.7 (31.3)	18.7 (31.3)	>37.47 (>62.5)
BAC	13.9±1.36 (49.03±4.80)	25.0 (88)	25.0 (88)	1.92±0.05 (6.76±1.86)	3.12 (11)	3.12 (11)	0.94±0.18 (3.31±0.62)

Colour code: ≤ 1 mg/L (■) = very toxic; 1 mg/L < toxic ≤ 10 mg/L (■); 10 mg/L < harmful ≤100 mg/L (■); > 100 mg/L (■) = not classified/not harmful.

4. DISCUSSION

4.1. Antimicrobial potency of 24 SAILs to E. coli and S. aureus.

Table 1 shows that, as a rule, the toxicity of studied SAILs was not depending on the cationic headgroup but on the length of the alkyl ester chain: the longer the chain the more toxic the compound. This tendency was observed also in our previous study concerning toxic effects of SAILs to *V. fischeri* [15]. As expected, all the C_2 SAILs, independent of the headgroup (pyridinum, imidazolium or cholinium), did not inhibit the bacterial growth (*E. coli* or *S. aureus*) even at very high concentrations (exceeding 1000 mg/L) (Table 1), i.e. these SAILs are not good candidates for antimicrobial use. C_4 and C_6 SAILs were of low to intermediate antimicrobial potency, i.e. inhibited bacterial growth from 65 to 916 mg/L concentrations, depending on toxicity endpoint and bacterium. C_8 and especially C_{10}-C_{14} SAILs proved efficient inhibiting the growth of bacteria at relatively low concentrations, from 1.39 to 64.4 mg/L, depending on toxicity endpoint and bacterial strain. These inhibitory values were already close to antimicrobial efficiency of benzalkonium chloride (BAC) - a well-known and widely used antimicrobial compound: EC_{50} values of BAC ranged from 1.9-13.9 mg/L and MIC and/or MBC from 3-25 mg/L (Table 1; Fig. 2). As a rule, the MIC and/or MBC values of studied SAILs were between 4 mg/L to > 8000 mg/L, up to 5-fold higher compared to respective EC_{50} values and dependent on the length of the alkyl ester chain (Table 1). Analogous results have been obtained with polyoxometalate ionic liquids (POM-ILs) by Kubo et al. [18].

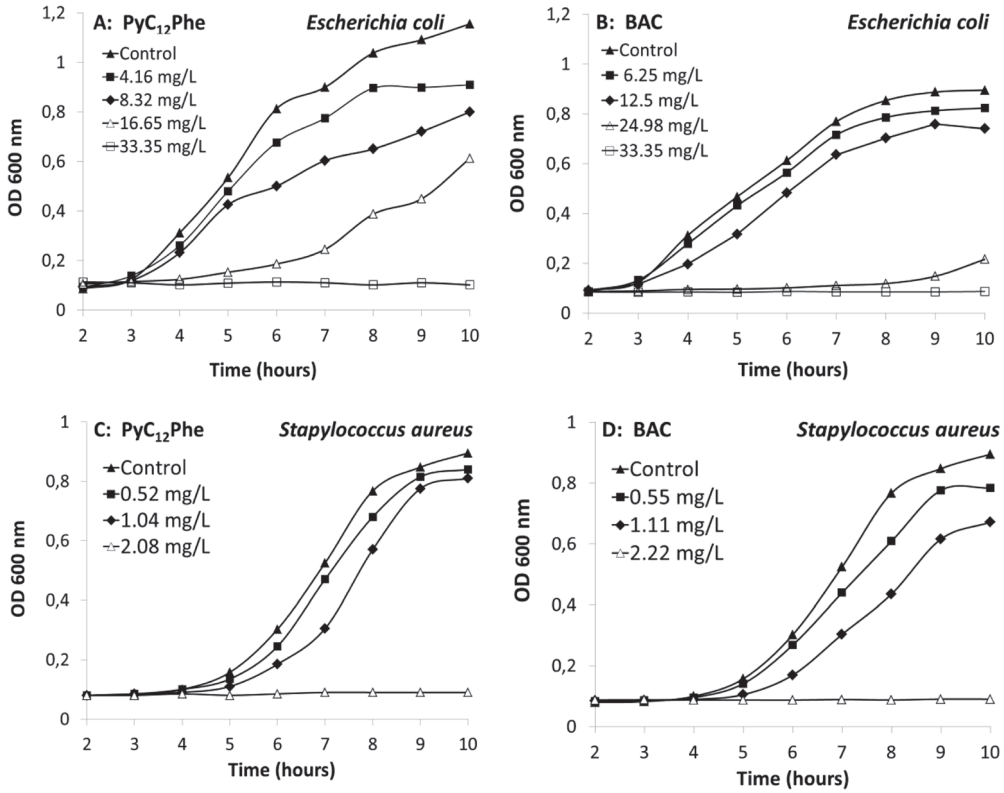

Fig. 2. The representative dose-response growth curves of *S. aureus* and *E. coli* for pyridinium C_{12} SAILs (A, C) and positive control benzalkonium chloride (BAC) (B, D).

The EC_{50} values for 24 SAILs tested on *E. coli* and *S. aureus* were in good correlation (log-log R^2=0.93) (Fig. 3A) although gram-positive *S. aureus* analysed in the growth inhibition assay in the same conditions and using the same toxicity endpoint was as average 7-fold more sensitive towards SAILs than gram-negative *E. coli*. Higher sensitivity of *S. aureu*s compared to *E. coli* towards different type of ILs - polyoxometalate (POM) ionic liquids - was also observed by Kubo et al. [18]. The gram-positive bacterium *S. aureus* is more susceptible to studied SAILs than *E. coli* most probably due to different structure of cell envelope of these bacteria (see 4.2). Interestingly, in the recent work of Kubo et al. [19] where we compared the sensitivity of *E. coli* and *S. aureus* towards nanosilver, the results were opposite, i.e. *E. coli* was more susceptible than *S. aureus*. Thus, the susceptibility of bacteria to different types of antimicrobials depends on type of the chemical and it is highly recommended that the testing for antimicrobials must be conducted with both, gram-positive and gram-negative bacteria.

As it was shown in the work of Kubo et al. [18], the antimicrobial potency of studied POM-ILs was dependent on the alkyl chain length of the cation, i.e. the longer the carbon chain the higher the antimicrobial potency. The same pattern was observed in case of library of SAILs analysed in the current work (Table 1; Fig. 3). Concerning the antimicrobial potency of C_{14} and C_{16} SAILs (Table 1) it could be noted that in the toxicity tests made in the current study they are less toxic than C_{12}, i.e. the elongation of the carbon chain was not accompanied by the increased toxicity. That is probably due the limited solubility of this compound and lower bioavailable fraction. Indeed, we observed that C_{14} and C_{16} were less soluble in the water than C_{12}. Therefore, the C_{12} SAILs are probably most suitable candidates for antimicrobial ILs with relatively high antimicrobial potency (Fig. 2) and reasonable solubility in water.

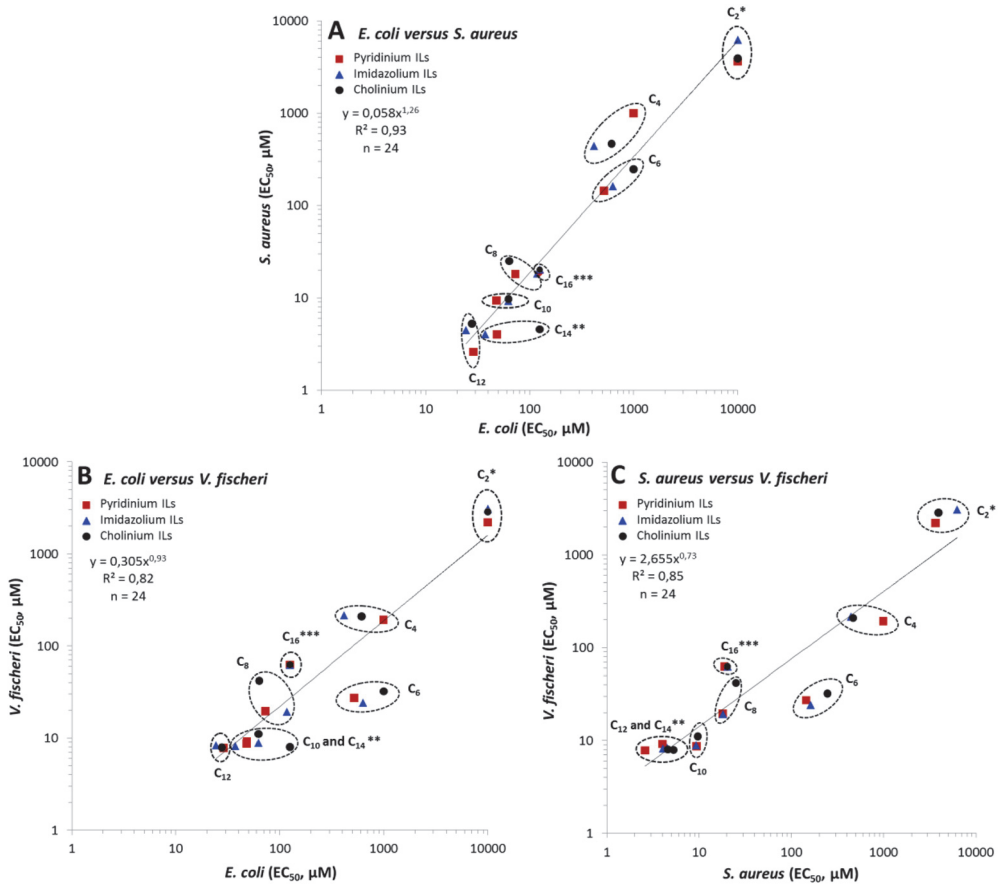

Fig. 3. The comparison of the toxicity of 24 SAILs towards different bacteria. A: *E. coli versus S. aureus*. Log-log R^2=0.93; B: *E. coli versus V. fischeri*. Log-log R^2=0.82; C: *S. aureus versus V. fischeri*. Log-log R^2=0.85. Data are plotted from Table 1. * EC_{50} of studied C_2 SAILs to *E. coli* > 10 000 µM; ** EC_{50} of $CholC_{14}Phe$ to *E. coli* > 125 µM; *** EC_{50} of studied C_{16} SAILs to *E. coli* > 125 µM and *V. fischeri* > 62 µM.

