
Bioavailability and biological properties of several selected ionic liquids

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To my grandma

Pentru Buna Maia

Table of Contents

Summary	VI
Zusammenfassung	VIII
Acknowledgements	XI
Acronyms and Symbols	XII
1. Introduction	1
1.1. Design of sustainable industrial chemicals.....	1
1.2. Model compounds: ionic liquids – a new promising class of solvents.....	3
1.3. Demands of the REACH process.....	5
1.4. Hazard and risk assessment: state of the art.....	8
1.5. Open questions, problem outlines and aims of the thesis.....	11
2. Theoretical considerations	14
2.1. Rational for selection of ionic liquids tested.....	14
2.2. Toxicity tests at mesocosm and microcosm level.....	14
2.2.1. Microcosm toxicity studies – state of the art.....	17
2.2.1.1. Indoor microcosms.....	17
2.2.1.2. Outdoor microcosms.....	18
2.2.1.3. Combined microcosm and field approach.....	19
2.2.1.4. Industrial chemicals – toxicity test at microcosm level.....	20
2.2.2. The Aqua Hab [®] testsystem – pros and cons.....	21
2.3. Monospecies tests.....	25
3. Experimental Part	29
3.1. Material and methods	29
3.1.1. Materials.....	29
3.1.2. Methods.....	34
3.1.2.1. Adsorption tests	34
3.1.2.2. Monospecies tests	35
3.1.2.2.1. Range finding tests.....	35

3.1.2.2.2. Influence of the excretion process on IM12Cl bioconcentration.....	39
3.1.2.2.3. Influence of light on IM12Cl bioconcentration.....	40
3.1.2.3. AquaHab[®] tests	40
3.1.2.3.1. Cation effect.....	40
3.1.2.3.2. Anion effect.....	42
3.1.2.4. Sample preparation for residue analysis.....	44
3.1.2.5. HPLC methods.....	44
3.2. Results and Discussions	46
3.2.1. Adsorption studies	46
3.2.1.1. Adsorption tests.....	46
3.2.1.2. Desorption tests.....	47
3.2.1.3. Saturation limit tests.....	48
3.2.2. Monospecies tests	49
3.2.2.1. Range finding tests.....	49
3.2.2.1.1. Cation effect.....	49
3.2.2.1.2. Anion effect.....	61
3.2.2.2. Influence of excretion on IM12Cl bioconcentration in fish.....	66
3.2.2.3. Influence of light on IM12Cl bioconcentration in plants.....	67
3.2.3. AquaHab studies	68
3.2.3.1. Cation effect.....	68
AquaHab [®] test 1.....	68
AquaHab [®] test 2 and 3.....	72
AquaHab [®] test 4.....	73
3.2.3.2. Anion effect.....	77
AquaHab [®] test 5.....	77
4. Conclusions and outlook.....	82
References.....	85
Appendix.....	102
Curriculum Vitae.....	120

Summary

Green Chemistry proposes the design of chemical products and processes that reduce or eliminate the use and the generation of hazardous substances. The new European Guideline (REACH) demands claim the elucidation of environmental fate pathways and (bio)transformation data as well as the bioaccumulation potential of chemicals in order to reduce the uncertainties in their hazard assessment.

Ionic liquids - model compounds with high ecotoxicological relevance for the future - were tested basically at the molecular and cellular level. Some of them have been tested on more complex and resource consuming single-species forming the second level of a flexible biological test battery. The next step of a tiered strategy to assess the hazard potential of a chemical compound should consist of multispecies tests at the microcosm scale having high ecological relevance for selected chemicals, which are of high interest with respect to their technological, economical and (eco)toxicological aspects.

Guided by the necessity to fulfill several research needs concerning the ecotoxicological hazard assessment of some selected ionic liquids (ILs) the main aims of the present work were defined.

A standardized closed aquatic ecosystem at laboratory scale termed AquaHab[®] designed by the company *OHB-System AG* (Bremen, Germany) was proposed as a tool for (eco)-toxicity investigations at microcosm level filling out the next levels of a flexible biological test battery.

The cation (IM12⁺, IM14⁺, Py2⁺, Py4⁺) and the anion (Cl and BTA) effects were first investigated in single-species tests. Data concerning the acute toxicity of the selected ionic liquids towards four species - *Ceratophyllum demersum* (plants), *Hyalella azteca* (crustaceans), *Biomphalaria glabrata* (snails), *Oryzias latipes* (fish) - were provided.

The cation effects indicated crustaceans as being the far most sensitive organism. In contrast, the snails demonstrated resistance even towards very high concentrations during short and long time exposure to the test chemical.

The anion effects were tested in single species tests with the BTA anion as lithium salt. *Hyalella azteca* showed a higher resistance towards the BTA anion as compared to previous published results on *Daphnia magna*. *Biomphalaria glabrata* showed higher sensitivity of the snails towards the BTA anion in contrast to their high resistance towards the tested cations (IM12⁺, IM14⁺, Py2⁺, Py4⁺). A clear indication of a mixture toxicity posed by the lithium cation and BTA anion towards snails and fishes was stated.

Additional working hypotheses were phrased: (i) distinct ILs have different bioconcentration times; (ii) IM12⁺ ion bioconcentration process is not influenced by the excretion process; (iii) photosynthesis might play a crucial role in the uptake of ILs by the plants. The necessity of further studies to confirm these hypotheses was emphasized.

The first hazard assessment of an ionic liquid performed at the microcosm level in a standardized closed aquatic multispecies system was carried out for IM12Cl. The IM12Cl was chosen to be tested in the AquaHab[®] system based on single-species results and due to its high importance for industrial applications.

In accordance with former results on metabolism and biological degradation indications for the hazard potential “persistence” and a high bioavailability of the IM12⁺ cation in the aquatic environment were found. Acute and subchronic effects were observed within the test period of six weeks. A first indication for a biomagnification potential of an ionic liquid was phrased - the IM12 cation can be transferred from one organism to another – here from fish to snail - within the food chain. But only a low bioaccumulation potential was suggested by the bioconcentration factors calculated from fish (BCF of 1.5 – 10).

The influence of the BTA anion - being the counterion in an IM12 ionic liquid - on the toxicity, bioconcentration and bioaccumulation of the IM12 cation at microcosm level was studied. A mixture toxicity posed by the IM12 cation and the BTA anion if exposed to fishes was evidenced. The investigation of the BTA anion fate in the organisms was proposed for future studies.

The data gained within this thesis further reduce the uncertainties within the ecotoxicological risk profile of the selected ILs.

AquaHab[®] proved to be a useful tool to investigate acute and subchronic ecotoxicological effects, bioconcentration, biomagnification, bioaccumulation and persistence of imidazolium ionic liquids in an aquatic multispecies system.

The present work thus serves as a further contribution to the development of a flexible ecotoxicological test battery. It is therefore an important component of a strategy which aims at a design of sustainable industrial chemicals. It increases the chances to detect hazards and risks for men and environment of a new chemical entity and its degradation products in the environment already within the development process of a new technology.

Zusammenfassung

Das Leitbild der „Green Chemistry“ beinhaltet als zentrales Element das gezielte Design chemischer Produkte und deren Produktionsprozesse, so dass die Verwendung und Synthese gefährlicher Substanzen vollständig vermieden oder zumindest deutlich reduziert werden kann. Die neue europäische Richtlinie zur Registrierung, Bewertung, Zulassung und Beschränkung chemischer Substanzen (REACH) verlangt ausdrücklich nach einer detaillierten Aufklärung des Schicksals von Chemikalien in der Umwelt. Dies beinhaltet sowohl Forschungsarbeiten zu der möglichen Transformation und Biotransformation von chemischen Substanzen in verschiedenen Umweltkompartimenten, als auch die Untersuchung des Bioakkumulationspotenzials dieser Stoffe. Solche Daten werden dringend benötigt, um die Unsicherheiten, mit denen eine Gefahrenpotenzialanalyse von Chemikalien stets verknüpft ist, schrittweise zu reduzieren.

Als Modellsubstanzen, für die aufgrund wachsender Märkte in naher Zukunft eine hohe ökotoxikologische Relevanz erwartet wird, wurden in dieser Arbeit Ionische Flüssigkeiten zunächst auf molekularer und zellulärer Ebene hinsichtlich ihrer toxikologischen Wirkungen getestet. Zusätzlich wurden ausgewählte Ionische Flüssigkeiten in aufwändigeren und komplexeren Monospezies tests untersucht. Diese Tests bilden nach den molekularen und zellulären Testsystemen die zweite Stufe einer flexiblen toxikologischen und ökotoxikologischen Testbatterie. Die nächst höhere Ebene einer solchen abgestuften Teststrategie bildet dann die Analyse des Gefahrenpotenzials einer Chemikalie in Multispezies testsystemen auf dem Level von Mikrokosmen. Solche Tests sind von hoher ökologischer Bedeutung allerdings auch sehr ressourcenintensiv und sollten daher auf gezielt ausgewählte Substanzen beschränkt werden, die eine große technologische, ökonomische und toxikologische Relevanz aufweisen.

Vor dem Hintergrund eines immer noch beschränkten Wissens zu dem ökotoxikologischen Gefahrenpotenzial, das von ausgewählten Ionischen Flüssigkeiten ausgeht, wurden in der vorliegenden Arbeit folgende Aspekte zur Schließung dieser Wissenslücken bearbeitet.

Ein von der Firma *OHB-System AG* (Bremen, Deutschland) entwickeltes geschlossenes aquatisches Ökosystem im Labormaßstab – das AquaHab[®] System – wurde als Testsystem für ökotoxikologische Untersuchungen auf Mikrokosmos-Level verwendet. Dieses Testsystem fungierte so als nächst höhere Stufe einer bereits bestehenden flexiblen biologischen Testbatterie, die als höchste Stufe Monospezies tests umfasst.

Zunächst wurden jedoch ausgewählte Kationen (IM12⁺, IM14⁺, Py2⁺ und Py4⁺) und Anionen (Cl⁻ und BTA) in Monospezies tests auf ihre akuten Toxizitäten hin untersucht. Als Testorganismen wurden dabei folgende vier Spezies verwendet: *Ceratophyllum demersum* (Pflanze), *Hyalella azteca* (Krustentier), *Biomphalaria glabrata* (Wasserschnecke) und *Oryzias latipes* (Fisch).

Die toxischen Effekte der Kationen erlaubten eine eindeutige Identifikation der Krustentiere als sensitivsten Testorganismus. Im Gegensatz dazu zeigten die Wasserschnecken hohe Resistenzen gegenüber den getesteten Substanzen, sogar gegenüber sehr hohen Konzentrationen der Kationen in Kurz- und Langzeit-Inkubationsexperimenten.

Die Anionen-Effekte wurden für das BTA Anion als Lithiumsalz in den Monospezies tests untersucht. Dabei zeigte *Hyalella azteca* eine höhere Resistenz gegenüber dem BTA Anion, verglichen mit Literaturdaten zu *Daphnia magna*. Die Wasserschnecke *Biomphalaria glabrata* zeigte eine höhere Empfindlichkeit gegenüber dem BTA Anion als gegenüber den getesteten Kationen (IM12⁺, IM14⁺, Py2⁺ und Py4⁺), gegen die sie eine hohe Resistenz zeigte. Für die Schnecken und die Fische konnten deutliche Hinweise auf Mischungstoxizitäten für das Lithium Kation in Kombination mit dem BTA als Anion gefunden werden.

Auf Grundlage dieser Monospeziesdaten wurden folgende Arbeitshypothesen für die weiteren Tests aufgestellt: (i) verschiedene Ionische Flüssigkeiten weisen unterschiedliche Biokonzentrationszeiten auf; (ii) die Biokonzentration des IM12⁺ Kations wird nicht durch Ausscheidungsprozesse beeinflusst; (iii) die Photosyntheseaktivität könnte eine entscheidende Rolle spielen bei der Aufnahme von Ionischen Flüssigkeiten in Pflanzen. Zur Klärung dieser Hypothesen wurde die Notwendigkeit weiterführender Tests deutlich gemacht.

So wurde zum ersten Mal eine Gefahrenpotenzialanalyse für eine Ionische Flüssigkeit auf Mikrokosmosebene in einem standardisierten, geschlossenen aquatischen Multispezies testsystem für IM12 Cl durchgeführt. Die Auswahl von IM12 Cl als Testsubstanz in dem AquaHab[®] System erfolgte aufgrund der toxikologischen Daten aus den Monospezies tests und wegen der hohen Relevanz von IM12 Cl für industrielle Anwendungen.

Übereinstimmend mit früheren Ergebnissen zum Metabolismus und Bioabbau des IM12⁺ Kations, konnten in dem hier untersuchten aquatischen Milieu Hinweise auf das Gefahrenpotenzial „Persistenz“ gekoppelt mit einer hohen Bioverfügbarkeit gefunden werden. In dem untersuchten Testzeitraum von sechs Wochen konnten sowohl akute als auch subchronische Effekte auf die Organismen beobachtet werden. Zusätzlich konnte die Übertragbarkeit des IM12⁺ Kations innerhalb der Nahrungskette – hier von der Schnecke zum Fisch – eindeutig nachgewiesen werden, eine essentielle Voraussetzung für ein mögliches Biomagnifikations-Potenzial dieses Kations. Allerdings konnte nach Analyse der Fische nur ein geringes Bioakkumulations-Potenzial (BCF im Bereich 1,5-10) für das IM12⁺ Kation berechnet werden.

Der Einfluss des BTA Anions, das als Gegenion zu IM12⁺ in Ionischen Flüssigkeiten Verwendung findet, auf die Toxizität, die Biokonzentration und die Bioakkumulation des IM12⁺ Kations wurde ebenfalls auf der Mikrokosmosebene untersucht. Dabei zeigten sich Hinweise auf Mischungstoxizitäten von dem BTA Anion und dem IM12⁺ Kation in den exponierten Fischen. Eine detaillierte Untersuchung des Verbleibs des BTA Anions in den Organismen stellt hier einen idealen Anknüpfungspunkt für weitere, über diese Arbeit hinausgehende Forschungsvorhaben dar.

Die in dieser Arbeit gewonnenen Daten tragen dazu bei, die Unsicherheiten in den ökotoxikologischen Risikoprofilen der hier untersuchten Ionischen Flüssigkeiten weiter zu reduzieren.

Das AquaHab[®] System hat sich als wertvolles aquatisches Multispezies-Testsystem zur Untersuchung der ökotoxikologisch relevanten Endpunkte akute und subchronische Toxizität, Biokonzentration, Biomagnifikation, Bioakkumulation und Persistenz der hier getesteten Imidazolium-Verbindungen erwiesen.

So konnte die vorliegende Arbeit auch einen wichtigen Beitrag zur Erweiterung und Weiterentwicklung der ökotoxikologischen Testbatterie leisten. Die hier präsentierten Ergebnisse und Überlegungen bilden einen wichtigen Baustein einer Strategie, die auf das gezielte Design nachhaltiger Industriechemikalien ausgerichtet ist. Eine solche Strategie erlaubt die Analyse und Aufdeckung möglicher Gefahrenpotenziale einer neuen Substanz für Mensch und Umwelt bereits in der frühen Entwicklungsphase und trägt somit zum prospektiven Entwicklungsprozess nachhaltiger Technologien bei.