4.2. Mechanism of antibacterial action: effect on cellular membranes

Bacteria are unicellular organisms that are separated from the surrounding environment by cell envelope consisting of various layers (cell wall, membrane) depending on the bacterial type (gram-negative *versus* gram-positive bacteria). However, the central barrier between the living and non-living is a biological membrane which basic structural unit is a lipid bilayer. As mentioned above, the type of the headgroup of studied 24 SAILs had a negligible effect on their antibacterial efficiency. However, the toxic effect was depending on alkyl ester chain length (Table 1). This is known as side chain effect [20] and the toxicity is assumed to be a manifestation of the interaction of SAILs with cell membranes. SAILs with longer alkyl ester chains probably more efficiently penetrate the cellular membrane's lipid bilayer, and the cell structure of the microorganism can be disrupted [21]. According to Yoo et al. [22], when the ILs cationic part begins to penetrate the cell membrane, the bending of the membrane starts yielding cell damage.

Based on the report of Ibsen et al. [2] there are two main stages for interaction of cationic amphiphiles with microorganisms: (i) first, the attachment of amphiphile to the target membrane - a process

governed by electrostatic interactions between the positively charged polar head of the surfactants and the negatively charged molecules of the bacterial membranes (lipopolysaccharides in gram-negative and lipoteichoic acid in gram-positive bacteria) [23]; (ii) following that, the hydrophobic alkyl chain of cationic amphiphiles interacts with the lipid bilayers of membranes. For that stage there is an optimum relationship between the hydrophobicity and polarity of the surfactant to facilitate the diffusion of the surfactant in the non-polar environment of lipid bilayer. Hence, cationic surfactants are generally more potent against gram-positive bacteria, which contain high amounts of negatively charged lipids [24]. Furthermore, it has been reported that there is an interaction between imidazolium heterocycle and the lipid head group in the cell membrane, while the side chain will interact with the lipid tail. This interaction can lead longer alkyl ester side chains to penetrate deeper into lipid bilayer [25]. Research by Cornmell et al. [26] has shown that the accumulation of ILs $[P_{6,6,6,14}]$ $[NTf_2]$ was found in the membrane of *E. coli* and not in the cytoplasm of bacteria. Therefore, cell membrane damage is assumed to be the main reason for SAILs antimicrobial action.

The effect of SAILs on biological membranes as a toxicity mechanism is supported also by the fact that growth inhibition data (EC_{50}; 20 h of incubation) for *E. coli* and *S. aureus* correlated well with 30-min bioluminescence inhibition data of *Vibrio fischeri* (Fig. 3B and 3C). The bioluminescence of *V. fischeri* is tightly connected to the vitality of its cellular membrane and the membrane-related processes such as energy production [27] and the decrease of the luminescence is proportional to the toxicity of chemical compound. Therefore, these data indicate that if toxic, SAILs mostly affect biological membranes and thus probably act universally on all types of microorganisms [28].

Research on this library of SAILs to refine design principles to enable the development of low microbial toxicity ILs has also been performed by Kapitanov et al. [3]. This included a preliminary toxicity evaluation where they used various microorganisms, including *E. coli* and *S. aureus*. In general, the trends observed were in agreement to our results reported herein: (i) the toxicity increased with the increase of the alkyl ester chain and was not dependent on the headgroup; (ii) the more efficient in inhibiting bacterial growth were C_{10}-C_{14} SAILs; (iii) *S. aureus* was more susceptible than *E. coli* to SAILs tested whereas the test format and test strains were different of these used in the current study.

Due to the importance of the membranes in ”guarding” the entrance of toxicants into the living cell, the octanol/water partition coefficient (K_{ow}) of studied 24 SAILs was compared to their antimicrobial potency. Indeed, the ability of a chemical to dissolve in lipids (a proxy for ability to cross the biological membrane’s lipid bilayer and enter the cell) is in correlation with its toxicity. According to Bradbury and Lipnick [29] about 70% of industrial organic chemicals are estimated to act *via* the narcosis mechanism the toxicity. Narcotic effects are estimated by the ability of a compound to interact with cellular membranes. Narcosis is ”baseline” toxicity of chemicals i.e. the toxicity which correlates with their hydrophobicity (for example, octanol/water partition coefficient, K_{ow}) and this parameter is widely used as a descriptor for QSARs [30, 31]. Therefore, consistent experimental toxicity data for different organisms are crucial in order to develop respective QSARs. As the research conducted by Stepnowski and Storoniak [32] has shown the relationship between the lipophilic properties of ionic liquids and their ability to penetrate cells, we compared the log K_{ow} values (taken from Kusumahastuti et al. [15]) with toxicity data (EC_{50} values; Table 1) of 24 SAILs to *E. coli* and *S. aureus* (Fig. 4). All log K_{ow} values of the studied ILs were estimated to be <4 and there was a positive correlation between the toxicity and log K_{ow} up to C_{12} (Fig. 4) but not further (for C_{14} and C_{16}). Also, the toxicity pattern of studied SAILs (Table 1; Fig. 5) indicates a hysteresis: toxicity of C_{14} and C_{16} was lower than that of C_{12} SAILs. Analogous pattern was observed in our previous study by Kapitanov et al. [3] and Kusumahastuti et al. [15] that all studied the toxicity of the same library of SAILs but with different bioassays. Also some previous studies [20, 33] have shown that the elongation of alkyl chain length up to specific lengths could decrease their toxicity; this phenomenon has been labelled as a “cut-off effect”.

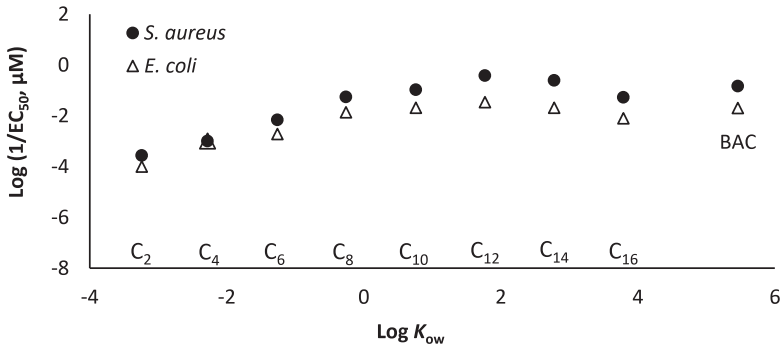

Fig. 4. Relation between Log K_{ow} (X-axis) and log (1/EC_{50}) (Y-axis) of 24 SAILs. Data of EC_{50} (μM) are taken from Table 1. The K_{ow} values were calculated using open-access online tool (https://www.molinspiration.com/services/logp.html) and taken from Kusumahastuti et al. [15].

To explain the latter irregularity, a contribution to the observed toxicity due to the critical micelle concentration of SAILs may be operational. Specifically, Kapitanov et al. [3] state that the effectiveness of surfactants as antimicrobial agents can be linked to critical micelle concentration (CMC). They found that the MIC_{95} values of C_{14} and C_{16} were above the CMC value. Therefore, their toxicity decreased. They proposed theories that the elongation of the side alkyl chain length affects the formation of micelles rather than their tendency to move to the surface of bacterial cells. This hypothesis was in agreement with Pernak et al. [34] who studied a series of alkoxymethylimidazolium ILs.

In summary, a systematic study of the antimicrobial potency of a library of 24 L-phenylalanine derived surface-active ionic liquids (SAILs; C_2-C_{16}) towards *E. coli* and *S. aureus* bacteria was performed using standard broth microdilution method ISO 20776-1:2006. The main results on the overall antimicrobial potency of studied SAILs to *E. coli* and *S. aureus* are depicted in Fig. 5: the short and medium chain length (C_2 to C_6) showed low toxicity but the SAILs with longer chain length (C_8 to C_{12}) exhibited antimicrobial activity to the bacteria screened, with the C_{12} SAILs being recommended as the most suitable candidates of our series for antimicrobial use.

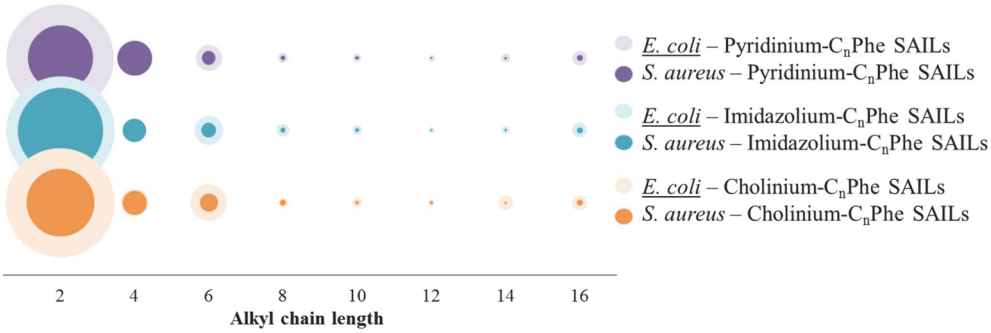

Fig. 5. Effect of the alkyl chain length (C_2-C_{16}) of studied SAILs (X-axis) on the overall antimicrobial potency (expressed as EC_{50} values, μM; Table 1) to *E. coli* and *S. aureus* bacteria (Y-axis). The smaller the bubble the more antimicrobial SAILs. Pyridinium, imidazolium and cholinium head groups are indicated with different colours.