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Acronyms and Symbols

ACN	Acetonitrile
BCF	Bioconcentration factor (depends on the partitioning of the chemical between the biota and the water)
BNFL	British Nuclear Fuels
BP	British Petroleum
CMR	carcinogens, mutagens, and toxic to reproduction
DDT	para-para-dichlordiphenyltrichlorethane
EC₅₀/ EC₂₅/ EC₁₀	Effective Concentration in mg/L or µg/L that produces a specific measurable effect in 50% / 25% / 10% of the test organisms within the stated study time
EtOH	Ethanol
EU	European Union
H₃PO₄	Phosphoric acid
HPLC	High-Performance-Liquid Chromatography
HPLC-MS	High-Performance Liquid Chromatography – Mass Spectrometric Detection
IC₅₀	Estimated concentration able to immobilize 50% of the organisms within the stated study time
ILs	Ionic Liquids
IM12 BTA or IM12 (CF₃SO₂)₂N	1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)amide
IM12 Cl	1-ethyl-3-methyl-1H-imidazolium chloride
IM14 Cl	1-butyl-3-methyl-1H-imidazolium chloride
IM18Cl	1-methyl-3-octyl-1H-imidazolium chloride
IUPAC	International Union of Pure and Applied Chemistry

KH₂PO₄	Potassium Dihydrogen Phosphate
LD₅₀/LC₅₀	Lethal Dose/Concentration in mg/L or µg/L to 50% of the test organisms within the stated study time
Li BTA or Li (CF₃SO₂)₂N	Lithium bis(trifluoromethylsulfonyl)amide
MeOH	Methanol
NA	No data available
NOEC	No observed effect concentration
PBT	persistent, bioaccumulative, and toxic
PEC	Predicted effect concentration
PNEC	Predicted no effect concentration
Py2 Cl	1-ethylpyridinium chloride
Py4 Cl	1-butylpyridinium chloride
Py8 Cl	1-octylpyridinium chloride
SAR	Structure-Activity-Relationships
SME	small and medium size enterprises
T-SAR	Thinking in Terms of Structure-Activity-Relationships
UFT	Centre of Environmental Research and Technology in Bremen, Germany
UV	Ultraviolet
vPvBs	very persistent, very bioaccumulative

1. Introduction

1.1. Design of sustainable industrial chemicals

Development which meets the demands of human beings living at present without endangers the chances of future generations to satisfy their needs means “**sustainable development**”.

According to the Organisation for Economic Cooperation and Development (OECD), industrial sustainability is defined as the continuous innovation, improvement and use of clean technologies to reduce pollution levels and consumption of resources. In practical terms, industrial sustainability means employing technologies and know-how to use less material and energy, maximizing renewable resources as inputs, minimizing generation of pollutants or harmful waste during product manufacture and use, and producing recyclable or biodegradable products (Jenck *et al.*, 2004).

Striving for a sustainable development is a necessary task for mankind. The globalized economy, but also the global society has intimate ties with chemical products and processes (Ranke *et al.*, 2007).

The chemical industry creates materials for multiple consumer markets. The global chemical industry represents a significant part of world trade and economic activity with 10 million employees and a combined turnover of some 1300 billion € excluding pharmaceuticals and at 1841 billion € including pharmaceuticals, representing 4–5% of world income (Jenck *et al.*, 2004).

So far there is a global *technology challenge*, especially in the chemical sector, as sustainability can be attained by the development of environmentally benign processes, integration of material constraints, costs and safety, and further increases in energy and material efficiency in producing goods and services (Sikdar, 2003). The challenge lies in improving the chemistry, the selection of raw and auxiliary materials and in the smarter design of chemical manufacturing facilities. But the aim of achieving sustainability cannot be achieved by technology alone.

The World Business Council for Sustainable Development’s “Business case for Sustainable Development” (<http://www.wbcsd.ch>) describes the following business incentives to promote sustainability:

- profitability, fiscal changes
- environmental conscience, legislation and regulations
- concerns from shareholders, employees, customers
- long term business viability related to public perception and image

Because of the close relationships between economy-society-chemical products and processes and because of some well-known examples of environmental disasters caused by the interactions of chemical products and processes with ecology (*e.g.* accumulation of

pesticides throughout the food chain –DDT, poisoning of people with cadmium- Itai-Itai disease, or with mercury-Minamata disease) chemistry has been explicitly addressed in the [Agenda 21 \(1993\)](#), which was a result of the UN conference on Environment and Development in Rio de Janeiro held in 1992.

For today's chemists, designing benign industrial chemicals is both a vision and a mission ([Jastorff et al., 2005](#)). To fulfill this mission an inter-/transdisciplinary thinking, communication and cooperation has to be adopted. The 12 principles of Green Chemistry ([Anastas, Warner, 1998](#)) offer guidelines as to how chemists can direct their efforts towards more sustainability in research and development of new chemical entities and products. For example:

- **Principle 1:** *It is better to prevent waste than to treat or clean up waste after it is formed;*
- **Principle 4:** *Chemical products should be designed to preserve efficacy of function while reducing toxicity;*
- **Principle 10:** *Chemical products should be designed so that at the end of their function they do not persist in the environment, and break down into innocuous degradation products;*
- **Principle 11:** *Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.*

When designing new materials, products or processes scientists and engineers are provided with a framework, beyond baseline engineering quality and safety specifications, based on 12 principles that consider environmental, economic and social factors ([Anastas, Zimmerman, 2003](#)):

- mass and energy in and outputs should be as inherently non-hazardous as possible
- prevention of waste is better than clean up
- minimize energy in separation/purification processes
- maximize mass, energy, volume and time efficiency in product/process
- output-pulled is preferred to input-pushed
- energy is main criterion for choice between recycle, reuse or disposal
- durability must be targeted (no eternal life)
- avoid one-size-fits-all, minimize excess
- minimize material diversity in multi-component products
- integration and interconnectivity are a way to industrial ecology
- design for performance in a commercial “after-life”
- favor mass and energy inputs from renewable sources

Sustainable development should bring about improvements in the economical, ecological and social conditions for present and future generations ([Jastorff et al., 2003b](#)).

Green Chemistry proposes design of chemical products and processes that reduce or eliminate the use and the generation of hazardous substances. The role of Green Chemistry is fundamental because it is a prerequisite shift in the perspective of chemistry. In the same time the problems can be addressed already at the molecular level. Molecular design of a chemical can reduce its impact on human health and the environment. In these sense the entire life cycle (physical hazards, toxicity, ecotoxicity, global changes) of a chemical has to be considered.

Taking the principles of Green Chemistry as a starting point the interdisciplinary "Project Team Ionic Liquids" from the Centre of Environmental Research and Technology (UFT) in Bremen, Germany, offered in 2003 a transdisciplinary strategy to assess potential risks and design sustainable products in the case of a new class of organic solvents called ionic liquids (Jastorff *et al.*, 2003b).

1.2. Model compounds: ionic liquids – a new promising class of solvents

Ionic liquids, salts of organic cations with melting points below 100 °C, are being widely investigated as replacements for volatile organic solvents in industrial and laboratory processes because they are thought to be "environmentally benign" (Masten, 2004). This class of compounds represent a fascinating group of new chemicals with the potential to improve development in organic chemistry and chemical technology (Wasserscheid, Welton, 2002; Seddon, 2002) stimulating a lot of research fields (Jastorff *et al.*, 2003b).

Ionic liquids have gained popularity in recent years (Welton, 1999; Holbrey, Seddon, 1999; Wasserscheid, Keim, 2000; Sheldon, 2001; Olivier-Bourbigou, Mogna, 2002; Zhao *et al.*, 2002; Dupont *et al.*, 2002; Chhikara *et al.*, 2004;) for their increasing use in the two important fields of chemistry - synthetic and biochemical - and their concept and history has been well documented (Hurley, Wier, 1951; Robinson, Osteryoung, 1979; Wilkes *et al.*, 1982; Hussey, 1983; Poole *et al.*, 1986; Carlin, Wilkes, 1990; Wilkes, Zawarotko, 1992; Chauvin *et al.*, 1990; Fuller *et al.*, 1994).

In the beginning the ionic liquids class of compounds was mainly investigated for their applications in electrochemical technologies and as solvents in electronic absorption spectroscopy for highly charged complex ions (Jain *et al.*, 2005).

Later on they have been used for example in: (i) liquid-liquid extraction processes (Wasserscheid *et al.*, 2003); (ii) organometallic reactions as recyclable alternatives to aprotic solvents (Scheeren *et al.*, 2003); (iii) biocatalysis (Retz *et al.*, 2002); (iv) catalytic cracking of polyethylene (Zavilla *et al.*, 2004); (v) radical polymerization (Hong *et al.*, 2002). Thus ionic liquids have been attracting the attention of the scientific community and a number of comprehensive reviews concerning the physicochemical properties of ILs and their applications in synthesis, catalysis and electrochemistry have been published (Marsch *et al.*, 2004; Welton, 2004; Chiappe, Pieraccini, 2005; Galinski *et al.*, 2006; Silvester, Compton, 2006; Chowdhury *et al.*, 2007; Parvulescu, Hardacre, 2007; Hough, Rogers, 2007; Winkel *et al.*, 2008; Chen *et al.*, 2008; Kobrak, 2008; Martius *et al.*, 2008; Greaves,

Drummond, 2008; Weingärtner, 2008). Therefore Plechkova and Seddon in their critical review from 2008 concluding about “*where does the future of ionic liquids lie*” were stating: *the field of ionic liquids is growing at a rate that was unpredictable even five years ago. The range of commercial applications is quite staggering; not just in the number, but in their wide diversity, arising from close cooperation between academia and industry [...]. Remarkably, a review of ionic liquids appears every two-to-three days, and papers are appearing faster than forty per week [...]. As ionic liquids can, in principle, replace conventional liquids wherever they are used, we have barely scratched the surface of the possible. The new few years will be truly fascinating*”.

Ionic liquids are salts that are generally liquid at room temperature. The different thermodynamic and kinetic behavior of the reactions in ionic liquids often leads to improved process performance. They are ‘designer solvents’ (Freemantle, 1998) as their physical properties such as *e.g.* melting point, viscosity, density and hydrophobicity can be modified according to the nature of the desired reactions by altering the chemical structure of their cations as well as their anions (Hagiwara, 2000).

Ionic liquids outclass other solvents in many organic reactions due to the variety of their very special properties (Jain *et al.*, 2005):

- They have essentially no vapor pressure and thus serve as potential replacements for volatile organic compounds in the chemical industry;
- They possess good thermal stability and do not decompose over a large temperature range, thereby making it feasible to carry out reactions requiring high temperature favorable in ionic liquids;
- They are able to dissolve a wide range of organic, inorganic and organometallic compounds;
- They serve as a good medium to solubilise gases such as H₂, CO, O₂ and CO₂ and many reactions are now being performed using ionic liquids together with supercritical CO₂;
- The solubility of ionic liquids depends upon the chemical structure of the cations and counteranions;
- They generally do not co-ordinate to metal complexes, enzymes and different organic substrates;
- Their ionic character enhances the reaction rates to a great extent in many reactions including microwaveassisted organic synthesis (Fry, 2003);
- Most of the ionic liquids can be stored without decomposition for a long period of time;
- They show a high degree of potential for enantioselective reactions as a significant impact on the reactivities and selectivities due to their polar and non-coordinating properties can be achieved. In addition, chiral ionic liquids have been used to control the stereoselectivity.
- The viscosity of 1-alkyl-3-methyl imidazolium salts can be decreased by using highly branched and compact alkyl chain, as well as by changing the nature of anion

(Swartling, 2000).

The viscosity decreases in the order: $\text{Cl}^- > \text{PF}_6^- > \text{BF}_4^- \approx \text{NO}_3^- > (\text{CF}_3\text{SO}_2)_2 \text{N}^-$

In spite of the impressive list of advantages of this new chemical class one important impact of a sustainable new technology and application based on ionic liquids is nearly not addressed: much more knowledge is needed to assess ionic liquids with regard to their environmental impact, if they are once released. They have to fulfill *e.g.* the principles 4 and 10 of green chemistry! An adequate product design for this promising group of chemicals therefore should consider not only the technological needs but from the very beginning of the creation (design and synthesis) of new chemical entities also their inherent toxicological and ecotoxicological hazards and risks (Jastorff *et al.*, 2003b).

In the beginning ionic liquids were considered in a green chemistry context due to their not existing or very low vapor pressure, their recovery facilitating recycling (Scammells *et al.*, 2002; Hemeon *et al.*, 2004) and their applicability to catalytic processes (Sheldon, 2001). However, it is the thermal stability that has opened the question concerning ionic liquids potential to accumulate in the environment (Nelson, 2002; Laird *et al.*, 2002; Swatloski *et al.*, 2003). Within the field of green chemistry it is unacceptable to produce large quantities of waste which have high ecotoxicity or biological activity (Freer, Curzon, 2003; Holbury, 2002) and the biodegradability becomes an essential chemical property (Scott, Jones, 2000). The factors which improved the biodegradation of surfactants have successfully been applied to ionic liquids (Gathergood *et al.*, 2004) but their technological properties have not been optimized yet.

Recycling is an important issue that addresses the economics of ionic liquids use, particularly for large scale applications. A capacity to contain and recycle ionic liquids also reduces concerns about ionic liquid disposal, biodegradation and toxicity (Scammells *et al.*, 2005).

For further risk assessment studies, more (eco)toxicological data and data on the exposure pathways (for selected technical applications), (bio)transformation and sorption processes as well as bioaccumulation studies are necessary. In general, more attention has to be drawn to suitable regeneration and/or recycling methods, taking into account the whole life cycle of ionic liquids. The adequate combination of the above elements and a dynamic communication and discussion of the results provides the opportunity for a really sustainable development of this fascinating group of chemical substances (Jastorff *et al.*, 2005).

1.3. Demands of the REACH process

After years of heated debate, European Union legislators agreed on a far-reaching proposal to review the way chemicals are approved in Europe, placing the obligation on companies manufacturing or importing chemical substances to prove their products are safe before they can be placed on the market. The system, called **Registration, Evaluation and Authorisation of Chemicals (REACH)**, aims to make chemicals safer for human health and

the environment and to stimulate innovation in the sector. It came into force in June 2007 with a phased implementation over the next decade and it has been described as the most complex legislation in the European Union's history and even the most important in the last 20 years. It is the strictest law to date regulating chemical substances and will impact industries throughout the world (Umweltbundesamt, 2007).

REACH introduces new registration requirement covering all substances supplied above 1 ton per year, and a new authorization criterion covering substances of very high concern (*e.g.* carcinogens). In the same time this new legislation creates a single system for what were previously described as “existing” and “new” substances and transfers responsibility for gathering data and carrying out initial risk assessments from the authorities to industry.

The main reasons which generated the need for the REACH legislation are:

- Over 30.000 substances on the EU market above 1 ton per year
- Very limited information available on hazards and risks to human health and the environment
- Current regulatory system has been very slow to produce results –less than 200 substances assessed properly over past 30 years
- Increasing public concern over risks of chemicals
- Need for better evidence base to address this concern
- Current system confusing for industry to understand and for authorities to administer

The basic elements on which REACH is working are very briefly presented within the Table I.