5. CONCLUSIONS

In this paper the antimicrobial activity of 24 L-phenylalanine based SAILs against clinically important gram-positive (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) bacteria are reported. The following conclusions can be drawn from the present study:

1. The antimicrobial potency of SAILs containing different cationic headgroups (pyridinium, imidazolium, and cholinium) increased with the length of the alkyl ester chain (from C_2 to C_{12}) and then levelled up (C_{14}) or decreased (C_{16}) and was not dependent on the type of head group.
2. The minimal inhibitory concentration (MIC) range varied from up to 8000 mg/L (C_2 SAILs) down to ~4 mg/L (C_{12} SAILs). Most efficient were C_{12} SAILs that inhibited the growth of gram-positive bacterium *S. aureus* and gram-negative bacterium *E. coli* at concentration of few milligrams per liter that is comparable to the antimicrobial efficiency of benzalkonium chloride - a well-known and widely used antimicrobial compound.
3. As a rule, gram-positive *S. aureus* bacteria were more susceptible to the SAILs than gram-negative *E. coli*.
4. The growth inhibition data (EC_{50}) for *E. coli* and *S. aureus* correlated well. Also, the EC_{50} values for above mentioned bacteria correlated well with 30-min bioluminescence inhibition data of *Vibrio fischeri* showing that the toxicity of these SAILs mostly affects biological membranes and thus probably affects universally all types of microorganisms.

Overall, the SAILs are promising antimicrobials, which toxicity towards bacteria can be tuned by modifying the alkyl ester chain properties.

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Ecotoxicity profiling of a library of 24 L-phenylalanine derived surface-active ionic liquids (SAILs)

Dewi K.A. Kusumahastuti [a,b], Mariliis Sihtmäe [a], Villem Aruoja [a], Nicholas Gathergood [b,c,*], Anne Kahru [a,d,**]

[a] Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, Tallinn, 12618, Estonia
[b] Department of Chemistry and Biotechnology, ERA Chair of Green Chemistry, Tallinn University of Technology, Ehitajate tee 5, 19086 Tallinn, Estonia
[c] School of Chemistry, University of Lincoln, Lincoln, Lincolnshire, LN6 7DL, UK
[d] Estonian Academy of Sciences, Kohtu 6, 10130, Tallinn, Estonia

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ABSTRACT

We evaluated the ecotoxicity of a library of 24 L-phenylalanine derived surface-active ionic liquids (SAILs) with various cationic head groups (pyridinium, Py; imidazolium, Imid and cholinium, Chol) and alkyl ester chains from C_2 to C_{16}. For toxicity evaluation we used 72-h algal growth inhibition assay (OECD 201) with *Raphidocelis subcapitata* and 24-h mortality test with aquatic crustaceans *Thamnocephalus platyurus* (ISO 14380:2011). The OECD 201 assay was applied to all 24 SAILs while the ISO 14380:2011 test was applied to a subset, specifically all eight pyridinium SAILs and C_6 and C_8 examples of the imidazolium and cholinium SAILs (total 12 SAILs). For the comparison, 30-min EC_{50} data (based on inhibition of bioluminescence) previously reported by this group for the 24 SAILs for marine bacteria *Vibrio fischeri* (ISO 21338:2010) were included and correlated to the algae and aquatic crustaceans data. According to the results of the multritrophic test battery only two studied SAILs - PyC_2 and $CholC_2$ – could be considered 'low toxicity', (i.e. were ranked not harmful, $L(E)C_{50} > 100$ mg/L by the most sensitive test - algal growth inhibition assay). *T. platyurus* proved about 100-times more tolerant to studied SAILs than algae. An alternative classification scale dependent on the average MW of the compound dataset (based on molar concentrations and not concentrations based on mg/L) was suggested to rank the compounds. When compared to the classification scale independent of the MW of the compound, a more accurate appraisal was achieved for suggesting the greener alternatives for certain commercial SAILs/surfactants.

1. Introduction

In order to preserve and protect the environment, in 2015 members of the United Nations implemented '*The sustainable Development Goals' (SDGs)*; drawing attention on actions to cease pollution, protect aquatic and terrestrial ecosystems while maintaining sustainable industry and economic growth. The aim of this movement is to end poverty, conserve the planet and ensure all people enjoy peace and prosperity by 2030 (UN, 2016). As the societal progress is unattainable without chemicals (including potentially harmful ones), the Green Chemistry concept was launched during 1990s and the publications in this field are constantly growing. In the Clarivate Analytics Web of Science database, about 2000 papers annually are published on this topic (keyword 'green chemistry'). Preventing (chemical) waste, using safer solvents, and designing harmless and degradable chemicals are some of the 12 green chemistry principles (Anastas and Warner, 1998) that all contribute to SDGs.

There are remarkable developments in the field of Green Chemistry over the last thirty years. One class of 'green' chemical substances receiving extensive attention is ionic liquids (ILs). Several thousands of different ILs have been described in the scientific literature and by chemical companies by 2019 (Schubert, 2020) and the estimation of the market size for ILs by 2021 is forecasted to be 39.6 million USD. ILs are salts, predominately consisting of organic cations, with the widely acknowledged property of a melting point below 100 °C. This property that is very valuable for several industrial technologies, especially if liquid at room temperature. In addition, due to the low volatility of ILs

* Corresponding author. School of Chemistry, University of Lincoln, Lincoln, Lincolnshire, LN6 7DL, UK.
** Corresponding author. Laboratory of Environmental Toxicology, National Institute of Chemical Physics and Biophysics, Akadeemia tee 23, 12618 Tallinn, Estonia.
E-mail addresses: ngathergood@lincoln.ac.uk (N. Gathergood), anne.kahru@kbfi.ee (A. Kahru).

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(i.e., unlikely to cause air pollution) they are considered as potentially 'environmentally benign' or 'green' alternatives to volatile organic solvents (De Los Ríos et al., 2013). Moreover, most ILs have high thermal, chemical, and electrochemical stability as well as tunable polarity and hydrophobicity (Gardas and Coutinho, 2009). Due to these properties ILs have diverse applications in modern science, ranging from solvents in organic synthesis and catalysis (Welton, 1999), liquid/liquid extraction of heavy metal ions (Wei et al., 2003), electrolytes for batteries (Osada et al., 2016), and even in the pharmaceutical field (Marrucho et al., 2014).

According to Schubert (2020) at 2017 the annual global production volume of ILs was of several hundreds of tons whereas < 1000 tons for well-known ILs. For the comparison, the annual industrial-scale production of organic solvents has been estimated at about 20 million tons (Clark et al., 2015). When a chemical is produced and used on a large industrial scale there is always a higher risk that it will be released to the environment either during its production, transport, use or disposal. Due to their low volatility, ILs do not cause air pollution, which is a positive property from the environmental safety view point. However, due to high stability (e.g. chemical, biochemical, thermal and electrochemical) which is often intended by design, and solubility in water, ILs may pollute water sources in nature due to accidental spills or via poorly treated effluents (Bubalo et al., 2017; Egorova and Ananikov, 2014). These groups have highlighted that the aquatic toxicity of ILs is a key concern which requires further study. When answering the question, 'How green are ILs?', aquatic toxicity studies are a fundamental consideration.

ILs are grouped into three groups (generations) based on their chemical structure and properties. The 'first-generation ILs' are ILs in which the cationic and anionic part were chosen to obtain a product of specific physical properties (Pernak et al., 2016) and consisted mostly of dialkyl-imidazolium and alkyl-pyridinium cations and metal halide anions (Egorova et al., 2017). These ILs reacted strongly with water and the requirement of strict anhydrous conditions restricted their wide adoption. The 'second generation ILs' are ILs developed to be conveniently used due to high stability (chemical, thermal and electrochemical), large liquid range, and reduced reactivity with water. The prevailing cations in these ILs are alkyl-pyridinium, dialkyl-imidazolium, phosphonium and ammonium and mostly commonly used anions are halides, hexafluorophosphate (PF_6) and tetrafluoroborate (BF_4) (Egorova et al., 2017). However, many of these ILs are characterized by high toxicity and low biodegradability (Egorova et al., 2017). Also a major concern is the breakdown of BF_4 and PF_6 under aqueous conditions into the toxic chemicals: HF, POF_3, $PO_2F_2^-$ and PO_3F^{2-} (Swatloski et al., 2003; Terborg et al., 2012; Freire et al., 2010). The 'third generation ILs' are referred to as task specific ILs where the chemical design is specifically directed towards a particular role or application. By judicious selection of functional groups to incorporate into the ILs' structure, the properties can be tailored to promote the desired outcome. This includes tuning the physical and chemical properties as well as the environmental features, i.e. low toxicity and biodegradability (Hough et al., 2007; Vieira et al., 2018). One green approach is to synthesize these ILs from natural and biodegradable components such as amino acids, choline or ions which biological activities are known (Egorova et al., 2017). Therefore, the ILs studied in the current work could be considered 'third generation ILs'. In addition, when considering anions for low toxicity third generation ILs, perfluorinated examples we have previously recommended these should be avoided (Gore et al., 2013; Myles et al., 2013).

Moreover, a particular class of ILs is surface-active ionic liquids (SAILs). SAILs are ILs that also have an activity of a surfactant as they contain large hydrophobic carbon chains either in cation part, anion part or both. Most often used cations in SAILs are imidazolium, pyrrolidinium, pyridinium, and quaternary ammonium. The long carbon chain of SAILs provides self-organizing properties for these compounds in aqueous solution which is analogous to conventional surfactants. To ensure the surface-active properties to the ILs the carbon side chains of the cations must be C_6–C_{17} and that of the anions C_{20} (that usually are derivatives of anionic surfactants) (for the references, see Vieira et al., 2018). Due to these interesting properties SAILs have been used in a wide range of products and applications, such as cosmetics, catalysis (Vieira et al., 2018; Cognigni et al., 2016), and oil recovery (Nandwani et al., 2019).

Kapitanov et al. (2019) have recently synthesized 24 SAILs derived from L-phenylalanine, with various cationic head groups (pyridinium, imidazolium, and cholinium) and the alkyl ester chains from C_2 to C_{16}. Biodegradation studies of these SAILs and phenylalanine derived ILs have been published by Kümmerer's group (Suk et al., 2020; Haiβ et al., 2016). Authors of the current paper have recently published data on toxicological properties of the above described library of SAILs using *Vibrio fischeri* bioassay (Kusumahastuti et al., 2019a) and two medically relevant bacteria, *Escherichia coli* and *Staphylococcus aureus* (Kusumahastuti et al., 2019b). The results of both our studies were in agreement that the toxicity of these SAILs was not dependent on the type of the cationic head group but on the side chain (the toxicity of the SAILs increased up to C_8–C_{10} and then plateaued). The toxicity of these SAILs (30-min EC_{50}) to *Vibrio fischeri* ranged from 4.2 mg/L, i.e. 7.8 μM (PyC_{12}Phe) to 1227 mg/L, i.e. 3096 μM) ($ImidC_2$Phe). The minimum inhibitory concentration (MIC) of these SAILs to *E. coli* and *S. aureus* ranged from ~1 mg/L (C_{12} SAILs) up to 8000 mg/L (C_2 SAILs). Interestingly, to *S. aureus* (a gram-positive bacterium) SAILs were about 7-fold more toxic/antibacterial than to gram-negative bacterium *E. coli*.

As in the previous study the test organisms were bacteria that are unicellular organisms with relatively simple cell organization, in the current study we continued to evaluate the toxic effects of these SAILs on environmentally relevant test organisms: algae and crustaceans, to evaluate how 'green' are these SAILs. Moreover, tests with algae (usually *Rapidocelis subcapitata* previous names also *Selenatrum capricornutum* and *Pseudokirchneriella subcapitata*) and aquatic crustaceans (usually *Daphnia magna*) are very important as the ecotoxicity data obtained with these tests are required in REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals; EC, 2006) for environmental hazard evaluation of chemicals produced from 1 to 10 tons annually. In this context algae are used as a proxy for primary producers and aquatic crustaceans/daphnids as a proxy for primary consumers. Here it is important to stress that *Vibrio fischeri* is also an environmentally relevant test organism as it is a naturally luminescent marine bacterium. Moreover, bacteria are important link in the ecosystems (degraders). In case of ILs bacteria are especially relevant test organism as ILs tend to resist to biodegradation – a process conducted largely by bacteria (Coleman and Gathergood, 2010). Moreover, the use of the test battery consisting of organisms from different biological complexity adds information on toxicological properties of chemicals and may shed light on toxicity mechanisms. It is important to note that although there is already remarkable amount of information on toxicity of ILs towards various organisms, including environmentally relevant ones, the data for algae and crustaceans are relatively scarce. In April 7, 2020 we performed the search in the WoS database for TOPIC: (ionic liquids AND toxic*) and retrieved 3376 papers. Within these papers 15.1% concerned *Vibrio fischeri* and *Aliivibrio fischeri* (the current taxonomical name for *V. fischeri*) (223 papers), 2.9% algae (101 papers), 2.6% *Daphnia* (88 papers) and 2.2% fish (76 papers). Reviews have been recently published in these areas (Kumari et al., 2020).

In our current study that is the follow-up of our previous two studies (Kusumahastuti et al., 2019a, 2019b) we aimed to analyse the ecotoxicity of the same library of 24 SAILs using freshwater algae *Raphidocelis subcapitata* and freshwater crustaceans *Thamnocephalus platyurus* to (i) obtain a homogenous toxicity data set for two environmentally relevant test species of different trophic level and biological complexity: algae and crustaceans. Together with the *Vibrio fischeri* EC_{50} values from our previous study (Kusumahastuti et al., 2019a) these data can be used (i) for toxicity ranking of these SAILs based on EC_{50} values obtained on

simplified food chain model organisms (primary producers, consumers, degraders) but also (ii) for QSAR studies to find out environmentally benign IL's chemical moieties; (iii) to compare the toxicity of these SAILs to unicellular (bacteria and algae) and multicellular organisms (crustaceans); (iv) to suggest the early warning (most sensitive) ecotoxicity test for the screening of the environmental hazard of novel ILs; (v) to suggest a screening test for initial screening of SAILs.

2. Materials and methods

2.1. SAILs

Twenty four L-phenylalanine SAILs were tested: PyC_2Phe, PyC_4Phe, PyC_6Phe, PyC_8Phe, PyC_{10}Phe, PyC_{12}Phe, PyC_{14}Phe, PyC_{16}Phe, $ImidC_2$Phe, $ImidC_4$Phe, $ImidC_6$Phe, $ImidC_8$Phe, $ImidC_{10}$Phe, $ImidC_{12}$Phe, $ImidC_{14}$Phe, $ImidC_{16}$Phe, $CholC_2$Phe, $CholC_4$Phe, $CholC_6$Phe, $CholC_8$Phe, $CholC_{10}$Phe, $CholC_{12}$Phe, $CholC_{14}$Phe, $CholC_{16}$Phe. Benzalkonium chloride (BAC) was used as a chemical reference (a positive control). A series of L-phenylalanine SAILs was synthesized in the Green Chemistry laboratories of Tallinn University of Technology. Full description of synthesis and characterization data (including physicochemical properties i.e. surface activity) is described elsewhere (Kapitanov et al., 2019; Jordan et al., 2016).

For the experiments, stock solutions of SAILs were prepared in algae medium and crustacean medium, respectively. All stock solutions of SAILs were freshly prepared and used on the day of toxicity test. All studied SAILs were soluble at the concentrations applied for testing.

2.2. Toxicity tests

Two toxicity tests were performed: the 24-h acute mortality test with *Thamnocephalus platyurus* (Thamnotoxkit F™) and the 72-h algal growth inhibition test with *Rapidocelis subcapitata* (Toxkit F) adhering to OECD 201 guidelines. The Toxkits were purchased from MicroBioTests, Inc. (Nazareth, Belgium).

2.2.1. Toxicity testing with aquatic crustaceans Thamnocephalus platyurus (fairy shrimp)

The 24-h acute mortality tests of *T. platyurus* were performed in artificial fresh water (AFW) according to the ISO 14380 (2011) guideline: Water quality - Determination of the acute toxicity to *Thamnocephalus platyurus*. Moderately hard water (mg/L in distilled water: $NaHCO_3$-96, $CaSO_4{\cdot}2H_2O$-60, $MgSO_4{\cdot}7H_2O$-123, and KCl-4 in pH 7.8 ± 0.2) was used as a test medium. Briefly, the larvae to perform the test were obtained after hatching of the cysts at 25 °C for 24-h under continuous illumination. Test plates (24-well polycarbonate plates; Falcon, Corning Incorporated, USA) with larvae (<24-h old) were exposed to different concentrations of SAILs (two-fold dilutions, 5 concentrations tested) and benzalkonium chloride, BAC, for 24-h at 25 °C in the dark. At least 3 independent tests with 3 parallels (10 larvae per 1 mL of test medium in each test well) for each concentration of the chemical and the control (test medium) were performed. Viable and dead (immobile) organisms were counted under dissection microscope. Mortality of the organisms was used as the toxicity endpoint. Evaluation of the toxicity was performed in two stages: (i) determination of 0–100% tolerance range of the test organisms to the respective chemicals and (ii) determination of the 50% effect values (LC_{50}).

2.2.2. Toxicity testing with unicellular green algae Rapidocelis subcapitata

The 72-h algal growth inhibition tests were conducted according to the OECD guideline 201 (OECD, 2011), standard test medium was used and pH was adjusted to 8.0 ± 0.1. The test procedure in detail is described in Aruoja et al. (2009). Briefly, the initial cell density of *R. subcapitata* was 10^4 cells/mL counted under light microscope using a Neubauer haemocytometer. The algae were exposed to the chemicals at 24 ± 0.1 °C for 72-h in glass scintillation vials each containing 5 mL of sample. The vials were shaken on a transparent table illuminated from below at 8000 lux. The toxicants were tested in five concentrations in a geometric series with a factor ≤3. There were three replicates of each sample alongside eight control replicates per experiment. All experiments were repeated at least twice. Growth inhibition of algae was calculated from algal biomass that was measured at least every 24 h by quantifying the fluorescence of chlorophyll in algal ethanol extracts (for details, see Joonas et al., 2017).

2.3. Calculation of toxicity values

L(E)C_{50} values were calculated using REGTOX software for Microsoft excel (Vindimian, 2016) using log-normal model.

2.4. Statistical analysis

All tests were performed in at least three independent experiments and triplicates in every single experiment. Statistical analysis was performed using SPSS 21.0 Edition for Windows. Results were expressed as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance, and Tukey's test was used to compare differences among all samples. A p value < 0.05 was considered statistically significant.

3. Results and discussion

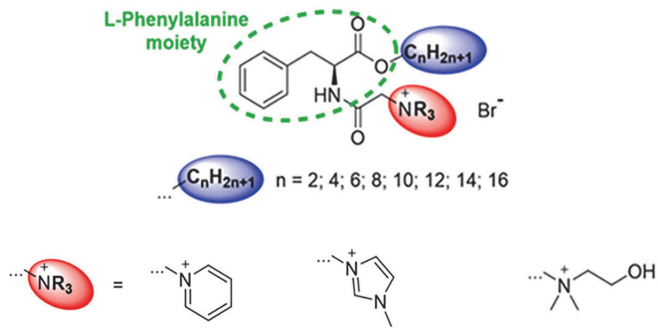
3.1. Library of SAILs and bioassays used

In this study, a library of 24 L-phenylalanine derived SAILs was evaluated for their potential environmental hazard using two bioassays with environmentally relevant test organisms of different food-chain level: unicellular green algae *Rapidocelis subcapitata* (representing primary producers) and aquatic crustaceans *Thamnocephalus platyurus* (representing consumers). *T. platyurus* assay was used instead of more commonly used 48-h *Daphnia magna* immobilization test as the *T. platyurus* assay needs smaller chemical volume and can be completed more rapidly (24-h). For the comparison and addition of bacterial data for this library of SAILS, our earlier data for bacteria *Vibrio fischeri* (representing degraders) are also included from Kusumahastuti et al. (2019a) (Fig. 1, Table 1).

The library comprised of three families of L-phenylalanine derived bromide SAILs based on pyridinium, imidazolium and cholinium cations and with varying alkyl chain from 2 (C_2) to 16 (C_{16}) carbons (Fig. 1). Algal test was applied for the analysis of all 24 SAILs as preliminary screening tests showed the high sensitivity of algae towards these SAILs that also have been analysed by use with *V. fischeri* test (Kusumahastuti et al., 2019a). Thus, we were interested to correlate the data of rapid (30-min) toxicity test with *V. fischeri* with the 72-h algal toxicity data obtained in this study. We did not use the crustacean test (24-h mortality assay) for the whole set of 24 SAILs as the initial testing showed that the sensitivity of crustacean test was much lower than that of the algal test. Thus, crustacean test was used for all 8 pyridinium-based SAILS (C_2–C_{16}) but in case of imidazolium and cholinium based SAILs only C_6 and C_8-SAILs were analysed for toxicity, to compare the effect of different cationic head group on toxicity to crustaceans (Fig. 1).