Table I. Key elements of REACH

REGISTRATION	a manufacturer or importer will need to register any substance they supply to the EU market above 1 ton per year
EVALUATION	the authorities will carry out annual in-depth evaluations (<i>i.e.</i> assessments) of substances flagged as being of potential high risk (<i>e.g.</i> on the basis of information provided at registration)
AUTHORISATION	the uses of substances of very high concern, <i>e.g.</i> CMRs (carcinogens, mutagens, and toxic to reproduction), PBTs (persistent, bioaccumulative, and toxic), and vPvBs (very persistent, very bioaccumulative) will require authorization
AGENCY	a new EU Chemicals Agency based in Helsinki, Finland will administer REACH, in co-operation with Member States' competent authorities

Most of REACH stipulations cover manufacturers and importers of chemicals, not the **downstream users**. Still downstream users have rights and obligations too.

- a. Rights:**
- to join a Substance Information Exchange Forum during the registration process
 - to request that their supplier's chemical safety assessment covers their use(s)
- b. Obligations:**
- implement risk reduction measures recommended by their suppliers
 - under certain circumstances might be obliged to carry out a risk assessment covering their particular use(s) of a chemical

The important dead-lines demanded by REACH are presented in short in Table II.

Table II. REACH implementation timeline (key dates)

1 December 2008	Deadline for all companies intending to register a substance to notify their intention to the EU chemicals Agency (pre-registration)
1 December 2010	Registration deadline for manufacturers/importers supplying a <ul style="list-style-type: none"> ▪ substance above 1.000 tons per year ▪ CMR substance above 1 ton per year ▪ PBT/vPvBs substance above 100 tons per year
1 June 2013	Registration deadline for manufacturers/importers supplying a substance above 100 tons per year
1 June 2018	Registration deadline for manufacturers/importers supplying a substance above 1 tons per year

Apart from the potential costs to industry and the complexity of the new law, REACH has also attracted concern because of the potential for a very significant increase in animal testing. Animal tests on vertebrates are allowed only once per one substance, and where suitable alternatives can not be used. If a company pays for these tests, it must sell the rights to the results for a "reasonable" price (but this is not defined). There are additional concerns that access to the necessary information might be very costly for potential registrants needing to pay for this.

1.4. Hazard and risk assessment: state of the art

There have been many attempts to give general definitions of the term "risk" and other terms describing potential damage. Each of these definitions has its advantages and its blind spots (Ranke, 2002). For the discussion of a risk assessment of chemicals it is important to be aware of the common distinction between:

- the **hazard** that a substance represents by its mere identity or its inherent properties :
 - physico-chemical character (e.g. pH, water solubility etc)
 - abiotic and biotic transformation mechanisms
 - acute and chronic toxicity
 - the influence on populations, biocoenosis and ecosystems (monospecies-tests, multispecies-tests, model-ecosystems)

- the **risk assessment** which is a result of the combination of:
 - effect assessment
 - hazard identification
 - dose/concentration-response/effect assessment
 - exposure assessment
 - application
 - amount and frequency of release
 - spatial distribution
 - environmental compartment (water, soil, air)
 - bioavailability
 - bioaccumulation

In the course of the collection of substances that were deemed on the European Market before 1981 in the European Inventory of Existing Chemical Substances (EINECS) it became clear that it was impossible to carry out a full risk assessment for all the 100 195 so-called existing substances in a reasonable time span, especially considering the requirements of the later published Technical Guidance Document (1996). Since that time, a considerable number of methods for the screening of these substances with respect to hazards and/or risks to human health and the environment have been described, with some of them specifically aiming at selecting priority substances for carrying out a full risk assessment (Ranke, 2002).

Comparative ecotoxicological risk profiles of chemical substances that can alternatively be used in a certain application have been previously proposed (Ranke and Jastorff, 2000; 2002). They are inspired by the idea of an assessment of the hazard potential of a chemical by persistence, bioaccumulation and toxicity (PBT assessment). However, a risk presupposes the probability of a release of a substance to some uncontrolled environment, and this release probability can already be taken as a first risk indicator when comparing substances. Therefore five risk indicators – as presented in Figure 1- were defined as essential components of an ecotoxicological risk profile: *release*, *spatiotemporal range*, *bioaccumulation*, *biological activity* and *uncertainty* (Ranke, 2002).

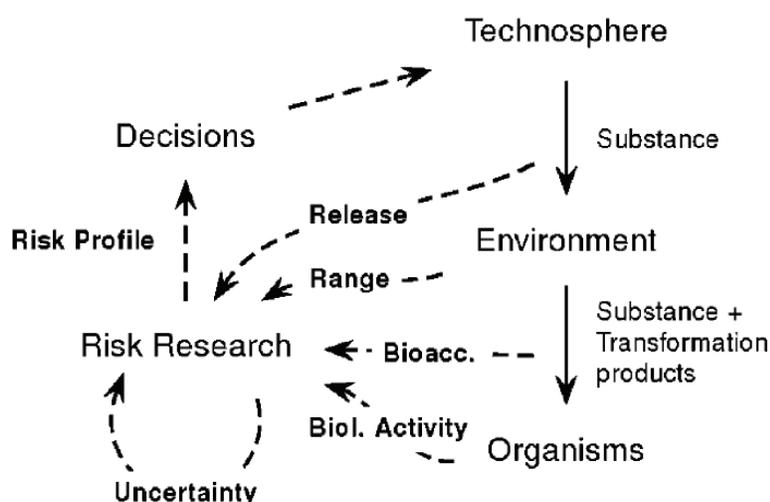


Figure 1. Risk management cycle and its correlation with the ecotoxicological risk indicators (adapted from Ranke, 2002)

The concept was applied by Jastorff *et al.* (2003b) to a preliminary comparison of two room temperature ionic liquids with the conventional solvent acetone in the course of a description of the product design strategy for ionic liquids. These preliminary risk profiles were later updated by Ranke *et al.* (2005).

The same risk indicators were used as a structural basis for a review on ionic liquids as sustainable products (Ranke *et al.*, 2008).

a. Release. The possible damages to organisms in the environment start with the release of the substance in question. Once this release has taken place, there is generally no control on the distribution and the effects of the substance any more. Consequently, the release of the chemical is the most crucial point of a risk analysis. The quality of the ecotoxicological risk analysis can never be better than the quality of the release information. The potential release is application specific rather than chemical specific. However certain intrinsic properties of chemicals (*e.g.* vapor pressure) make a release more or less likely (Ranke *et al.*, 2008).

b. Spatiotemporal range. The tendency of the potentially released substance and its environmental transformation products to spread in space and time is component of an ecotoxicological risk. The quantification of this component is generally a very complex task because the environment itself is made up of so many different chemical milieus and there are so many possibilities for transport and reaction. Therefore for quantifying the spatiotemporal range situation specific methods can be used if an appropriate environmental fate

model is lacking or the substance specific data are not available (Ranke *et al.*, 2008).

c. Bioaccumulation. Describing the *bioconcentration* from the surrounding media as well as *biomagnification* along the food chain, the bioaccumulation potential of a substance is frequently evaluated using either the partitioning constant between 1-octanol and water ($\log K_{ow}$) or the bioconcentration factor (BCF), ideally describing the steady-state concentration in fish tissue in relation to the concentration in the surrounding medium. The BCF always contains information about the kinetics of uptake and purging processes like metabolism and excretion. The K_{ow} is of a more limited relevance for the evaluation of bioaccumulation (Ranke, Jastorff, 2000). The affinity of a chemical substance to organisms (Table III) is commonly expressed by the bioconcentration factor BCF determined by laboratory tests with fish, or from concentrations measured in environmental samples, where the uptake by feeding is additionally possible.

Table III. Scheme for evaluating the bioaccumulation tendency
(adapted from Ranke, Jastorff, 2000)

BCF	< 30	30-100	100-1000	>1000
K_{ow}	< 2,8	2,8 – 3,5	3,5 – 4,8	>4,8

Compounds with BCF value greater than 1000 have high bioaccumulation potential. BCF values between 1000 and 250 indicate moderate potential, and BCF values less than 250 indicate low potential (Allen, Shonnard, 2002). The remaining uncertainty caused by the limited significance of $\log K_{ow}$ and BCF for an evaluation of true bioaccumulation has to be kept in mind (Ranke *et al.*, 2008).

d. Biological activity. The role of the indicator biological activity is to express the relation between the substances accumulated in the organisms and their effects. Therefore, the most accurate type of data for its assessment is the *internal effect concentration* (Sijm, Hermens, 2000) which should be considered for the substance, its transformation products and metabolites which have been proven relevant in the antecedent assessment parts. It is better to estimate relevant risk indicators than to work with less relevant data, even if they are more easily obtained and better defined (Ranke *et al.*, 2008). The severity of these effects is a function of the released amount, the spatiotemporal range, bioaccumulation and the biological activity of the substance. Obviously, a calculation of this severity for all organisms is impossible, not only because of a lack of data about substances, organisms and environment, but also because of the complex

interrelations between them (Ranke, 2002).

Additional data that can influence the indicator biological activity would be information about biochemical mechanisms of observed noxious effects.

e. Uncertainty. A risk evaluation is complete if not only the relevant facts for the assessment are reported, but also the knowledge about the relevance of these facts is communicated, and if the information about the size of gaps in knowledge and interpretation is conveyed (Ranke, 2002). In the case of ecotoxicological risk profiles the uncertainty indicator is defined as the resulting uncertainty from the evaluation of the four preceding risk indicators (Ranke et al., 2007).

1.5. Open questions, problem outlines and aims of the thesis

By the mid 1990s, the basic understanding of the ionic liquid concept was well known in a narrow scientific community, mostly electrochemists. However there was a suggestion that ionic liquids could be used for green chemistry and industrial chemistry (Seddon, 1996a, b; 1997). There was no public hint for industrial applications. But behind the scenes several industries (*e.g.* BP, BNFL, Unilever) were filing patents related directly to potential uses of ionic liquids for large scale green industrial processes (Abdul-Sada *et al.*, 1995a, b, c; Fields *et al.*, 1998; Roberts *et al.*, 1998a, b; Davey *et al.* 1999; Fields *et al.* 1999; Thied *et al.* 1999). Thus, by late 1990s, once these patents were published an increasing interest for green chemistry arose.

In 2000, at a crucial meeting in Crete, a strategy for the development of ionic liquids for industrial applications was planned in detail and the criteria that would have to be met were set (Green Industrial Applications of Ionic Liquids, 2002). Among the requirements which were underlined as being absolutely necessary i) useful applications to be studied; ii) a new paradigm in thinking about synthesis in general combined with green chemistry; iii) cooperation between science/engineering/business from the very beginning in the field development; iv) readily available, well characterized ionic liquids, free of intellectual property; v) cost/benefit, economic and life-cycle analysis; vi) regulatory road blocks to ionic liquids implementation; vii) public free, verified, web-database of physical, thermodynamic and related data – not process specific; viii) increase in the number but especially in the area of expertise of ionic liquids researchers; ix) international collaboration, communication and education regarding the results; x) development of a brochure for advancing the understanding of ionic liquids and their applications; the immediately need of toxicity, biodegradation, bioaccumulation, safety, health and environment impact data has been explicitly addressed.

Thus in 2003 within the “Centre of Environmental Science and Environmental Technology” (UFT) from Bremen, Germany, based on a fruitful collaboration between academia and industry, a multidisciplinary working group developed a strategy which aims at an environmental risk assessment of chemicals, using a combination of structure-activity

relationship (SAR), toxicological and eco-toxicological test and modelling (Jastorff *et al.*, 2003b). In short the following tools were proposed:

- interdisciplinary theoretical and work-sharing experimental collaboration
- selection of lead chemicals according to the “test-kit-concept”
- ecotoxicological test battery on different levels of complexity (*e.g.* enzymes, cells, organisms, microcosm and mesocosm studies)
- assessment of the molecular interaction potential, shape and conformational flexibility, chemical and biochemical reactivity of a chemical entity from a systematic algorithm
- evaluation of qualitative and quantitative structure-activity relations (SAR/QSAR)
- theoretical assessment of presumable transformation products due to metabolic reactions based on T-SAR (Thinking in Terms of Structure Activity Relationships)
- multidimensional risk analysis (release, spatiotemporal range, bioaccumulation, biological activity and uncertainty)

During the next years several toxicity and ecotoxicity papers concerning the effect of different types of ionic liquids – especially imidazolium and pyridinium classes of ionic liquids – were published (*e.g.* Ranke *et al.*, 2003; Stock *et al.*, 2004; Stepnowski *et al.*, 2004; Matsumoto *et al.*, 2004; Skladanowski *et al.*, 2005; Docherty, Kulpa, 2005; Stolte *et al.*, 2006). The chemicals were tested basically at the molecular and cellular level. The influence of the structural modifications of the selected classes of ionic liquids on their biological activity was described in detail. For a compilation of existing knowledge gained so far in the field of structure-activity relations *see* Ranke *et al.*, 2007.

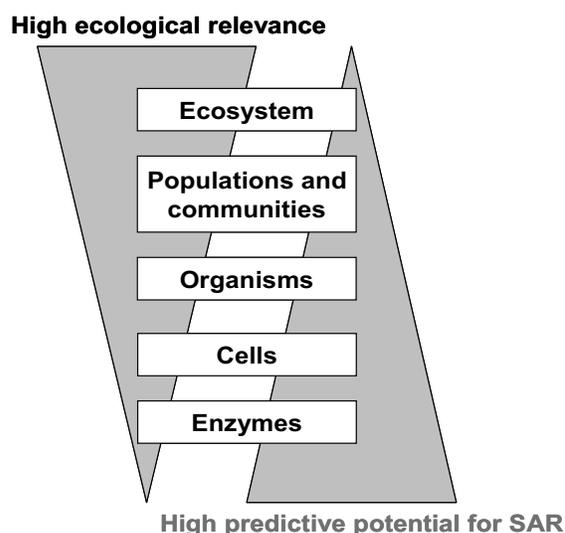


Figure 2. Levels of complexity for the evaluation of the biological activity of chemicals (modified from Jastorff *et al.*, 2003b)

In a publication from 2005 (Jastorff *et al.*, 2005) it has been shown that considerable progress has been achieved concerning the assessment of the general biological activity of ionic liquids. This progress has focused until that time on screening methods for larger sets of compounds, and on the class of imidazolium based ionic liquids. Some of these have been tested in more complex and resource consuming single-species tests (Bernot *et al.*, 2005a, b), forming the second level of a flexible biological test battery as previously presented by Jastorff *et al.* 2003b (Figure 2).