3.2. Toxicity of SAILs to algae, crustaceans and bacteria. Toxicity ranking of SAILs

According European Union (EU) REACH Regulation chemicals on the EU market must be analysed for their safety (EC, 2006). As different organisms/test species have different sensitivity to chemicals (Kahru and Dubourguier, 2010), including ILs (Costa et al., 2017), a suite of tests with organisms of different trophic levels is usually recommended and used for the evaluation of environmental hazard of chemicals (Kahru et al., 2000). For REACH, usually the number and type of



Test species	Pyridinium (Py)	Imidazolium (Imid)	Cholinium (Chol)
Algae *R. subcapitata*	$C_2 - C_{16}$	$C_2 - C_{16}$	$C_2 - C_{16}$
Bacteria *V. fischeri*	$C_2 - C_{16}$	$C_2 - C_{16}$	$C_2 - C_{16}$
Crustaceans *T. platyurus*	$C_2 - C_{16}$	C_6 & C_8	C_6 & C_8

Fig. 1. A series of L-phenylalanine derived SAILs studied for toxicity. PyC_nPhe, $ImidC_nPhe$ and $CholC_nPhe$. All SAILs are Br^- salts. Data for *V. fischeri* were taken and Figure is modified from Kusumahastuti et al. (2019a).

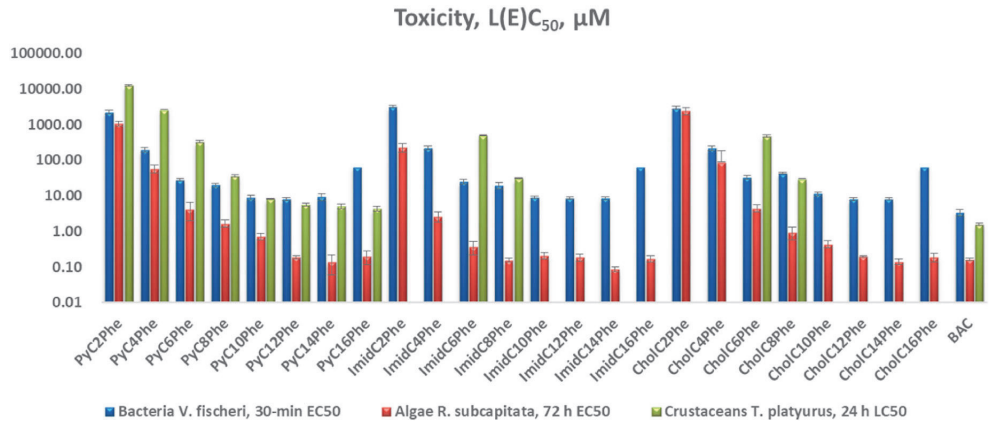


Fig. 2. Toxicity (72-h EC_{50}, 24-h LC_{50} and 30-min EC_{50} values; µM, average values ± SD) of 24 pyridinium, imidazolium and cholinium substituted phenylalanine-derived bromide SAILs and used positive control (benzalkonium chloride; BAC) towards algae *Rapidocelis subcapitata,* crustaceans *Thamnocephalus platyurus* (this work) and bacteria *Vibrio fischeri* (from Kusumahastuti et al., 2019a). Data are plotted from Table 1. See also Fig. A2 (dose-response curves) in the Electronic Supplementary Information.

standardized ecotoxicity tests (by OECD, ISO, US EPA, ASTM) that should be used (ECHA, 2017) depends on the annual production and/or sales amounts. As a rule, the acute toxicity to invertebrates (such as crustaceans *Daphnia magna*) and/or growth inhibition of aquatic plants (e.g. green microalgae) are required when the annual production exceeds 1 ton. In addition, acute toxicity test with fish and activated sludge respiration inhibition tests are mandatory when the annual tonnage exceeds 10 t and additional tests are needed at higher tonnage levels.

In the current study we performed SAILs toxicity studies using 72-h algal growth inhibition assay (OECD 201) with *Raphidocelis subcapitata* and 24-h mortality test with aquatic crustaceans *Thamnocephalus platyurus* (ISO 14380:2011). For the comparison, 30-min EC_{50} data (based on inhibition of bioluminescence) for these 24 SAILs from the literature for marine bacteria *Vibrio fischeri* (ISO 21338:2010) were added (Table 1). All toxicity values (EC_{50} or LC_{50}) in Table 1 are presented both, in mg/L as these values are used for hazard ranking or chemicals as well as in µM that is informative for future QSAR analysis.

Comparing the EC_{50} values of SAILs obtained using the multritrophic test battery (algae, crustaceans, bacteria) it was obvious that the algal growth inhibition assay was the most sensitive test of the battery (Table 1). Therefore, the EC_{50} value of the algal test (mg/L) was used for ranking as algae proved currently the weakest link the simplified food-chain model and determined the toxicity ranking of the studied SAILs. Thus, according to the results of currently applied test battery only two SAILs of the current library - PyC_2 and $CholC_2$ – could be strictly considered 'low toxicity' as they were ranked as 'not harmful' (EC_{50} > 100 mg/L). PyC_4 and $CholC_4$ were ranked 'harmful' (EC_{50} 10–100 mg/L) and PyC_6, $ImidC_4$ and $CholC_6$ ranked 'toxic' (EC_{50} 1–10 mg/L). Twelve studied SAILs ranked as 'very toxic' (EC_{50} > 0.1–1 mg/L). Importantly, four SAILs – PyC_{14}, $ImidC_8$, $ImidC_{13}$ and $CholC_{14}$ ranked as 'extremely

Table 1
Toxicity results (72-h EC_{50}, 24-h LC_{50} and 30-min EC_{50} values) and a simplified hazard classification scheme for pyridinium, imidazolium and cholinium substituted phenylalanine derived bromide SAILs and used positive control (benzalkonium chloride; BAC) according to the toxicity data obtained using algae *Rapidocelis subcapitata,* crustaceans *Thamnocephalus platyurus* (this work) and bacteria *Vibrio fischeri* (from Kusumahastuti et al., 2019a). See also Fig. 2 below and Fig. A2 (dose-response curves) in the Electronic Supplementary Information.

SAILs	Algae *Rapidocelis subcapitata*		Bacteria *Vibrio fischeri* (data taken from Kusumahastuti et al., 2019a)		Crustaceans *Thamnocephalus platyurus*		Toxicity ranking	
	72-h EC_{50}±SD, mg/L	72-h EC_{50}±SD, μM	30-min EC_{50}±SD, mg/L	30-min EC_{50}±SD, μM	24-h LC_{50}±SD, mg/L	24-h LC_{50}±SD, μM	mg/L*	μM**
PyC$_2$Phe	417±65.5	1061±166	866±110	2203±281	4801±300	12209±763	Non toxic	Non toxic
PyC$_4$Phe	23.7±5.52	56.2±13.1	80.1±13.4	192±31.8	1078±5.53	2559±13.1	Harmful	Harmful
PyC$_6$Phe	1.86±0.99	4.14±2.20	12.3±0.73	27.3±1.62	144±8.81	321±19.6	Toxic	Toxic
PyC$_8$Phe	0.78±0.17	1.63±0.36	9.32±1.21	19.5±2.53	16.9±1.07	35.3±2.24	Very toxic	Very toxic
PyC$_{10}$Phe	0.35±0.07	0.70±0.13	4.40±0.62	8.71±1.23	4.08±0.13	8.06±0.26	Very toxic	Very toxic
PyC$_{12}$Phe	0.10±0.01	0.18±0.02	4.17±0.44	7.82±0.82	2.91±0.34	5.46±0.63	Extremely toxic	Extremely toxic
PyC$_{14}$Phe	0.07±0.04	0.13±0.07	5.14±1.22	9.16±2.17	2.75±0.39	4.89±0.70	Extremely toxic	Extremely toxic
PyC$_{16}$Phe	0.11±0.05	0.19±0.08	>36.9	> 62.5	2.48±0.33	4.21±0.56	Very toxic	Extremely toxic
ImidC$_2$Phe	88.1±23.1	222±58	1227±112	3096±282	n.t.	n.t.	Harmful	Non toxic
ImidC$_4$Phe	1.07±0.33	2.53±0.78	91.7±10.7	216±25.2	n.t.	n.t.	Toxic	Toxic
ImidC$_6$Phe	0.16±0.07	0.36±0.15	11.±1.58	24.3±3.50	223±6.94	494±15.3	Very toxic	Very toxic
ImidC$_8$Phe	0.07±0.01	0.15±0.02	9.31±1.90	19.4±3.95	14.4±0.45	29.9±0.94	Extremely toxic	Extremely toxic
ImidC$_{10}$Phe	0.10±0.02	0.20±0.04	4.52±0.42	8.89±0.83	n.t.	n.t.	Extremely toxic	Extremely toxic
ImidC$_{12}$Phe	0.10±0.02	0.18±0.04	4.49±0.33	8.36±0.62	n.t.	n.t.	Extremely toxic	Extremely toxic
ImidC$_{14}$Phe	0.05±0.01	0.08±0.01	4.64±0.47	8.22±0.83	n.t.	n.t.	Extremely toxic	Extremely toxic
ImidC$_{16}$Phe	0.10±0.02	0.17±0.03	>37.0	> 62.5	n.t.	n.t.	Extremely toxic	Extremely toxic
CholC$_2$Phe	960±212	2381±525	1153±109	2858±271	n.t.	n.t.	Non toxic	Non toxic
CholC$_4$Phe	37.4±38.5	86.7±89	90.1±14.8	209±34.2	n.t.	n.t.	Harmful	Harmful
CholC$_6$Phe	2.01±0.49	4.38±1.07	14.8±2.02	32.3±4.40	215±15.5	470±33.9	Toxic	Toxic
CholC$_8$Phe	0.44±0.17	0.91±0.36	20.5±1.23	42.0±2.51	14.1±0.00	28.8±0.00	Very toxic	Very toxic
CholC$_{10}$Phe	0.22±0.05	0.43±0.10	5.72±0.77	11.1±1.49	n.t.	n.t.	Very toxic	Very toxic
CholC$_{12}$Phe	0.11±0.01	0.19±0.01	4.29±0.44	7.90±0.81	n.t.	n.t.	Very toxic	Extremely toxic
CholC$_{14}$Phe	0.08±0.01	0.14±0.03	4.59±0.42	8.03±0.73	n.t.	n.t.	Extremely toxic	Extremely toxic
CholC$_{16}$Phe	0.11±0.03	0.18±0.05	>37.5	> 62.5	n.t.	n.t.	Very toxic	Extremely toxic
BAC	0.04±0.00	0.16±0.01	0.94±0.18	3.31±0.62	0.44±0.03	1.54±0.10	Extremely toxic	Extremely toxic