However, monospecies-tests are not suitable for inferring effects of a chemical agent in real ecosystems (Genoni, 1992), because interactions of chemicals with ecosystems (or in other words: with biotic and abiotic factors within the system) or at least part of ecosystems as well as chronic effects cannot be observed (Smolka, Weidemann, 1995; Cairns *et al.*, 1992). Therefore small multispecies-systems can serve to bridge the gap between the rapid monospecies tests and real ecosystems (Slenzka *et al.*, 2001). Thus, the next step of a tiered strategy to assess the hazard potential of a chemical compound should consist of multispecies tests at the level of populations and communities having high ecological relevance for selected chemicals, which are of high interest with respect to their technological, economical and (eco)toxicological aspects. Hence, for the technologically promising ionic liquids more long-term-effect studies, the elucidation of environmental fate pathways and (bio)transformation as well as bioaccumulation studies are necessary to reduce the existing uncertainties in their hazard assessment.

In light of the data presented above and the necessity to fulfill several research needs concerning the ecotoxicological hazard assessment of several ionic liquids (ILs) exhibiting high interest for the large-scale technological applications the main aims of the present work were defined as follows:

- to propose and implement an useful tool for (eco)-toxicity investigations at the level of populations and communities (microcosm scale) filling out the levels of a flexible biological test battery
- to follow and understand the impact of selected ILs on microcosm scale
- to gain useful data about acute, sublethal and chronic effects of the selected ILs on biological organisms
- to generate a standard bioconcentration study with selected ILs
- to provide information about the bioaccumulation of the selected ILs
- to gather knowledge about the biomagnification potential of the selected ILs
- to reduce the uncertainties within the ecotoxicological risk profile of the selected ILs

It is obvious that this work does not intend to handle in an exhaustive manner the previously mentioned tasks but to contribute to the need of knowledge stated in the specialized literature in this field.

2. Theoretical considerations

2.1. Rational for selection of ionic liquids tested

Owing to their nomination by the United States National Toxicology Program (NTP) for toxicological testing based on their widespread interest as possible alternatives to organic solvents (Masten *et al.*, 2004) the four ionic liquids selected to be tested in the present work are (see Figure 3): 1-ethyl-3-methyl-1H-imidazolium chloride (**IM12Cl**), 1-butyl-3-methyl-1H-imidazolium chloride (**IM14Cl**), 1-ethylpyridinium chloride (**Py2Cl**), 1-butylpyridinium chloride (**Py4Cl**). These structures were previously investigated in studies on molecular and cellular level (e.g. Ranke *et al.*, 2003; Stock *et al.*, 2004; Stepnowski *et al.*, 2004; Matsumoto *et al.*, 2004; Skladanowski *et al.*, 2005; Docherty, Kulpa, 2005; Stolte *et al.*, 2006). By maintaining the anion (chloride) and the head group (*i.e.* imidazolium and pyridinium) the side chain effect can be probed.

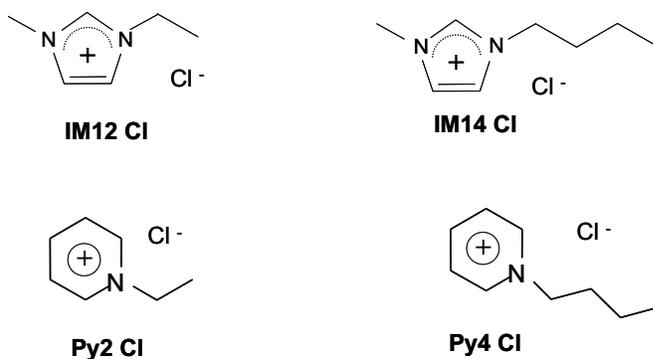


Figure 3. Ionic liquids selected to be tested in the present work

2.2. Toxicity tests at mesocosm and microcosm level

Mesocosms have been used in aquatic ecotoxicology for approximately 20 years and were sometimes claimed to be essential tools, especially for regulatory purposes. The term *aquatic mesocosm* describes indoor and outdoor artificial streams or experimental ponds and enclosures. The originality of mesocosms is mainly based on the combination of ecological realism, achieved by introduction of the basic components of natural ecosystems, and facilitated access to a number of physicochemical, biological, and toxicological parameters that can be controlled to some extent. This characteristic determines various features of the systems such as the minimal size required, initial physicochemical and biological composition, or choice of model species for ecotoxicological investigations. The choice of the experimental design should be based on the objectives of the study rather than

on theoretical considerations (Caquet *et al.*, 2000).

The use of mesocosms refines the classical methods of ecotoxicological risk assessment because mesocosms provide conditions for a better understanding of environmentally relevant effects of chemicals. They make it possible to assess effects of contaminants by looking at the parts (individuals, populations, communities) and the whole (ecosystems) simultaneously. Ecotoxicological investigations in mesocosms do not entirely replace the use of laboratory animals. However, they allow tests to be performed on species that are not of major societal concern, but which play key roles in the structure and function of ecosystems. In this respect, mesocosms allow nondestructive measurements of integrated endpoints. They also appear as potent tools to predict changes at the highest levels of organization (population, community, and ecosystem) from measurements of individual endpoints. However, after a period of extensive use, regulatory studies using large-scale mesocosms were more or less abandoned at the beginning of the 1990s, mainly because their cost-effectiveness was questionable (Caquet *et al.*, 2000).

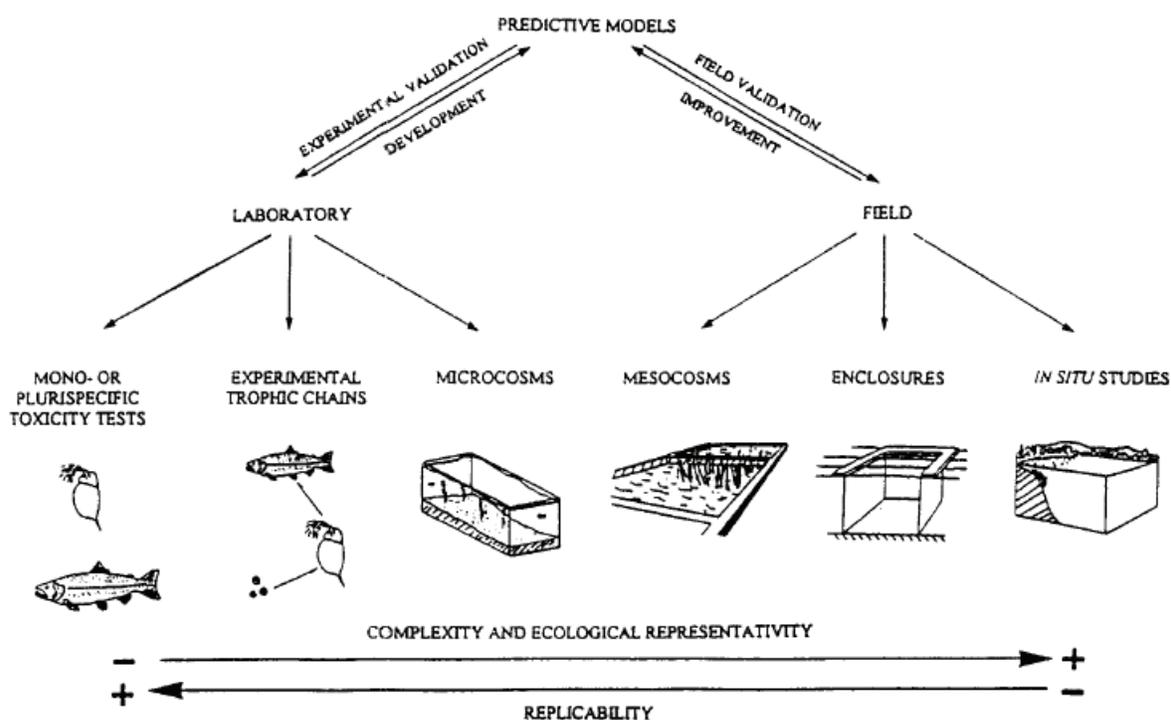


Figure 4. Schematic representation of methodologies and complementary approaches used in environmental toxicology and ecotoxicology (Caquet *et al.*, 1996).

Mesocosms are considered as an intermediate level of study between laboratory and field (Figure 4). These offer more realistic ecological conditions than laboratory tests and allow simultaneous studies on the fate and biological effects of pollutants (Odum, 1984; Cairns, 1988; Crossland, 1994). Because many biological characteristics of such ecosystems depend on their size it was suggested to characterize experimental devices according to their water volume. It was therefore proposed that the term “mesocosm” should be used for

artificial ponds bigger than 15m³ (SETAC-Europe, 1992; Heimbach, 1994). However this limit makes no reference to relevant ecological parameters such as stability and self-sustainability. The term mesocosm would therefore better characterize artificial ecosystems placed under natural environmental (climatic) conditions, which display sufficient complexity and stability to be self-sustainable (Caquet *et al.*, 1996).

Realism, representativity, and replicability of mesocosms are critical for evaluating their usefulness in both risk and impact assessment procedures. Each natural ecosystem is unique because its structure and function mainly depend on local factors. Therefore, there is a conceptual opposition between realism and replicability when applied to mesocosms. Considering the objectives of most mesocosm studies, replicability should be preferred to realism. Replicability may be achieved, in part, by a relative simplification of the systems (Caquet *et al.*, 2000).

Microcosms started to gain considerable interest as predictors of transport, transformation and fate of potentially toxic organics in aquatic systems. Generic microcosms of varying complexity can serve as useful tools for studying partitioning and kinetics of organic chemicals. Microcosms facilitate determination of mass balances and recovery of transformation products for further study (Rodgers *et al.*, 1983).

Depending upon the question or the hypothesis addressed, microcosm construction may range from simple to complex with one to several compartments. Through careful microcosm design, processes controlling the fate of a particular chemical in nature may be isolated and examined in detail. Fates of a variety of chemicals can be compared and contrasted efficiently in replicate microcosms. Such simplified systems can provide feedback information for theoretical model testing and validation (Rodgers *et al.*, 1983).

Microcosm studies can be indoor or outdoor and the objectives and general conduct can be similar for both types of model ecosystems.

Model ecosystems (microcosms) containing water, sediment, and communities of plants and invertebrates from natural ponds can be established in aquaria in the laboratory, and can be used to measure higher-level ecological effects of chemicals. Effects on phytoplankton, periphyton, zooplankton, or macroinvertebrates can be measured, depending on the objectives of the investigation. The results may help the registrant in two ways:

- First, it is usually found that aquatic populations and communities can tolerate chemical exposures higher than the LD₅₀ or NOEC (No Observed Effect Concentration = the highest concentration of a substance – usually in water – which did not result in an observable effect on the tested species) of sensitive test species, due to factors such as population recovery and replacement of species. Microcosm results can therefore be used to justify a higher PNEC (Predicted No Effect Concentration = predicted concentration of a chemical in environment at which no effect should be observed)
- Second, results of microcosm studies are considered by most regulatory authorities to be more representative of natural conditions than results of conventional toxicity tests

2.2.1. Microcosm toxicity studies – state of the art

The up to date available literature dealing with toxicity tests performed at microcosm level has been scrutinized. A brief review is presented based on the type of microcosm (in terms of location), the different type of organisms and experimental time employed in the evocated investigations and the class of chemical substances which have been tested so far in higher-tier approaches within environmental risk assessment. More details about the mentioned studies are presented in the *Appendix*.

2.2.1.1. Indoor microcosms

Indoor microcosm studies are intended to measure chemical effects on populations and communities under simulated natural conditions.

Scrutinizing the open literature dealing with hazard and risk assessment of various classes of chemicals at microcosm (indoor as well as outdoor) level it is evident that the major concern is posed by pesticides. They have been assessed using: i) aquatic indoor microcosm (Soursa, Fisher, 1986; Cedergreen *et al.*, 2004; Coutellec *et al.*, 2008) including phototrophic flagellates and predatory ciliates for 13 days (Liebig *et al.*, 2008); ii) freshwater microcosms (Flum, Shannon, 1987; Leeuwangh *et al.*, 1994) hosting zooplankton and phytoplankton, periphyton, macroinvertebrates, macrophytes for 14 weeks (Cuppen *et al.*, 2002), 13 weeks (van Wijngaarden *et al.*, 2004) or 4 weeks (Daam, Van den Brink, 2007); iii) artificial indoor streams comprising simple lotic food web for 72 days (Brust *et al.*, 2001); iv) natural soil microcosms including earthworms for 5 weeks (Reinecke, S., Reinecke, A., 2007); v) integrated soil microcosm incorporating earthworms, enchytraeids, and microarthropods (Burrows, Edwards, 2004).

The risk assessment of heavy metals ions was also widely investigated at microcosm level. Studies have been carried out for 6 weeks employing natural biofilms containing diatom assemblages (Moris *et al.*, 2008), for 30-32 days on meiofauna and nematode communities (Millward *et al.*, 2001; Gyedu-Ababio, Baird, 2006; Hedfi *et al.*, 2007), for 20 days on sediments hosting polychaete worms (Lee J.S., Lee J.H., 2005). The influence of heavy metals ions on crustaceans (Gagneten, Vila, 2001), flagellate alga *Euglena gracilis*, ciliate protozoan *Tetrahymena thermophila* and the bacterium *Escherichia coli* (Fuma *et al.*, 2003) was also assessed.

Hydrocarbons were investigated in: i) a multiple component laboratory-scaled microcosm containing water, sediment (soil + microbiota), plants (aquatic macrophytes and algae), and animals (zooplanktonic and zoobenthic invertebrates) (Johnson, Romanenko, 1989); ii) freshwater-marsh-microcosms (including crustaceans and fishes) (Bhattacharyya *et al.*, 2003); iii) soil microcosms comprising fungi (Mollea *et al.*, 2005).

Natural marine sediment, hosting indigenous microorganism has been used for 11 weeks in a microcosm study by Näslund *et al.* (2008) to assess pharmaceuticals effects in the system. The same class of chemicals was investigated in a 7 weeks test using a microcosm containing *Folsomia candida* (springtails) (Noël *et al.*, 2006).

Mohamed and Hussein (2006) conducted a microcosm investigation to study the depuration of hepatotoxin in tilapia fish (*Oreochromis niloticus*). Closed soil microcosm comprising earthworms (*Peryonix excavatus*, *Eisenia andrei*) was used by An (2005) to assess volatile organic compounds (methyl *tert*-butyl ether) in a short term (72 hours) exposure test. Blue mussels, *Mytilus edulis*, were exposed for 4 days to antifouling biocides in a seawater microcosm (Devier *et al.*, 2003).

2.2.1.2. Outdoor microcosms

Pesticides were broadly investigated at outdoor microcosm level, too. The employed test systems comprised: i) phytoplankton and periphyton for 16 weeks (Hense *et al.*, 2003) and 11 months (Rand *et al.*, 2001) tests; ii) freshwater invertebrates in a 122 days experiment (van Wijngaarden *et al.*, 2009) and an 8 weeks (Scott, Kaushik, 2000) test including Culicidae larvae as well iii) a wide array of freshwater taxa (*i.e.* macroinvertebrates, zooplankton, phytoplankton, macrophytes) in an 11 months test (Roessink *et al.*, 2006 a,b) and an 86 days test using additionally fishes (Rand, 2004); iv) natural marine microbial communities (Stachowski-Haberkorn *et al.*, 2008a) in a 7 days field experiment; v) in-situ field microcosm hosting oyster spat *Crassostrea gigas* (Stachowski-Haberkorn *et al.*, 2008b) in a 13 days experiment; vi) a plankton-dominated microcosm (Daam *et al.*, 2009) and vii) a mesotrophic and eutrophic ditch microcosm (Roessink *et al.*, 2005).

Using field and stream microcosms including macroinvertebrates (Ephemeroptera, Plecoptera, Trichoptera) Clark and Clements (2006) followed the repercussion of heavy metals presence in the ecosystem. For the same purpose Bahrndorff *et al.* (2006) used for 10 weeks microcosms comprising 27 species of chironomids (Diptera). Furthermore, outdoor microcosm experiments (consisting of macroinvertebrates assemblages including Ephemeroptera, Plecoptera, Trichoptera, Chironomids taxa) were performed for 10 days by Kashian *et al.* (2004) and wetlands studies at microcosm scale for 62 days by Gillespie *et al.*, (1999).

Hydrocarbons effects on the ecosystem were investigated using outdoor microcosms as well. Mahmoudi *et al.* (2005) accomplished a 90 days test on a free living nematode community. Benthic salt marsh communities (including *e.g.* nematode, crustaceans and algal biomass) were used for 21 days (Carman *et al.*, 2000) and freshwater microcosms comprising zooplankton and phytoplankton for 83 days (Sibley *et al.*, 2001; 2004). Field-base microcosms hosting indigenous benthic macroinvertebrates (Anson *et al.*, 2008) and microcosms including rainbow trout (*Oncorhynchus mykiss*) (Karrow *et al.*, 2001) were also used for hydrocarbons' risk assessment.

Beyrem *et al.* (2007) designed a 90 days test to study the influence of a mixture (metal and hydrocarbon) on a free living nematode community.

The impact of surfactants on the surrounding environment was studied for 42 days (Hanson *et al.*, 2005) and for 285 days (Boudreau *et al.*, 2003) in outdoor microcosms comprising aquatic macrophytes and for 39 days (Oakes *et al.*, 2004) on fathead minnow (*Pimephales promelas*) under microcosm conditions. Using the same type of microcosm as

for surfactants Hanson and co-workers investigated also the effects induced by halogenated organic compounds under semi-natural field conditions (Hanson *et al.*, 2001; 2002a; 2002b).

Brain and co-workers (2004; 2005) employed aquatic microcosms under semi-field conditions to examine pharmaceuticals influence on macrophytes over a 35 days period. Antifouling biocides' effects were studied by Sargian *et al.* (2005) in a 5 days study on natural planktonic conglomeration including marine phytoplankton and bacteria.

2.2.1.3. Combined microcosm and field approach

Results obtained for pesticides hazard and risk assessment studies on pesticides in laboratory microcosm studies were compared with conclusions drawn from the field studies and proved to be consistent (George, Liber, 2007). Furthermore, the conclusions of two other comparative approaches emphasized that microcosm studies employing a field-relevant design may be linked successfully to field risk assessment results (Schulz *et al.*, 2002; Thiere and Schulz, 2004).

From the above presented mini-review on toxicity tests at microcosm level it can be concluded that the terminology “microcosm” is a very ample and flexible one in terms of type of organisms involved, dimension (*see* corresponding details in *Appendix*) and experimental time required. While the indoor/laboratory microcosms are used in shorter experimental set-ups (mostly expressed in weeks) subsuming shorter acclimatization periods the outdoor microcosms studies require much longer pre-treatment periods (*e.g.* between 4 weeks - Hense *et al.*, 2003 and 8 months - Roessink *et al.*, 2006 a,b) resulting in a considerable experimental effort.

Very rare in the previous studies the employed microcosm systems were standardized. Therefore in order to provide a higher level of reproducibility combined with an adequate experimental effort and dealing with relevant aspects at ecosystem level standardized multispecies test systems on laboratory scale are needed. This requirement is in conformity with the legislation in course *i.e.* German Chemical Law (ChemG) and especially the new European Guideline (REACH)(*see* subchapter 1.3.) demanding a set of ecotoxicological base data regarding effects and fate of chemicals (Slenzka *et al.*, 2008).

Even if microcosm studies are not specifically addressed in regulatory demands it is clear that such tests are necessary in order to obtain a risk assessment of the industrial chemicals as close to reality as possible. Such studies are recommended to be used (Campbell *et al.*, 1999) when planning mesocosms (ponds) which should be used according to the regulatory German law for plants protection EWG 91/414/EWG – as an example.

2.2.1.4. Industrial chemicals – toxicity tests at microcosm level

On the web site of The European chemical Substances Information System (ESIS) (<http://ecb.jrc.ec.europa.eu/esis/index.php?PGM=dat>) can be found an extract of data from the IUCLID -International Uniform Chemical Information Database - on high production volume chemicals reported by European Industry in the frame of the European existing chemicals risk assessment programme. This extract refers to 2604 substances out of 10400 total substances currently incorporated in IUCLID and includes also data on environmental fate and pathways, ecotoxicological or toxicological properties of the selected substances.

Furthermore a list of priority substances selected out of the 2604 is mentioned by the Umweltbundesamt (www.uba.de) as being in the course of the risk assessment carried out by the Member States. The first priority list comprises 42 substances. For these substances a literature screening on microcosm studies has been done.

Thus it was found that most of the studies involving microcosms tests with these substances refer to biodegradation investigations *e.g.*: i) aniline degradation in riverbed sediments (Wu *et al.*, 2007); ii) benzene degradation in microcosms filled with different solids (sand, lava, Amberlite XAD-7) (Herrmann *et al.*, 2008) or soil and groundwater microcosms (Burland *et al.*, 1999); iii) naphthalene biodegradation in soil microcosms (Mollea *et al.*, 2005, Miller *et al.*, 1997) or simulated microcosms in the presence and absence of indigenous microflora (Pathak *et al.*, 2009); iv) trichloroethylene degradation in groundwater microcosms (Timmis *et al.*, 1993; Hopkins *et al.*, 1993).

Only very few toxicity studies at microcosm level were reported.

To study effects of sub-lethal concentrations of dibutyl phthalate on *Escherichia coli* H10407 microcosms of sterile Chesapeake Bay water were used (Palmer *et al.*, 1984). The tests were lasting for 19 days.

Yount *et al.*, 1987 used generic mixed-flask microcosms to evaluate ecosystem responses to *aniline*. Toxicity was determined on both an acute and chronic basis using changes in ecosystem-level variables (pH and dissolved oxygen levels) as indicators of effect. The calculated toxicity values were compared with reported toxicity data on bacteria, algae, protozoa and Cladocera to evaluate the relative sensitivity of the method. The minimum effect concentrations determined were generally lower than reported literature values, suggesting that the method does not sacrifice sensitivity in providing an integrated picture of ecosystem-level effects.

The changes of the bacterial community within the soil after *acrylonitrile* addition were investigated by Baxter *et al.*, 2006.

The differential inhibitory response to toxicity of *phenolic compounds* on microbial processes in a groundwater-based system was studied (Wu *et al.*, 2006). Wu and coworkers prepared microcosms in 1-liter glass bottles with 1-inch Teflon gas-tight caps and equipped with both liquid and gas sampling ports to permit periodic sampling.

The literature screening showed that up to day no data on standardized microcosm studies of first priority industrial chemicals - as mentioned by Umweltbundesamt - were published.

2.2.2. The AquaHab[®] testsystem – pros and cons

As emphasized by Crane already in 1997 aquatic multispecies tests began to be increasingly used to assess the fate and effects of chemicals in Europe (Crane, 1997). The strong need of more relevant standardized systems on laboratory scale for the prospective risk assessment of chemicals and drugs in general was accentuated again by Slenzka *et al.*, 2008. This is a very important issue especially in the context of REACH demands which claim the elucidation of environmental fate pathways and (bio)transformation data as well as bioaccumulation potential of chemicals in order to reduce the uncertainties in their hazard assessment.

Therefore a closed aquatic multispecies system on laboratory scale (termed AquaHab[®]) initially designed by the company *OHB-System AG* (Bremen, Germany) based on a system for the space research was subsequently developed for the ecotoxicological risk assessment of the fate and the effect of a chemical (Slenzka *et al.*, 2001; 2003; Dünne *et al.*, 2003, Slenzka *et al.*, 2008). The three years lasting development, verification and test phase showed the general suitability of AquaHab[®] for ecotoxicological purposes in general and clarified its benefits in contrast to other complementary test methods (Slenzka *et al.*, 2008).

The system consists of a biological and a control unit (*see* Figure 5A). The biological unit (A) comprises organisms representing different trophic levels (*see* Table IV): i) bacteria (nitrifying and denitrifying species) as destruents; ii) plants (*Ceratophyllum demersum*, hornweed – possessing an extraordinary nitrate absorbance ability) as producers; iii) gastropods (*Biomphalaria glabrata*) representing primary consumers – graze algae on plant surface; iv) fishes (*Oryzias latipes* – model species in ecotoxicology) as secondary consumers. Crustaceans (*Hyalella azteca* – commonly used species in ecotoxicology) act as saprophagous organisms. They feed on decaying plant and animal feces. The gastropodous act as saprophagous as well feeding on decaying fishes. The ecotoxicological relevance of the organisms is shown in several papers, as *e.g.* Cohen *et al.* (1994) and Toussaint *et al.* (2001) for *Oryzias latipes*, Day-Kristin *et al.* (1998) for *Hyalella azteca*, Furushima *et al.* (1991) and Jurberg *et al.* (1995) for *Biomphalaria glabrata* as well as Pflugmacher *et al.* (2000) for *Ceratophyllum demersum*.

The control unit (*see* Figure 5B) is designed for continuous data (*e.g.* oxygen and carbon dioxide concentration, pH, temperature, illumination) acquisition and storage as well as for the regulation of the oxygen concentration and the temperature.

The main assumed advantages of the AquaHab[®] system are (Dünne, 2005):

- its closure against external impacts allowing the balance of the tested chemicals and their metabolites;
- the reproducibility of results is assured by the control unit which allows a standardized test set-up;
- the possibility to perform longer test runs – chronic aspects can be investigated;
- the organisms from different trophic level parallel integrated in the system permit studies on relevant aspects of the ecosystem (*e.g.* bioaccumulation)

All the properties mentioned above are essential for the ecotoxicological risk assessment

of chemicals.

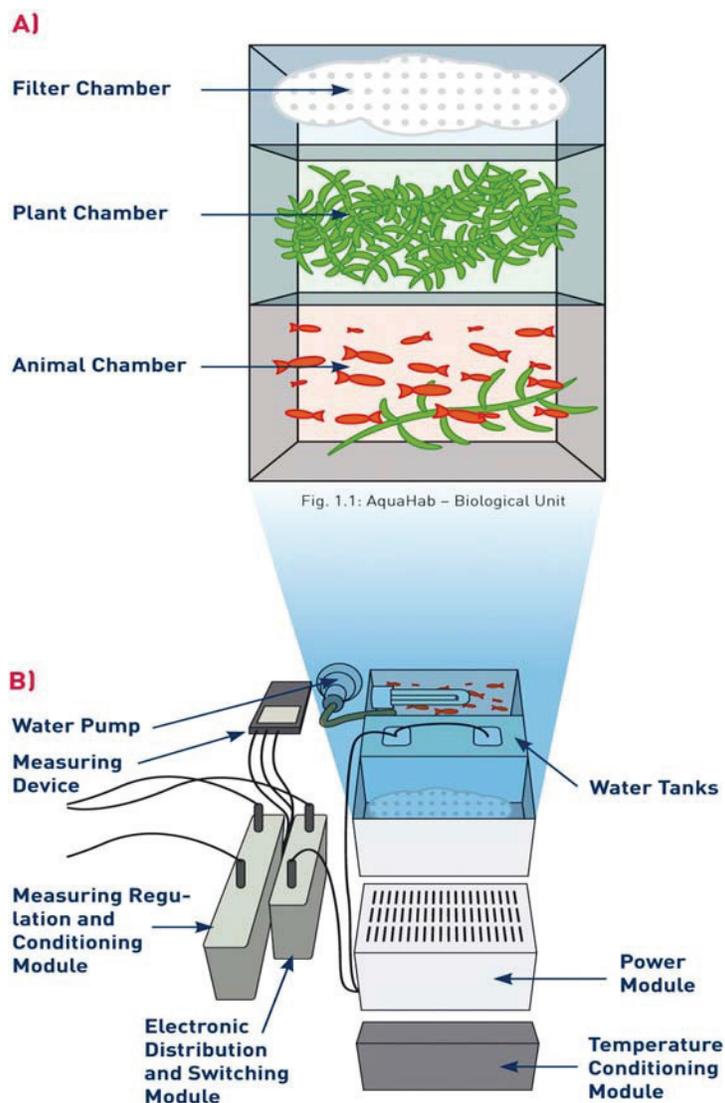
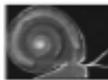


Figure 5. Aqua Hab[®] - system overview (Slenzka *et al.*, 2008)

Since AquaHab[®] is a complex test system studies performed using it are much more time and cost consuming than monospecies tests. But in the same time it delivers also much more data which are reproducible in contrast to outdoor systems as *e.g.* ponds or artificial lakes. Outdoor systems deliver relevant test results, however of limited reproducibility and amount of test run replicating in general. Therefore the careful selection of lead compounds out of the multitude of possible ones to be tested in AquaHab[®] becomes an important step.

Table IV. Species inhabiting the biological unit of the AquaHab[®] testsystem

Organism group	Represented trophic level	Species	Integrated Amount
Fish	Secondary Consumer	<i>Oryzias latipes</i> 	20 Individuals in Animal Chamber
Snails	Primary Consumer	<i>Biomphalaria glabrata</i> 	9 Individuals each in Animal and Plant Chamber
Plants	Producer	<i>Ceratophyllum demersum</i> 	5 g in Animal Chamber 25 g in Plant Chamber
Crustaceans	Saprophagous	<i>Hyalella azteca</i> 	180 Individuals in Animal Chamber 60 Individuals in Plant Chamber
Bacteria	Destruents	Nitrifying and denitrifying species	Not determined

Experimental options

- **Concentration monitoring via HPLC**

AquaHab[®] system permits frequent water sampling from the biological unit and thus the concentration of the tested substances in the system can be followed in time by HPLC analysis.

- **Metabolisation and chemical stability**

The analysis of the transformation products of the applied substance in the water and the biomass is possible in AquaHab[®]. Thus further assessment of observed effects is enabled, leading to a better understanding of the environmental behavior of the applied chemical. Furthermore a possible expected transformation of the applied chemical can be assessed in detail and the kind of transformation products (produced by organisms of different trophic levels and thus representing different biochemical and physiological tools) can be identified as well (Slenzka *et al.*, 2008).

- **Residue analysis**

Despite the limited available sample volume and biomass, residue analysis of the organisms enables the determination of very low concentration of the applied chemicals. This is of utmost importance in regard of a potential evaluation of low (chronic) effect concentration of chemicals, as well as for analyzing the effect on non-target organisms (Slenzka *et al.*, 2008).