* Evaluation grid applied by Sanderson et al., 2003 and Blaise et al., 2008 was used. Classification is based on median L(E)C_{50} value of the most sensitive organism used: <0.1 mg/L = extremely toxic to aquatic organisms; >0.1–1mg/L = very toxic to aquatic organisms; >1–10 mg/L = toxic to aquatic organisms; >10–100 mg/L = harmful to aquatic organisms; >100 mg/L = non-toxic to aquatic organisms. n.t. – not tested.
** Evaluation grid applied Sanderson et al., 2003 and Blaise et al., 2008 based on conversion of concentration units to molarity using an average MW of 496 for the SAILs studied. Classification is based on median L(E)C_{50} value of the most sensitive organism used: <0.2 μM = extremely toxic to aquatic organisms; >0.2–2 μM = very toxic to aquatic organisms; >2–20 μM = toxic to aquatic organisms; >20–200 μM = harmful to aquatic organisms; >200 μM = non-toxic to aquatic organisms. n.t. – not tested.

toxic' (EC_{50} < 0.1 mg/L) analogously to well known biocide benzalkonium chloride (Table 1). Analogous trends in sensitivity of aquatic organisms towards ILs were obtained by Pretti et al. (2009) analysing the toxicity of 18 ILs to fish *Danio rerio*, crustaceans *D. magna* and algae *P. subcapitata* and showing that algal tests was most sensitive and fish test least sensitive. Costa et al. (2017) have also concluded that ecotoxicity test organisms of different trophic levels differed in sensitivity to ILs. It is important to note that high toxicity of ILs to algae cannot be ignored, as microalgae account for ~ 50% of global primary production (Field et al., 1998).

3.2.1. *Alternative interpretation of table of toxcity data obtained with algae, crustaceans and bacteria*

The standard units used to rank the SAILs (from extremely toxic to non-toxic) in the Table of Toxicity Data are mg/L. An alternative unit of concentration is molarity (mM) which represents the number of molecules of a compound per unit concentration, rather than the mass per unit concentration. Although concentration values (in mg/L) of a SAIL, or any compound, can be converted into molar concentrations, from a ranking perspective the selection of the scale is critical. This is discussed in more detail below by evaluating a subset of the SAILs from Table 1.

We have previously suggested that the C_6 and C_8 derivatives

(PyC_6Phe, $ImidC_6Phe$, $CholC_6Phe$, PyC_8Phe, $ImidC_8Phe$, $CholC_8Phe$) are suitable as green surfactants (Kapitanov et al., 2019). Table A1 compares the toxicity of 6 SAILs towards algae with the toxicity of 12 commercially available cationic surfactants. This data can be analysed in several ways: colour coding, toxicity results in molarity units and toxicity results in mg/L units. Before beginning this analysis one must ask the question, 'How are we going to move forward based on the outcome?'. The goal of green chemistry is to promote the use of safer chemicals and as such we wish to establish which of our SAILs are safer (based on algal toxicity data as algae proved most sensitive organism group towards SAILs analysed in the current study; Table 1) than commercially available cationic surfactants (see Table A1 and Figure A1 in the Electronic Supplementary Information for their chemical structures).

$ImidC_8Phe$ is rated as 'extremely toxic', the same as BAC, and is not recommended as a substitute for any of the commercial surfactants in the study. PyC_8Phe, $ImidC_6Phe$, and $CholC_8Phe$ are rated 'very toxic' and thus are suggested as a suitable substitution for BAC and STAB only. PyC_6Phe and $CholC_6Phe$ are classified as 'toxic' and are recommended for investigation as substitutes of the 'very toxic' surfactants in Table A1 (i.e. D1622, D1222, CTAB, TTAB, EDDAB, EDHAB, CPB and Hyamine) as well as BAC and STAB.

It is important to note that the recommendations above are based on the data available and some inconsistencies could lead to errors. For instance, the literature EC_{50} values reported are for different algae (i.e. *C. pyrenoidosa, C. vulgaris, S. capricornutum, N. gaditana, C. pyrenoidosa)* and in two studies a 96-h test instead of 72-h was used as the exposure time of algae to ILs. The mg/L units used in the classification scale are independent of the MW of the compounds, and thus the colour coding and comparison of the concentration (mg/L) of compounds with significantly different MW concentrations can be misleading. For instance, the MW range of SAILs MW is from 393 to 599, 1 mg/L is 2.54 and 1.67 μM, respectively, which is not an insignificant difference (52%), that the scale classification does not take into account. To improve the accuracy of the classification scale for our set of SAILs, the average MW was used. This value 496, results in 1 mg/L being converted to 2 μM. The classification scale is set at: <0.2 μM = extremely toxic to aquatic organisms; >0.2–2 μM = very toxic to aquatic organisms; >2–20 μM = toxic to aquatic organisms; >20–200 μM = harmful to aquatic organisms; >200 μM = non-toxic to aquatic organisms. We propose that this enables a more accurate comparison of our SAILs compounds toxicity based on molarity concentration units. It is acknowledged that compounds at the extremes of the MWs within the range 393–599 are more susceptible to erroneous classification. However, this is a more reliable scale than basing classification on mg/L units. Several surfactants in Table A1 have different classifications depending on which classification scale (mg/L or μM) is used (e.g. STAB, CTAB and ($[C_{12}mim]Cl$).

Analysis of the toxicity values in molarity units demonstrates that the classification scale (molarity units) provides a more accurate assessment. Despite these limitations/provisos we propose that the new average MW based classification scale is a more useful guide to rank chemical toxicity of a set of compounds. The recommendations in the previous paragraph are based on prudent consideration of all contributing insight: colour coding, toxicity results in molarity units and toxicity results in mg/L units.

Finally, for comparison with commonly used methylimidazolium salts, toxicity data for algae is included in Table A1. PyC_2Phe and $CholC_2Phe$ are classed as 'non-toxic' and lower toxicity than ([C4mim]Cl) classified as 'harmful', and the more toxic C_8 and C_{12} analogues. All the C_2 and C_4 SAILs (except $ImidC_2Phe$) have lower toxicity towards algae than the C_8 methylimidazolium salts.

3.3. Effect of the type of cationic head group and length of the alkyl side chain on toxicity of SAILs

Interestingly, SAILs with imidazolium head group were up to 35 times more toxic to algae than pyridinium and cholinium head groups. The toxicity was depending on the alkyl chain. Whereas, the difference was most remarkable for C_4 SAILs (22-35-fold difference for pyridinium- and cholinium SAILs, respectively). There was no significant difference in algae toxicities for C_{10}–C_{16} SAILs (Table 1). It is apparent that the imidazolium head group results in additional toxicity of the C_2–C_8 SAILs to algae, compared with pyridinium and cholinium-based SAILs with the same C-chain length (hydrophobicity). Data showing the higher algal toxicity of imidazolium SAILs compared to Py and Chol SAILs are depicted in Fig. 3A. For the comparison, the toxicity data towards crustaceans *T. platyurus* are also shown (Fig. 3B) showing that toxicity of SAILs to *T. platyurus* depended on alkyl side chain length but not on nature of the cationic head group (Fig. 3B). Notably, the effect of cationic head group on toxicity of these 24 SAILs was also not observed when bacteria *Vibrio fischeri* were used as test organisms (Table 1; Kusumahastuti et al., 2019a).

Our results obtained with algae are in agreement with the findings of a recent study on 'second generation ILs' by Xia et al. (2018). They reported that the imidazolium ILs (1-hexyl-3-methylimidazolium nitrate and 1-hexyl-3-methylimidazolium chloride were more toxic to unicellular green algae *Scenedesmus obliquus* than the pyridinium ILs (*N*-hexyl-3-metylpyridinium chloride and *N*-hexyl-3-methylpyridinium bromide). The 48-h EC_{50} values based on algal growth inhibition were very low, 1.4–1.5 mg/L (6–7 μM) for imidazolium ILs and 2.2–3 mg/L (10–11 μM) for pyridinium ILs. The same group also studied the mechanism of toxic action of these ILs and showed that the inhibition of algal growth by ILs was accompanied by damage in photosystem II, increase in cell membrane permeability and damages in cellular ultrastructure and cell wall.

Higher toxicity of imidazolium-based ILs compared to ILs with the morpholinium or pyridinium ring has been reported also by (Bubalo et al., 2017). A study by (Petkovic et al., 2009) compared the toxic effects of 16 ILs based on imidazolium, pyridinium, or cholinium cation to fungi *Penicillium* sp. and imidazolium ILs showed the highest toxicity. However, it was noted that these fungi were generally very tolerant towards the ILs screened with the benchmark dose of studied ILs set at 50 mM.

Concerning the **effect of alkyl side chain length** on the toxicity of 24 SAILs to algae, the toxicity was following the same pattern as for other test organisms: the toxicity increased with the number of carbon atoms in the alkyl chain (Fig. 3). The same tendency was observed by (Latała et al., 2009) who evaluated the toxic effects of imidazolium and pyridinium based ILs (C_2–C_{10}) towards two green algae (*Chlorella vulgaris and Oocystis submarina*) and two diatoms (*Cyclotella meneghiniana* and *Skeletonema marinoi*). They also reported the increase of toxicity in parallel to the increase of length of the alkyl side chain in case of all algal species but no significant differences between toxicity of imidazolium and pyridinium ILs of similar lipophilicity. The diatoms were more sensitive and the sensitivity depended on the cell size. Toxicity of ILs based on imidazolium salt with bromide anion to algae *Selenastrum capricornutum* was studied by (Cho et al., 2007). They showed that the ILs had high toxicity to algae that was strongly dependent on the alkyl side chain length: the longer alkyl chain resulted in higher algal growth inhibition.