- **Adsorption to system components**

The consisting materials of AquaHab[®] system were initially chosen to be chemically inert (Dünne *et al.*, 2003) preventing: i) production of presumable transformation products by a chemical reaction between the tested chemical and system consisting materials; ii) adsorption of the chemical at the system surfaces. Even so later studies performed in order to calibrate the system for its use in ecotoxicological tests in which the well-known biocide Irgarol[®] 1051 (2-methylthio-4-*tert*-butylamino-cyclopropylamino-6-(1,3,5-Triazin)) was applied demonstrated that the plastic material used in the system adsorbed a considerable amount of the tested chemical. Therefore during following investigations more inert consisting materials for the water loop had to be considered (Slenzka *et al.*, 2008). Still, performing adsorption pretest with each of the consisting materials of AquaHab[®] and the chemical of interest is a prerequisite.

- **Identification of subchronic and chronic effects, biomagnification, bioconcentration, bioaccumulation, persistence**

Being a self-sustainable test system, AquaHab[®] allows for long time test runs. Thus identification of *subchronic effects*- adverse effects on organisms which can be observed in tests lasting for less than 1/10 of the lifetime of the exposed organism (van Leeuwen, Hermens, 1995) - and *chronic effects* - adverse effects on organisms which recur frequently or develop slowly over an extended period, usually upon repeated or continuous exposure, sometimes lasting for the entire life of the exposed organism - is feasible.

Assessment of respiration and photosynthesis activities based on the oxygen curve is also possible in AquaHab[®] (Slenzka *et al.*, 2008). The *respiratory activity* is an integrated endpoint of organisms, populations and biocoenoses and is correlated to metabolic processes within them which are potentially being influenced by chemicals (Holler *et al.*, 1996). The respiratory activity is a well suited endpoint for the determination of toxicity (Maki, 1979; Haubenstricker, 1990). The *photosynthetic activity* indicates mainly effects of sublethal concentrations of photosynthesis inhibitors (Holler *et al.*, 1996).

Bioaccumulation (subsuming the *bioconcentration* from the surrounding media and the *biomagnification* potential along the food chain) and *persistence* (defining substances with high stabilities against biotic and abiotic transformations) as the key processes determining the fate of a chemical in the environment can also be studied in AquaHab[®].

2.3. Monospecies tests

Performing acute toxicity monospecies pretests with each of the species inhabiting the AquaHab[®] system is a prerequisite in order to find out the organisms resistance to the selected chemicals and to define an appropriate concentration to be tested in the more complex and time consuming AquaHab[®] tests.

The influence of each type of organism on the chemical concentration in water can be determined in monospecies tests via HPLC measurements of the water samples. By residue analysis of the organisms at the end of the acute toxicity test the bioconcentration potential of the chemical under investigation and possible biotransformation products can be identified.

Prior to monospecies test design a literature survey on already existing ionic liquids acute toxicity tests on aquatic plants, invertebrates and vertebrates has been done. An overview of the available literature existing at the beginning of this thesis is presented in Table V for those ionic liquids which have been selected to be investigated also in the present work and in Table XXII from *Appendix* for any other ionic liquids which have been tested on aquatic plants, invertebrates and vertebrates.

Table V. Acute toxicity of selected ionic liquids tested on different organisms groups

Organism Group	Specie	Ionic liquids tested	Method	Endpoint	Reference
Plants	<i>Selanastrum capricornutum</i> (algae)	IM14 Cl	Algal growth inhibition (72h)	48h EC ₅₀ = 38,5 mg/L = 220 µM	Wells and Combe, 2006
		Py4 Cl		48h EC ₅₀ = 63 mg/L = 366 µM	
Crustaceans	<i>Daphnia magna</i>	IM14 Cl	Acute toxicity (48h)	LC ₅₀ = 14,8 mg/L = 84 µM	Bernot <i>et al.</i> , 2005a
	<i>Daphnia magna</i>	IM14 Cl	Acute toxicity (48h)	EC ₅₀ = 6,5 mg/L = 37 µM	Wells and Combe, 2006
		Py4 Cl		EC ₅₀ = 20 mg/L = 116 µM	
		IM14 Cl	Immobilisation test (24h)	IC ₅₀ = 12,3 mg/L = 70,8 µM	Garcia <i>et al.</i> , 2005
Gastropodes (Snails)	<i>Physa acuta</i>	IM14 Br	Acute toxicity (96h -static)	LC ₅₀ = 229 mg/L = 1044 µM	Bernot <i>et al.</i> , 2005b

As presented in Table XXII from *Appendix* until the end of 2006 there were only eight publications dealing with monospecies toxicity tests on aquatic plants, invertebrates and vertebrates of the new class of chemical substances ionic liquids. The aquatic organisms used within these investigations were i) plants: the green algae *Oocystis submarina*, the diatom *Cyclotella meneghiniana* (Latała *et al.*, 2005), the algae *Selenastum capricornutum* (Wells, Combe, 2006) and the aquatic plant *Lemna minor* (Jastorff *et al.*, 2005); ii) invertebrates: the crustaceans *Daphnia magna* (Garcia *et al.*, 2005; Wells, Combe, 2006; Bernot *et al.*, 2005a; Couling *et al.*, 2006) and the gastropods *Physa acuta* (Bernot *et al.*, 2005b); iii) vertebrates: the zebra fishes *Danio rerio* (Pretti *et al.*, 2005). The considered endpoints are presented in the same table from *Appendix*.

The chemical structure (*see* Tables VI and VII) of the ionic liquids tested in acute toxicity monospecies test contained i) as head groups: predominant imidazolium and pyridinium but also aminopyridinium, piperidinopyridinium, pyrrolidinium, phosphonium and ammonium; ii) as side chain: ethyl, butyl, hexyl, octyl, benzyl, dodecyl, tetradecyl, hexadecyl, octadecyl, hydroxyethyl, stearyl, cocos, polyethylene- and polypropyleneglycol groups; iii) as anion: bromide, chloride, tetrafluoroborate, hexafluorophosphate, diethylphosphate, bis(trifluoromethylsulfonyl)imide, methylsulfate, tosylate, nitrate, trifluoromethane-sulfonate, and dicyanamide.

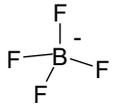
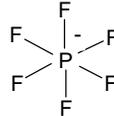
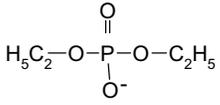
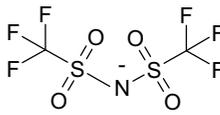
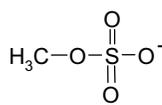
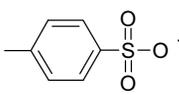
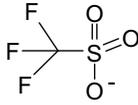
In another two reports acute toxicity data were published for pyridinium cationic surfactants (Grabinska-Sota, Kalka, 2003) and quaternary imidazolium and pyrrolidinium compounds (Demberelnyamba *et al.*, 2004) which contain similar structural elements as ionic liquids. These cationic surfactants were tested on the algae *Scenedesmus quadricauda*, the crustacean species *Daphnia magna* and the fish *Lebistes reticulatus*. The anion was always chloride and the side chains were dodecyl, dodecylthiomethyl, dodecylthiomethyl, methyl and dimethyl. The antimicrobial activity of the quaternary imidazolium and pyrrolidinium compounds (having no other structural elements as the one presented in Tables VI and VII) was tested on the algal bacterium *Chlorella regularis*.

Table VI. Name and structure of the cations used in tests on different organism groups

Symbol	Name	Structure (Headgroup)
H	Hydrogen	H ⁺
Na	Sodium	Na ⁺
IM12	1- <i>n</i> -Ethyl-3-methylimidazolium	
IM14	1- <i>n</i> -Butyl-3-methylimidazolium	
IM16	1- <i>n</i> -Hexyl-3-methylimidazolium	
IM18	1- <i>n</i> -Octyl-3-methylimidazolium	
IM14-2 Me	1- <i>n</i> -Butyl-2,3-dimethylimidazolium	
IM16-2 Me	1- <i>n</i> -Hexyl-2,3-dimethylimidazolium	
IM1-1Ph	1- <i>n</i> -Benzyl-3-methylimidazolium	
IM1-12	1- <i>n</i> -Dodecyl-3-methylimidazolium	
IM1-16	1- <i>n</i> -Hexadecyl-3-methylimidazolium	
IM1-18	1- <i>n</i> -Octadecyl-3-methylimidazolium	
Py4	1- <i>n</i> - Butylpyridinium	
Py6	1- <i>n</i> - Hexylpyridinium	
Py4-3 Me	1- <i>n</i> - Butyl-3-methylpyridinium	
Py6-3 Me	1- <i>n</i> - Hexyl-3methylpyridinium	
Py8-3 Me	1- <i>n</i> - Octyl-3methylpyridinium	
Py4-3Me-5Me	1- <i>n</i> - Butyl-3,5-dimethylpyridinium	
Py6-4(Me2N)	1- <i>n</i> - Hexyl-4-dimethylaminopyridinium	
Py6-3-Me-4(Me2N)	1- <i>n</i> - Hexyl-3-methyl-4-dimethylaminopyridinium	
Py6-4Pip	1- <i>n</i> - Hexyl-4-piperidinopyridinium	
Pyrr4	Butyl-pyrrolidinium	
P444-2	Tributyl-ethyl- phosphonium	
P4444	Tetrabutyl phosphonium	
P666-14	Trihexyl-tertradecyl- phosphonium	
N4444	Tetrabutyl ammonium	
N888-1	Trioctyl-methyl- ammonium	
EcoEng [®] 500 ^a	PEG-5 cocomonium methylsulfate R ₁ = -CH ₃ ; R ₂ ,R ₃ = -CH ₂ CH ₂ (OCH ₂ CH ₂) ₄ -OH; R ₄ =-CH ₃	
AMMOENG [®] 110 ^b	R ₁ ,R ₄ =-C ₂ H ₅ ; R ₂ = -CH ₂ CH ₂ (OCH ₂ CHCH ₃) _n OH; R ₃ =-CH ₃	
AMMOENG [®] 112 ^c	R ₁ ,R ₄ =-C ₂ H ₅ ; R ₂ = -CH ₂ CH ₂ (OCH ₂ CHCH ₃) _n OH; R ₃ =-(CH ₂) ₂ OH	
AMMOENG [®] 100 ^d	R ₁ = cocos; R ₂ ,R ₃ = -CH ₂ CH ₂ (OCH ₂ CH ₂) _n OH; R ₄ =-CH ₃	
AMMOENG [®] 130 ^c	R ₁ =-C ₂ H ₅ ; R ₂ ,R ₃ = stearyl; R ₄ =-CH ₃	

^{a, b, c, d, e} trade names; anion = ^{a, d} MeSO₄, ^{b, c} Cl⁻; ^c H₂PO₄⁻;

Table VII. Name and structure of the anions used in tests on different organism groups

Symbol	Name	Structure
Br	Bromide	Br^-
Cl	Chloride	Cl^-
BF_4	Tetrafluoroborate	
PF_6	Hexafluorophosphate	
$(\text{EtO})_2\text{PO}_2$	Diethylphosphate	
$\text{N}(\text{SO}_2\text{CF}_3)_2$	Bis(trifluoromethylsulfonyl)imide BTA	
MeSO_4	Methyl sulfate	
$\text{Me-4-C}_6\text{H}_4\text{SO}_3$	Tosylate	
NO_3	Nitrate	NO_3^-
CF_3SO_3	Trifluoromethanesulfonate	
$\text{N}(\text{CN})_2$	Dicyanamide	$(\text{CN})_2\text{N}^-$

3. Experimental Part

3.1. Material and methods

3.1.1. Materials

Chemicals and solvents

The tested ionic liquids were received as generous gifts from the companies Merck KGaA (Darmstadt, Germany): IM12Cl, IM14Cl, Py2Cl, Py4Cl, IM18Cl (1-methyl-3-octyl-1H-imidazolium chloride), Py8Cl (1-octylpyridinium chloride) and Iolitec (Denzlingen, Germany): IM12 BTA (1-ethyl-3-methylimidazolium bis(trifluoromethylsulfonyl)amide). Li BTA (lithium bis(trifluoromethylsulfonyl)amide, LiCl, acetonitrile, methanol and ethanol were purchased by Sigma-Aldrich Cooperation (Deisenhofen, Germany). H₃PO₄ and KH₂PO₄ salts were provided by Merck KGaA (Darmstadt, Germany). The water used for preparations was deionized water processed in UFT.

Rapid test-kits for water hardness and nitrite content determinations were purchased by Tetra GmbH (Melle, Germany) and Merck KGaA (Darmstadt, Germany) respectively.

Materials tested for adsorption

AquaHab[®] consisting materials used in preliminary tests are presented in Table VIII. Their structural formulae are shown in Table IX.

Table VIII. Materials used in adsorption preliminary tests

Nr.	Material	Form	Abbreviation	Dimmension ^a	Amount per sample
1.	Polycarbonate - Macrolone	Plate	PC	76,7*16,5;2mm thick Aside = 1265,55 mm ² Atotal = 2531 mm ²	1
2.	Polyurethane	Tube	PU	6,4*9,6 mm;70mm long Ainside = 1406,7 mm ² Aoutside = 2110 mm ² Atotal = 3516 mm ²	1
3.	Silicone	Mat/slice	Si	Dm: 50mm; 1mm thick Aside = 1962 mm ² Atotal = 3924 mm ²	1
4.	Polyester	Filter cotton	FW	Variable	5 g
5.	Polypropylene	Round	PP	Dm: 50mm; 1mm thick Aside = 1962 mm ² Atotal = 3924 mm ²	1
6.	Aluminium	Adhesive foil	AL	Dm: 50 mm Aside = 1952 mm ² Atotal = 3924 mm ²	1
7.	Polyoxymethylene	Connectors Sensors	POM	Non-geometrical form	1
8.	Polytetra fluoro-ethylene, Teflon	Adhesive foil	TF-F	72*27mm; 25µm thick Aside = 1944 mm ² Atotal = 3888 mm ²	2
9.	Polytetra fluoro-ethylene, Teflon	Plate	TF-P	Dm: 50mm; 1mm thick Aside = 1962 mm ² Atotal = 3924 mm ²	1
10.	Polytetra fluoro-ethylene, Teflon	Tube	TF-R	6*1*140mm Ainside = 2637,6 mm ² Aoutside = 439,6 mm ² Atotal = 3077,2 mm ²	2
11.	Stainless steel	Tube	ES	76,7*16,5;1mm thick Aside = 1265,55 mm ² Atotal = 2531 mm ²	2
12.	Zeolite	Filter Stones	FS	Variable	15 g
13.	Volcanic stones	Lava stones	LS	Variable	13 g

Table IX. Chemical structure of the consisting materials from AquaHab[®] system
(adapted from Dünne, 2005)

Material	Component in	Proportion within the whole AquaHab [®] Surface	Structure
Polycarbonate	Water tank Separation walls and filters	high	
Polyurethane	Tubes	high	
Zeolite	Filter stones	high	Mineral, volcanic origin from - SiO ₂ , TiO ₂ , Al ₂ O ₃ , Fe ₂ O ₃ , MnO, CaO, K ₂ O, CO ₂ , FeO, MgO, Na ₂ O,
Silicone	Wall and lattice adhesive	medium	undefined
Polyester	Filter cotton	high	undefined
Polypropylene	Lattice filter	medium	
Aluminium	Dim-out separation wall	medium	--
Polyoxy- methylene	Connectors Sensors	low	
Polytetra fluoro- ethylene, Teflon	Water pump Closures	low	
Stainless steel	Water pump Flow-through Cooling unit	very low	--
Lava stones	Filter Stones	high	undefined

Biological material (organisms)

The organisms used for the monospecies tests were hatched in the *OHB System AG Bremen* laboratory. They had the characteristics and the origin presented in Table X.