Moreover, a report by (Ventura et al., 2013) showed that both butyl ester of pyridinium and imidazolium ILs were more toxic than methyl ester pyridinium and imidazolium derivatives towards algae, which agrees with the trend of this current work. $ImidC_6Phe$, $ImidC_8Phe$, $ImidC_{10}Phe$, and $ImidC_{14}Phe$ SAILs in our study was safer towards algae compared to imidazolium ILs with the same alkyl chain reported by the Centre for Environmental Research and Sustainable Technology (UFT) (Ventura et al., 2013). Additionally, $ImidC_8Phe$ in this study is 50 times less toxic than $ImidC_{14}$ and PyC_6Phe is 1000 times less toxic than

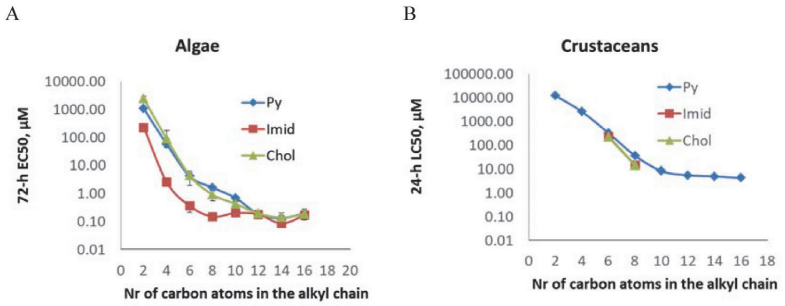


Fig. 3. Toxicity of the studied SAILSs with pyrimidinium, imidazolium and cholinium head groups to algae *Rapidocelis subcapitata* (A) and to crustaceans *Thamnocephalus platyurus* (B). Data are presented as L(E)C_{50} values (μM) average values ± SD *versus* the length of the alkyl side chain and are plotted from Table 1.

$ImidC_{14}$ mentioned in Table 7 of (Ventura et al., 2013).

As stated above, for the **aquatic crustaceans *T. platyurus*** the studied SAILs were much less toxic to algae than BAC: all studied C_2–C_6 SAILs had LC_{50} values > 100 mg/L, C_8 SAILs had LC_{50} values about 15 mg/L and C_{10}–C_{16} SAILs with pyridinium head group had LC_{50} values from 2 to 4 mg/L (Table 1, Fig. 3, right panel). Despite of the different toxicities, the L(E)C_{50} values correlated well, log-log $R^2 = 0.75$) (Fig. 4).

As in case of *V. fischeri* (Table 1 and Kusumahastuti et al., 2019a) the toxicity of studied SAILs to *T. platyurus* did not depend on the cationic head group's type but only on the alkyl side chain length (hydrophobicity) (Fig. 3B). Also, the toxicity of studies SAILs to *V. fischeri* and *T. platyurus* were relatively comparable with *V. fischeri* slightly more sensitive (Table 1; Fig. 5).

3.4. Mechanism of toxic action of SAILs

As in the case of all chemicals, their toxicity is intrinsically linked to their chemical structure and one of the most important parameters in the prediction of the chemical's toxicity (i.e. cause harm to living cells), is its ability to cross the biological membrane. This is the main unit of living

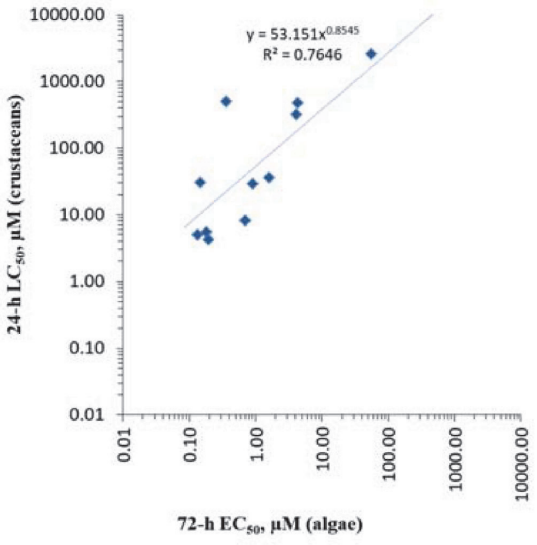


Fig. 4. Toxicity of studied 12 SAILs to algae *R. subcapitata* (72-h EC_{50}, μM) versus toxicity to crustaceans *T. platyurus* (24-h LC_{50}, μM). A log-log plot; R^2 value 0.76 ($p < 0.05$). Data are plotted from Table 1.

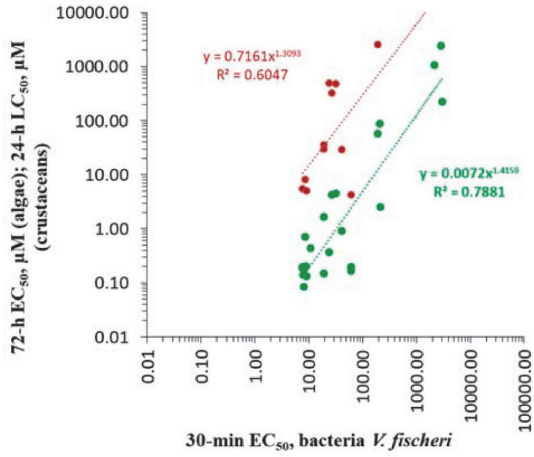


Fig. 5. Toxicity of studied SAILs to bacteria *Vibrio fischeri* (30-min EC_{50}, μM, 24 SAILs) *versus* toxicity to algae *R. subcapitata* (green dots; 72-h EC_{50}, μM, 24 SAILs; log-log $R^2 = 0.79$; $p < 0.05$) and crustaceans *T. platyurus* (red dots; 24-h LC_{50}, μM, 12 SAILs; log-log $R^2 = 0.60$; $p < 0.05$). A log-log plot. Data are plotted from Table 1.

cells, separating cellular interior (with biologically crucial targets such as mitochondria, chloroplasts, and nucleus – depending on the cell type) and the external environment. In the case of unicellular organisms, such as bacteria and algae inhabiting various environmental niches, the exterior also means the abiotic environment, i.e. water, soil or sediment. Thus, the hydrophobicity of the chemical, characterized by the octanol-water partition coefficient, log P, which predicts the potential of the chemical to cross the lipid bilayer of the biological membrane, is widely used in QSAR analysis for the successful prediction of toxicity of industrial organic chemicals. As an example, we recently showed that the algal toxicity of 50 non-polar narcotic industrial compounds relevant for REACH regulation (phenols and anilines) tightly correlated with chemicals' hydrophobicity, log P ($R^2 = 0.95$) (Aruoja et al., 2014). Analogously, the main **toxicity mechanism** proposed for ILs is the disruption of the integrity of biological membranes (Docherty and Kulpa, 2005) and has been shown to be dependent on alkyl side chain length, i.e. hydrophobicity, of ILs often presented in the cationic part of ILs (see above). Indeed, the longer alkyl side chain will increase the lipophilicity of ILs increasing interaction of ILs with both cell membrane phospholipid bilayer and hydrophobic domains of membrane proteins. These interactions lead to the disruption of membrane's physiological functions, causing the leakage of intracellular content and, consequently

leading to cell death (Bubalo et al., 2017). As mentioned above, this toxicity mechanism is especially relevant for unicellular organisms such as bacteria or unicellular algae being in close contact with the ILs during the exposure.

A strong correlation between ILs toxicity and the length of the alkyl side chain has been found in various unicellular species such as bacteria *Escherichia coli*, *Staphylococcus aureus* and *Vibrio fischeri* (Kusumahastuti et al., 2019a, 2019b), unicellular algae *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii* (Kulacki and Lamberti, 2008), but also for multicellular species such as nematodes *Caenorhabditis elegans* (Swatloski et al., 2004) and crustaceans *Daphnia magna* (Couling et al., 2006). The chloroplasts that are the centers of the photosynthesis in plant cells and also have a phospholipid bilayer can be affected by ILs by the same mode of action, resulting in inhibition of photosynthesis (Couling et al., 2006) as described also above.

The lipophilicity of the SAILs is dependent on to the two hydrocarbon groups present: the benzyl substituent of phenylalanine subunit and the alkyl chain. Of note these are also the two key hydrocarbon groups found in BACs. Our original design concept was that it would be possible to prepare safer cationic surfactants where reducing the lipophilicity of the alkyl chain by reducing the chain length, could be offset by the inclusion of an aromatic ring from phenylalanine (Haiβ et al., 2016). The significant contribution of the phenyl ring to surface activity was confirmed by comparing with the alanine derivates, specifically the C_6 cholinium, imidazolium and pyridinium ILs (Kapitanov et al., 2019). An assessment of the surface activity properties of the series of 24 SAILs (e.g. critical micelle concentration, CMC) and discussion about the relationship to toxicity is also reported by Kapitanov and coworkers. We have also reported the relationship between LogK_{ow} and toxicity (*Vibrio fischeri*) for the 24 SAILs (Kusumahastuti et al., 2019a).

In addition to the length of the alkyl side chain of ILs also the nature of the cation (e.g. type of heterocyclic ring) may influence the toxic effects. For example, ILs with imidazolium rings have shown to be more toxic than ILs with morpholinium or pyridinium ring (Egorova and Ananikov, 2014). We also observed superior toxicity of imidazolium-based SAILs to algae compared to pyridinium and cholinium SAILs (Fig. 3A). For crustaceans such as *D. magna* as well as nematodes (*C. elegans*) having the nervous system, the inhibition of acetylcholinesterase activity by ILs can also be one mechanism of toxic action of ILs as shown by *in vitro* experiments on interaction of ILs with AChE by (Stock et al., 2004).