Table X. Source of the organisms used in monospecies tests and AquaHab[®] tests (adapted from Dünne, 2005)

Species	Life/condition stage	Original Source
<i>Ceratophyllum demersum</i>	scions	Working group Prof. Blüm Ruhr University, Bochum
<i>Hyaella azteca</i>	adults	B. Posseckert, Berlin
<i>Biomphalaria glabrata</i>	intact and brown shell	J. Marxen, working group Prof. Becker, Hamburg University
<i>Oryzias latipes</i> *	robust individuals	Dr. Scholz, UFZ Leipzig, Dresden University

* Hybrid between drR (Yamamoto, 1953) and Hd-rR.YHNI (Matsuda *et al.*, 1997)

Equipment

- **Adsorption tests**

The following equipment was used: 500ml beakers, large form from VWR International GmbH (Darmstadt, Germany), aluminium foil and GFL 3019 shaker (Burwedel, Germany).

- **Monospecies tests**

The monospecies tests were carried out in a controlled climate room (temperature and light conditions could be adjusted). For the different types of organisms appropriate glass recipients were used: 250 ml Erlenmeyer flasks, narrow neck, for plants tests, 50 ml beakers, large form, for crustacean tests, 1000ml beakers, large form, for snails and fish tests. All of them were obtained from VWR International GmbH (Darmstadt, Germany). Thermometer, pH-, conductivity- and oxygen-meter (InoLab Multilevel 1 WTW, Weilheim, Germany) were used as well as plastic transparent foil.

▪ AquaHab[®] Tests

The AquaHab[®] test-system (*see* subchapter 2.2.2. – Figure 5 overview) consists of:

- A) The biological unit: 8,2 L water tank (29 x 14,5 x 20,5 cm) hosting plants- *Ceratophyllum demersum*, crustaceans – *Hyalella azteca*, snails-*Biomphalaria glabrata* and fish- Japanese Medaka, *Oryzias latipes*). Separation walls divide the tank in an animal (50 % of the total volume), a plant chamber (30% of the total volume) as well as a filter chamber (20% of the total volume). They are optical separators but the water flow is not disturbed. The animal chamber integrates: 5g plant, 180 crustaceans, 9 snails and 20 fish. The plant chamber incorporates: 25g plant, 60 crustaceans and 9 snails. The filter chamber contains: 20g (dry weight) filter cotton and 160g (wet weight) filter stones. A bacterial community was added *via* a commercial available product. It contains several bacteria species including denitrifying ones, but details are kept confidential by the providing company.
- B) The control and support unit: a self suctioning water pump is the driving element of the water loop. Within the water loop is integrated the measuring, regulation and conditioning module. The following data are acquired by respective sensors and partly controlled via the data management unit (laptop PC):
- Temperature: $25 \pm 0.5^{\circ} \text{C}$
 - Plant chamber illumination: switched on/off at 4,5 and 6,5 mg/L oxygen according to the actual $[\text{O}_2]$
 - Animal chamber illumination: light/dark 16/8 h
 - O_2 concentrations, pH-value, temperature
 - The temperature conditioning module regulates the temperature in the required range.

▪ Sample preparation for residue analysis

1,5 and 2 ml cups, tips, Eppendorf-cup-homogenizer, bench top microcentrifuge, thermomixer from Eppendorf Company (Hamburg, Germany) were used for sample preparation. For plants pulverization a bead mill Pulverisette 6 from Fritsch GmbH (Idar-Oberstein, Germany) with five Sintercorundum balls was employed.

▪ HPLC system

The HPLC system used was a VWR Hitachi system containing the L-2130 HTA-pump, L-2130 degasser, L-2200 autosampler, L-2300 column oven, L-2450 diodearray-detector and EZChrom Elite software.

For the analysis of the ionic liquids cations in the water samples the Multospher 100 Si-5 μ 125 x 4,6mm column with a guard column (packing material: high quality and purity silicagel) obtained from CS-Chromatographie Service GmbH was used.

The ionic liquids cations in the biomass extracts were analyzed on a cation exchanger column (250/3 NUCLEOSIL 100-5 SA) with a guard column (packing material: spherical standard silica with benzene sulphonic acid modifications) purchased from Macherey-Nagel (Dürren, Germany).

3.1.2. Methods

All the test solutions were prepared using standardized water (pH =7, conductivity around 640 $\mu\text{S}/\text{cm}$). The pH was adjusted by CO_2 . The standardized water was prepared according to the international guidelines. It contains different mineral salts ($\text{CaCl}_2 \times 2 \text{H}_2\text{O}$; $\text{MgSO}_4 \times 2 \text{H}_2\text{O}$; NaHCO_3 ; KCl) to simulate fresh water and thus guaranteeing the best living conditions for the organisms.

3.1.2.1. Adsorption/ desorption/ saturation limit tests

The **adsorption tests** between the ILs and the surface of the AquaHab[®] consisting materials (*i.e.* polycarbonate, polyurethane, silicone, polyester, polypropylene, aluminium, polyoxymethylene, teflon, stainless steel, filter materials) were carried out as follows: 500 ml beakers were filled with 400 ml of standardized water containing each IL at a final concentration of 250 μM . Two IL mixtures were used: imidazolium mixture (IM12Cl + IM14Cl) and pyridinium mixture (Py2Cl + Py4Cl). The 0,01 M stock solutions for each IL and later on the two IL mixtures were prepared using standardized water. One piece of material (polycarbonate, polyurethane, silicone, polypropylene, aluminium, polyoxymethylene, teflon-plate) or two pieces of material (teflon-tube, teflon-adhesive foil, stainless steel) with defined surface area (*see* subchapter 3.1.1. – Table VIII) were first cleaned with a solvent mixture (*i.e.* pentane: hexane: methanol: t-buthylmethylether: iso-propanol 1:1:1:1:1) in order to remove any grease rests from the material surface. After drying they were placed in the beakers. In the case of zeolite 15 g dry weight (20 stones with similar dimensions) were put into each beaker. To test lava stones and filter cotton 13g (dry weight) and 5g (dry weight) respectively were used. Two replicates for each type of material and IL mixture were performed. The beakers were covered with aluminum foil and shaken continuously for one week. Water samples were taken at $t=0$ and after 22 hours, 96 hours and 7 days. The amount of ionic liquids in the water was quantified by HPLC analysis.

Desorption tests were accomplished with the filter stones, polypropylene and silicon materials at the end of the adsorption tests. The materials were taken out from the solution containing the IL mixtures and dried at room temperature. 500 ml beakers were filled with 400 ml standardized water and the materials were added. The beakers were covered with aluminium foil and shaken continuously for one week. Water samples were taken at $t=0$ and after 22 hours, 96 hours and 7 days. The amount of ionic liquids in the water was quantified by HPLC analysis.

For the **saturation limit tests** with filter stones 0,02M stock solutions of IM12Cl, IM14Cl, Py2Cl and Py4Cl were prepared. Mixtures of IM12Cl + IM14Cl and Py2Cl + Py4Cl at 500, 1000 and 10.000 μM final concentration in standardized water were used. Two replicates per each concentration and IL mixture were tested on 20g filter stones in 500ml beakers filled with 400 ml test solution.

3.1.2.2. Monospecies tests

3.1.2.2.1. Range finding tests

The selected ionic liquids were tested using different types of tests as presented in Table XII in species-depending concentrations (*see* Table XI). The monospecies tests were performed in pre-defined conditions of light and temperature. The uptake of ionic liquids by the tested organisms was determined *via* HPLC analysis of the test solution. At the end of the acute toxicity tests extracts from the tested organisms were prepared for residue analysis.

Table XI. Accomplished acute toxicity monospecies tests

Species	Test	Concentrations tested (μM)
<i>Ceratophyllum demersum</i> (plants)	Acute Toxicity – 168 hours	25; 125; 250; 500; 2500; 5000
<i>Hyaella azteca</i> (crustaceans)	Acute Toxicity – 96 hours – 168 hours	10; 25; 50; 125; 250; 500 50; 75; 100
<i>Biomphalaria glabrata</i> (snails)	Acute Toxicity – 48 hours – 192 hours	25; 125; 250; 500; 2500; 5000 500; 5000; 10000
<i>Oryzias latipes</i> (fishes)	Acute Toxicity – 96 hours – 192 hours	250; 500 5000

Table XII. Single species tests general table

Test Type	Exposure for 7 days	Acute 96 hours	Acute 24 hours	Acute 96 hours
Organisms	<i>Ceratophyllum demersum</i> (plant)	<i>Hyaella azteca</i> (crustaceans)	<i>Biomphalaria glabrata</i> (snails)	<i>Oryzias latipes</i> (fish)
Age of tests organisms	Scions	Adults	Mature	Juvenile
Dimensions/ Fresh weight of test organisms	5 cm 100 – 500 mg	2 - 4 mm 2 - 4,5 mg	5 -10 mm Ø 70 - 130 mg	2 - 3 cm 100 – 400 mg
Number of organisms per test vessel	1	1	3	1
Experimental Design				
Test vessel type and size	250 ml Erlenmeyer	50 ml beakers	1000 ml beaker	1000 ml beaker
Test solution volume	100 ml	40 ml	500 ml	500 ml
Number of replicates per tested concentration	3 6 controls	10 10 controls	2 6 controls	2 2 controls
Feeding regime	-	None	None	None
Test duration	168 ± 2 hours	96 hours	48 hours ^a	96 hours
Physical and Chemical Parameters	pH = 7-8 Water temperature: 21-23°C; Illumination rhythmus: light/dark 16/8h; Light intensity: 7/10-13 µmol/m ² /sec (17-21 µmol/m ² /sec for plants)			
Endpoint	Length Fresh weight Visual evaluation	Death	Inactivity Changes in colour	Death
References	Garg, Chandra, 1990 Fomin <i>et al.</i> , 2003	McDonald <i>et al.</i> , 1997 ASTM, 1989	Giovanelli <i>et al.</i> , 2002 WHO, 1983	OECD 203

^a period of exposition to the tested chemicals was 24 hours followed by another 24 hours recovery period

Ceratophyllum demersum (plants)

The 7 days toxicity test with *Ceratophyllum demersum* was adapted from the method proposed by [Formin et al., 2003](#) for *Myriophyllum aquaticum* (brazilian water milfoil - used in indoor and outdoor aquaria). Young shoots of *C. demersum* (5 cm and 100 - 500mg fresh weight) were used. For each IL tested and each concentration 3 scions were employed. In addition 6 scions were exposed to a control treatment (only standardized water).

Before starting the tests longer plant segments were relocated in 1L beakers filled with standardized water (six plants per beaker) for two days, under the test conditions: temperature (23-24°C), light intensity (17-21 $\mu\text{mol}/\text{m}^2/\text{sec}$) and day-night rhythm (16h/8h). Out of these plant segments 5 cm scions were cut and placed in 250 ml Erlenmeyer flasks filled with 100 ml IL test solutions (25; 125; 250; 500; 2500 and 5000 μM , obtained by dilution of 0,01M stock solutions with standardized water). The Erlenmeyer flasks were covered with transparent plastic foil.

Water samples for monitoring the IL concentration in each Erlenmeyer flask were taken at $t=0$, after 24, 96 hours and after 7 days and analyzed *via* HPLC. At the end of test the scions were harvested from the test solution and gently dabbed of with a tissue paper. The length, fresh weight and morphological characteristics (visual rating) were determined. Subsequently the young shoots were dried at 80°C for 2 hours and stored under dark and dry conditions for further residue analysis.

Hyalella azteca (crustaceans)

Acute toxicity tests conducted with *Hyalella azteca* were adapted from established methods ([ASTM, 1989](#)). Adults *H. azteca* (2-4 mm and 2- 4,5 mg fresh weight) were tested in 96-h static acute toxicity tests. 10 animals were exposed per concentration and ionic liquid tested. As controls 10 animals were tested in standardized water. No food was provided during the whole test period.

Two days prior to testing the appropriate number of mature amphipods were transferred with a plastic pipette into 1L beakers (around 30 individuals per beaker) filled with standardized water. The beakers were placed for acclimatization of the animals under the test conditions: temperature (23-24°C), light intensity (10-11 $\mu\text{mol}/\text{m}^2/\text{sec}$) and day-night rhythm (16h/8h). During the first preliminary tests it was observed that *H. azteca* exhibit cannibalism when 5 animals were placed in a single test vessel (effect reported by [Arthur, 1980](#) and [McDonald et al., 1997](#) as well). Thus further amphipods tests were performed in a single-animal per exposure-vessel system. 50 ml beakers filled with 40 ml test solution were used. The beakers were covered with transparent plastic foil.

Stock solutions (0,01M) for each ionic liquid tested were prepared in advance. The dilution factor between the tested concentration was 2 and 2,5. The six exposure levels were 10; 25; 50; 125; 250 and 500 μM .

The number of affected amphipods was monitored at 24 hours intervals. The effect criterion for *H. azteca* was death as defined by the American Society for Testing and materials (ASTM, 1989). The IL concentration in the test solutions was monitored *via* HPLC measurements. At the end of test the organisms were frozen at -80°C in 1,5 ml plastic cups for further residue analysis.

According to the same working protocol *H. azteca* were also exposed to 50, 75 and 100µM of IM12Cl for 7 days.

***Biomphalaria glabrata* (snails)**

The acute toxicity experiments with *Biomphalaria glabrata* were adapted from Giovanelli *et al.*, 2002, considering the World Health Organisation guideline for evaluation of a plant molluscicide (WHO, 1983). The tests were performed with 5 to 10 mm diameters *B. glabrata* adults (70- 130 mg fresh weight). For each concentration and type of ionic liquid six animals were exposed. Additionally six animals were exposed to control conditions (standardized water). No food was provided during the whole test period.

Before starting the test the snails were transferred in 1L beakers filled with standardized water (10 snails per beaker) for 2 days under the test conditions of temperature (23-24°C), light intensity (10-11 µmol/m²/sec) and day-night rhythm (16h/8h). After the acclimatization period the animals were relocated in 1000 ml beakers filled with 500 ml test solution (3 animals per beaker). The beakers were covered with transparent plastic foil.

The tested exposure levels (25; 125; 250; 500; 2500 and 5000µM) were prepared from stock solutions (0,01M). The period of exposure to the IL dilutions and controls was 24 hours. Thereafter the snails in each replicate were placed in 500 ml clean standardized water (without chemicals) for another 24 hours (recovery period). At the end of the first 24hours the changes in color and the number of mollusks withdrawn into their shells was recorded. If the snails remained inactive at the end of the recovery period (after 48 h) they were considered dead.

Water samples for monitoring the IL concentration in each beaker were taken at t =0, after 24 and 48 hours and analyzed *via* HPLC. At the end of test the animals were frozen at - 80°C in 2 ml plastic cups for further residue analysis.