3.5. Correlation of the toxcity data obtained with algae, crustaceans and bacteria. Suggestion of the toxicity screen

Given the enormous number of different combinations of ILs (see above), cheap and rapid toxicity tests are needed to evaluate the potential harmful effects of ILs to aquatic ecosystems as ILs are soluble in water and all (industrial) chemicals sooner or later end-up in the aquatic waterbodies if waste flows are not well controlled and/or wastewater treatment not enough efficient (Bubalo et al., 2017; Egorova and Ananikov, 2014). The tests with algae, crustaceans, fish and bacteria are a proxy of the aquatic ecosystem represented by organisms from different food-chain levels (producers, consumers and decomposers). It is obvious that the enormous amount of different ILs does allow massive toxicity testing as toxicity tests are relatively expensive and time-consuming. To avoid the future high testing costs, the ILs could be screened for toxicity already at the early stage of their development, to exclude combinations that are, for example, too toxic.

For toxicological screening of big chemical libraries at least median throughput assays are needed such as *Vibrio fischeri* bioluminescence inhibition test that we used for the toxicological evaluation of 24 SAILs in our previous paper (Kusumahastuti et al., 2019a). Although the algal growth inhibition test was remarkably more sensitive towards SAILs than *V. fischeri* test and also crustacean mortality test with *T. platyurus*, the *V. fischeri* toxicity data (EC_{50} values) for above described 24 SAILs correlated reasonably well with both, algal and crustacean toxicity data (Fig. 4) as for all these organisms the main predictor of toxic action was the length of the alkyl side chain of the SAILs, i.e., its lipophilicity.

Thus, at least for this type of SAILs rapid *V. fischeri* test could be successfully used for initial toxicity profiling. Moreover, the *Vibrio fischeri* toxicity data for SAILs can most probably used for toxicity prediction of SAIls to algae and crustaceans, via building the respective QSAR models, as toxicity data for chemicals towards algae and crustaceans are compulsory for REACH regulation.

4. Conclusions

This study provides new algal toxicity data for a library of 24 L-phenylalanine derived SAILs and new crustacean toxicity data for 12 SAILs.

According to the results of the multritrophic test battery only two studied SAILs - PyC_2 and $CholC_2$ – could be considered 'low toxicity', i.e. were ranked not harmful (i.e. $L(E)C_{50} > 100$ mg/L by the most sensitive test (algal growth inhibition assay) in the simplified food chain model comprised of algae, crustaceans and bacteria. Both these ILs we propose are green chemicals based on the data herein and the green chemistry evaluation previously performed (Kapitanov et al., 2019) (Suk et al., 2020).

PyC_2Phe and $CholC_2$Phe are classed as lower toxicity than ([C_4mim]Cl classified as 'harmful', and the more toxic C_8 and C_{12} analogues. All the C_2 and C_4 SAILs (except ImidC_2Phe) have lower toxicity towards algae than the C_8 methylimidazolium salts, ([C_8mim]Cl, ([C_8mim]BF_4 and ([C_8mim]NO_3).

An alternative classification scale dependent on the average MW of the compound dataset (based on molar concentrations and not concentrations based on mg/L) was suggested to rank the compounds. When compared to the classification scale independent of the MW of the compound, a more accurate appraisal was achieved for suggesting the greener alternatives for certain commercial SAILs. The recommendations for the greener surfactant alternatives were based on prudent consideration of all contributing insight: toxicity results in molarity units and toxicity results in mg/L units and the respective colour-codings.

As a result, PyC_6Phe and $CholC_6$Phe were classified as 'toxic' and thus could be recommended for investigation as substitutes of the 'very toxic' surfactants D1622, D1222, CTAB, TTAB, EDDAB, EDHAB, CPB and Hyamine, as well as 'extremely toxic' BAC and STAB. SAILs PyC_6Phe and $CholC_6$Phe were up to 27× less toxic to algae than BAC ($EC_{50} = 0.16$ μM).

PyC_8Phe, ImidC_6Phe and. $CholC_8$Phe were rated 'very toxic' and thus could be suggested as a suitable substitution for BAC and STAB only. ImidC_8Phe was rated as 'extremely toxic', the same as BAC, and thus is not recommended as a substitute for any of the commercial surfactants in the study. Most of these SAILs proved very toxic to algae and this must be considered in the production, use and disposal of this type of SAILs.

The toxicity was dependent mostly on length of the alkyl side chain and in case of algae also on cationic head (imidazolium-ILs more toxic than pyridinium- and cholinium-ILs). Algal and crustacean toxicity values for given SAILs correlated tightly with toxicity values obtained using rapid (30-min) *Vibrio fischeri* test. Due to that *V. fischeri* test could be recommended as a screening test before conducting more time-consuming and laborious algal 72-h and crustacean 24-h or 48-h assays.

Author contributions

Conceptualization: Anne Kahru, Nicholas Gathergood, and Mariliis Sihtmäe; testing and data reduction: Dewi K. A. Kusumahastuti, Villem Aruoja, Nicholas Gathergood, Anne Kahru; original draft preparation: Anne Kahru, Dewi K.A.Kusumahastuti, Nicholas Gathergood; review and editing: Anne Kahru and Nicholas Gathergood; visualization, Dewi K.A.Kusumahastuti, Nicholas Gathergood, Anne Kahru; supervision:

Anne Kahru, Nicholas Gathergood, and Mariliis Sihtmäe; funding acquisition: Anne Kahru, Nicholas Gathergood.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2020.100369.

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Curriculum vitae

Personal data

Name	Dewi Kurnianingsih Arum Kusumahastuti
Date of birth	22.04.1983
Place of birth	Salatiga
Citizenship	Indonesian

Contact data

E-mail	dewi.arum@taltech.ee or dewi.hastuti@uksw.edu

Education

2016–2021	Estonia, Tallinn University of Technology, Department of Chemistry and Biotechnology – PhD
2008–2010	South Korea, Kosin University, Department of Chemistry – MS
2001–2006	Indonesia, Universitas Kristen Satya Wacana, Department of Chemistry – S.Si
1998–2001	Indonesia, SMUN 3 Salatiga

Training

October 2020	Estonia, Principles and Techniques for the Extraction of Bioactive Compounds
July 2018	Italy, Green Chemistry Summer School

Language competence

Javanese	Native (fluent)
Bahasa Indonesia	Native (fluent)
English	Good
Korean	Beginner

Professional employment

2019–2021	Estonia, National Institute of Chemical Physics and Biophysics, Laboratory of Environmental Toxicology, early stage researcher
2016–2019	Estonia, Tallinn University of Technology, Green Chemistry ERA Chair, early stage researcher
2014–2015	South Korea, Kyungpook National University, Department of Education (Chemistry), early stage researcher
2011–present	Indonesia, Universitas Kristen Satya Wacana, Department of Chemistry, lecturer and researcher
2010–2011	Indonesia, Universitas Ma Chung, lecturer and student affair staff
2008–2010	South Korea, Kosin University, Department of Chemistry, research assistant
2006–2008	Indonesia, Yayasan Info International, education staff

Elulookirjeldus

Isikuandmed

Nimi	Dewi Kurnianingsih Arum Kusumahastuti
Sünniaeg	22.04.1983
Sünnikoht	Salatiga, Indoneesia
Kodakondsus	Indoneesia

Kontaktandmed

E-mail	dewi.arum@taltech.ee or dewi.hastuti@uksw.edu

Harduskäik

2016–2021	Tallinna Tehnikaülikool, Loodusteaduskond, Keemia ja biotehnoloogia instituut, doktorant
2008–2010	Lõuna-Korea, Kosini Ülikool, Keemiateaduskond, MSc
2001–2006	Satya Wacana Kristlik Ülikool (Indoneesia), Keemiateaduskond, BSc
1998–2001	SMUN 3 Salatiga Keskkool (Indoneesia)

Täiendusõpe

oktoober 2020	Eesti, Maaülikool, Bioaktiivsete ühendite ekstraheerimise põhimõtted ja tehnikad
september 2018	Eesti, KBFI ja TalTech, Nanotehnoloogiast nanomeditsiinini töötuba
juuli 2018	Itaalia, Veneetsia Ca' Foscari ülikool, Rohelise keemia suvekool
aug–sept 2017	Indoneesia, Bandungi Tehnoloogiainstituut, Immunoloogia ja mikroobide patogeneesi suvekool

Keeleteoskus

jaava keel	emakeel
indoneesia keel	emakeel
inglise keel	kesktase
korea keel	algtase

Teenistuskäik

2019–2020	Keemilise ja Bioloogilise Füüsika Instituut, Keskkonnatoksikoloogia laboratoorium, nooremteadur
2016–2019	Tallinna Tehnikaülikool, Rohelise keemia õppetool, nooremteadur
2014–2015	Kyungpook Rahvuslik Ülikool (Lõuna-Korea), Keemiateaduskond, nooremteadur
2011–present	Satya Wacana Kristlik Ülikool (Indoneesia), Keemiateaduskond, lektor ja nooreteadur
2010–2011	Ma Chung Ülikool (Indoneesia), lektor ja üliõpilasesinduse töötaja
2008–2010	Kosini Ülikool (Lõuna-Korea), Keemiateaduskond, assistent
2006–2008	Indoneesia Rahvusvaheline Sihtasutus (Yayasan Info Internasional), administraator

Tunnustused

2019	Biomeditsiini ja biotehnoloogia doktorikooli toetus Rohelise ja säästva keemia konverentsil (Saksamaa) osalemiseks
2019	Doktorikooli toetus Põhja- ja Baltimaade ning Indoneesia teadlaste konverentsil (Taani) osalemiseks
2018	DoRa Pluss 1.1 doktorantide õpirände toetus IUPACi Rohelise keemia konverentsil (Tai) osalemiseks
2018	Biomeditsiini ja biotehnoloogia doktorikooli toetus Rohelise keemia suvekoolis (Itaalia) osalemiseks
2017	DoRa Pluss 1.1 doktorantide õpirände toetus Bandungi Tehnoloogiainstituudi Immunoloogia ja mikroobide patogeneesi suvekoolis (Indoneesia) osalemiseks

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