The resistance observed for the *Biomphalaria glabrata* species during the first preliminary tests yielded in an extension of the exposure period to 8 days (no recovery period). These modified monospecies tests aimed to find out the snails' tolerance limit towards the ionic liquids under scrutiny. Three exposure levels were investigated: 500; 5000 and 10000µM (prepared out of 0,02M stock solutions).

Oryzias latipes (fish)

The 96 hours acute toxicity test with *Oryzias latipes* was adapted from OECD guideline Nr. 203. Juvenile *O. latipes* (2-3 cm and 0,1 – 0,4g fresh weight, males and females) were used for the experiments. For each concentration and ionic liquid tested 2 animals were exposed. Additionally 2 or 3 fish were exposed to a control treatment (only standardized water). No food was provided during the whole test period.

Two days prior to testing the fish were transferred into 1L beakers (2 fish per beaker) filled with standardized water. The beakers were placed for organisms' acclimatization under the test conditions of temperature (23-24°C), light intensity (7-13 $\mu\text{mol}/\text{m}^2/\text{sec}$) and day-night rhythmus (16h/8h). After 48 hours the fish were shifted in 1000 ml beakers filled with 500 ml test solution (1 fish per beaker). The beakers were not covered.

Stock solutions (0,01M) for each ionic liquid tested were prepared in advance. The two tested exposure levels were 250 and 500 μM .

Mortalities were recorded at 24, 48, 72 and 96 hours. The pH, temperature, dissolved oxygen, water hardness, nitrite content and conductivity of the test solutions in each beaker were monitored at 24 hours intervals and compared with the same parameters determined for the standardized water at $t=0$. Water samples for monitoring the IL concentration in each beaker were taken each 24 hours and analyzed *via* HPLC. At the end of test the animals were collected from the test solution, gently dabbed of with a tissue paper and the head was cut with a scalpel. They were stored at -80°C in 2 ml plastic cups for further residue analysis.

Oryzias latipes' resistance towards the selected ILs was investigated by exposing them according to the same working protocol also to 5000 μM ILs for 8 days.

3.1.2.2.2. Influence of the excretion process on the IM12Cl bioconcentration in fish

A 10 days experiment was designed to study the influence of the excretion process on the IM12Cl bioconcentration in *Oryzias latipes* (fish). Five organisms (2,7–3 cm and 0,12 – 0,19g fresh weight) were exposed to 5000 μM IM12Cl (test solution was prepared from a 0,02M stock solution) and two organisms as a control treatment to standardized water only. The exposure concentration for this test was chosen based on range finding test results: the 8 days acute toxicity test at a concentration of 5000 μM of IM12Cl exhibited a very good survival rate; furthermore the residue analysis of the fish from range finding tests proved that an exposure concentration of 5000 μM yielded in a good detectability by means of HPLC.

The acclimatization step and the conditions of temperature, light intensity and day-night rhythmus were the same as described for *Oryzias latipes* range finding tests (*see* 3.1.2.2.1). Each of the five fishes was placed in 1000 ml not covered beaker filled with 500 ml test solution. Each two days one fish was taken out from the test solution and prepared for

storage (*see* 3.1.2.2.1) and further residue analysis. The control fishes were processed at the end of test. The uptake of IM12Cl by the organisms was determined *via* HPLC analysis.

3.1.2.2.3. Influence of light on the IM12Cl bioconcentration in plants

The influence of light on the IM12Cl uptake by *Ceratophyllum demersum* was investigated in a two weeks experiment. Four scions (≈ 15 cm, $1,5 \pm 0,1$ g fresh weight) of *C. demersum* were transferred to a 500 ml Erlenmeyer flasks, narrow neck (one shoot per flask) filled with 250 ml of 300 μ M IM12Cl (obtained from 0,02M stock solution). The flasks were covered with plastic foil. Two of them were placed under dark condition (completely closed box) and two under constant light (light intensity 7-10 μ mol/m²/sec). The IM12Cl concentration in each vessel was monitored *via* HPLC by sampling the test solution each 48 hours.

After two weeks the scions were collected from the test solution and gently dabbed of with a tissue paper. The length, fresh weight and morphological and color characteristics (visual rating) were determined. Later on the young shoots were dried at 80°C for 2 hours and stored under dark and dry conditions for further residue analysis.

3.1.2.3. AquaHab[®] tests

3.1.2.3.1. Cation effect

AquaHab[®] test 1: 250 μ M IM12 Cl / 5 weeks (species were added one after the other)

The IM12Cl 250 μ M **biotest solution** was prepared from 20mM stock solution in an external covered aquarium two days before starting the test.

The AquaHab[®] **water tank** was prepared for the experiment as follows: the tank was cleaned with tap water; clean tap water was pumped through the whole system (three times 10-15 minutes each) and the flow rate was adjusted to 410 ml/min. The filter materials (lava stones and filter cotton) were washed a few times with clean tap water. 20 g (dry weight) filter cotton and 160 g (wet weight) lava stones were placed in the filter chamber. The biotest solution (pH= 7,6 and conductivity 703 μ S/cm) was introduced to the AquaHab[®] tank.

Adding the organisms: (a) for one week the system was running without any organisms; (b) in the second week 30 g *C. demersum* (fresh weight) was added to the system: 25 g in the plant chamber (three young shoots 57-67 cm and 5-11g fresh weight) and 5g in the animal chamber (one young shoot - 27cm); (c) in the third week 240 adult *H. azteca* (2-4

mm and 2- 4,5 mg fresh weight) were introduced (60 individuals in plant chamber and 180 individuals in animal chamber); (d) in the fourth week 18 snails, *B. glabrata* (6-9 mm diameter and 75-110mg fresh weight) were added to the system (9 organisms each to plant and animal chamber); (e) in the fifth week 20 fishes (*O. latipes*) - 2-2,8 cm and 95-195 mg fresh weight – were added to the animal chamber. No food was provided during the whole test period.

The **cation concentration** in the system was monitored *via* HPLC measurements during the whole test period: (a) first week (only the filter materials were in the system) samples were taken from each chamber at $t=0$, after 10 and 30min, and subsequently after 1, 2, 4, 8, 24, 48, 72 and 96 hours and after 7 days; (b) 2nd week (adding the plants): tap water was added to the system in order to substitute the test solution lost through water sampling; water samples were then taken from each chamber after 24, 96 hours and 7 days; (c) 3rd week (adding the crustaceans): water samples were taken (*see* (b)); (d) 4th week (adding the snails): water samples were taken (*see* (b)); (e) 5th week (adding the fishes): water samples were taken (*see* b). Because the plant in the animal chamber grew a lot it was partially removed (until the amount remained the initial one – 5g). The calculated deficit of IL was ca. 0,2%. The nitrite content was determined each 2-3 days after the fishes were added to the system.

Mortalities, changes in behavior (swimming, consuming), morphological changes were registered every 24 hours.

To control the quality of the sensors included in the control unit of the AquaHab[®] system at the end of test the flow rate, the pH, the oxygen and nitrite content and the temperature of the biotest solution were verified once more this time using new external sensors.

Organisms sampling and analysis: at the end of test period all plants and snails were prepared for the residue analysis; 5 of the fishes being exposed for 1 week to IM12Cl were prepared for residue analysis, 15 fishes were placed for “recovery” in an external aquarium filled with fresh (clean) water including plants and snails both not being exposed to IL before. Each two weeks 5 fishes from the “recovery” aquarium were taken out and processed for residue analysis. After 6 weeks all the snails from the “recovery” aquarium were prepared for residue analysis as well.

A hypothetic IM12Cl adsorption by the system materials was checked at the end of experiment. After removal of the biotest solution and raising the system three times with tap water, the water tank was filled again with tap water and the control unit was put in function. Water samples were collected at $t=0$, after 24 hours and 7 days.

A **desorption test from filter materials** was performed at the end of Aqua Hab[®] Test 1. The lava stones and filter cotton were first let to dry and then placed in 1000 ml beakers filled with fresh deionized water (500 ml in the beaker with lava stones and 1000 ml in the beaker with filter cotton). The beakers were covered with aluminum foil and shaken for 1 week. Water samples collected at $t=0$, after 24, 72 hours and after 7 days were analyzed *via* HPLC.

AquaHab[®] test 2 and 3: 250 μ M IM18Cl and 200 μ M Py8Cl / 2 weeks (no organisms)

The *biotest solutions* were prepared from stock solutions of ILs (20 mM) in two external covered aquaria two days before starting the test. Two AquaHab[®] systems were used in parallel: one for IM18Cl and one for Py8Cl.

Prior to the test the AquaHab[®] *water tanks* were prepared and the filter materials were introduced to the system as described for AquaHab[®] test 1. No organisms were used in these tests.

In both systems the *IM18⁺ and Py8⁺ concentration* was monitored *via* HPLC, water samples were taken from each chamber every 1-3 days.

At the end of tests *desorption tests* from filter materials were performed as described in AquaHab[®] test 1.

AquaHab[®] test 4: 100 μ M IM12Cl / 6 weeks (all organisms added at the beginning)

The IM12Cl 100 μ M *biotest solution* (pH= 7,5 and conductivity 645 μ S/cm) was prepared from a stock solution of IL (20 mM) in an external covered aquarium before starting the test.

The AquaHab[®] *water tank* was prepared as described for AquaHab[®] test 1.

Adding the organisms: (a) for one day the system was running without any organisms; (b) at the second day two young shoots of *C. demersum* were added to the system (one in the animal chamber and one in the plant chamber) and left there for one day in order to populate the system with bacteria (the two plant shoots originated from an aquarium populated with bacteria and were not rinsed with clean water before adding them to the AquaHab[®] system); (c) at the third day the two young shoots of *C. demersum* were removed from the AquaHab[®] system and the organisms were introduced to the system: 30g *C. demersum* (fresh weight) - 25g in the plant chamber (three young shoots 64-70 cm and 7-9g fresh weight) and 5g in the animal chamber (one young shoot – 50 cm), 240 adult *H. azteca* (2-4mm and 2- 4,5 mg fresh weight) - 60 individuals in the plant chamber and 180 individuals in the animal chamber, 18 snails, *B. glabrata* (6-8 mm diameter and 65-90mg fresh weight) - 9 organisms each in the plant and animal chamber, 20 fish (*O. latipes*) - 2,1-2,8 cm and 80-170 mg fresh weight – in the animal chamber. 10 mg of fish food (TetraMin Baby) was provided once per week during the whole test period.

The *IM12⁺ concentration* in the system was monitored *via* HPLC measurements during the whole test period. Every 2-3 days water samples from each chamber were taken. The *nitrite content* was determined each 2-3 days. Small quantities of tap water were added to the system once per week (when opening the aquarium for organisms' sampling) in order to substitute the lack of biotest solution caused by water sampling.

Mortalities, changes in behavior (swimming, consuming), morphological changes were

registered each 24 hours.

Organisms sampling and analysis: every week 3 fishes (different combinations of male and female) were taken out from the test system and processed to residue analysis. The plants and snails were analyzed at the end of test.

A **desorption test** from filter materials was performed at the end of test using the same working protocol as described for AquaHab[®] test 1.

3.1.2.3.2. Anion effect

AquaHab[®] test 5: 100 μ M IM12 BTA / 5 weeks (all organisms added from the beginning)

The IM12BTA 100 μ M **biotest solution** (pH= 7,5 and conductivity 638 μ S/cm) was prepared from a stock solution of IL (10 mM) in an external covered aquarium before starting the test.

The AquaHab[®] **water tank** was prepared as described for AquaHab[®] test 1.

Adding the organisms: all the organisms were introduced to the system at the beginning of the experiment according to the same working protocol used in AquaHab[®] test 4. The characteristics of the used organisms were: (a) the *C. demersum* young shoots – three in the plant chamber (33- 45 cm / 6-11,5 g fresh weight) and one in the animal chamber (25 cm / 5 g fresh weight); (b) the 240 amphipods, *H. azteca* - 2-4 mm / 2- 4,5 mg fresh weight; (c) the 18 snails, *B. glabrata* - 5-7 mm diameter / 50-60 mg fresh weight; (d) the 20 fishes (*O. latipes*) - 2- 3,3 cm / 70-260 mg fresh weight. Fish food - 10 mg TetraMin Baby once per week - was provided during the whole test period.

The **IM12⁺ and the nitrite concentration** were monitored as described for AquaHab[®] test 4. Small quantities of tap water were added to the system once per week (when opening the aquarium for organisms' sampling) in order to substitute the lack of biotest solution caused by water sampling.

Mortalities, changes in behavior (swimming, consuming), morphological changes were registered each 24 hours.

Organisms sampling and analysis: according to the AquaHab[®] test 4 working protocol.

A **desorption test** from filter materials was performed at the end of test using the same working protocol as for AquaHab[®] test 1.

3.1.2.4. Sample's preparation for residue analysis

▪ Sample's smashing/powdering

Plants - the dried scions from the monospecies tests were pulverized by introducing them to liquid nitrogen and subsequently crushing the material manually in a mortar. The *C. demersum* samples collected at the end of the AquaHab[®] tests were dried at 80°C for 24 hours and later on pulverized (2 times 3 minutes) in a bead mill with five Sintercorundum balls. The difference in the methods chosen to powder the samples was determined by the difference in the dried biomass quantity.

Crustaceans – the fresh biomass was smashed manually using an Eppendorf-Cup-homogenizer.

Snails – first for each snail shell and flesh were separated and subsequently the shells and flesh crushed in the same way as described for the crustaceans.

Fish – first the fresh biomass was cut into very small pieces using a scalpel and afterwards the same method as for crustaceans and snails was used.

▪ Extraction method

As presented in Figure 6 the IL cations were extracted from the smashed samples with 1 mL methanol. The extracts were stirred (1400 rpm) in thermomixer at room temperature for 24 h and centrifuged afterwards for 20 minutes (14,5 x 1000 rpm). The biomass was stored at -20°C. The supernatant was transferred to another Eppendorf cup and left under the hood for evaporation. The remaining residues containing the ILs were dissolved in a 250µL methanol-water mixture (1:1) each. The solution was stirred for 24h and centrifuged afterwards. The supernatant was analyzed *via* HPLC.

3.1.2.5. HPLC methods

The following optimized standard conditions were used throughout all experiments.

▪ For the water samples

For the determination of the IM12 cation in the water samples a Multospher 100 Si-5µ 125 x 4,6mm column with guard column (the same material) was used. The mobile phase consisted of 70% acetonitrile (HPLC grade) and 30% aqueous 10 mM KH₂PO₄ buffer (pH= 4,5) . A flow rate of 1 mL min⁻¹, a temperature of 40°C and a detection wavelength of 212 nm were employed.

▪ For the organisms' extracts

For the determination of the IM12 cation in the biomass samples a cation exchanger column (250/3 NUCLEOSIL 100-5 SA) with guard column (the same material) was utilized. The mobile phase consisted of 55% acetonitrile (HPLC grade) and 45% aqueous 35mM KH_2PO_4 / 3,9 mM H_3PO_4 buffer (pH =3,5). A flow rate of 0,7 mL min^{-1} , a temperature of 40°C and a detection wavelength of 212 nm were employed. The method was adapted from [Stolte *et al.*, 2007](#).

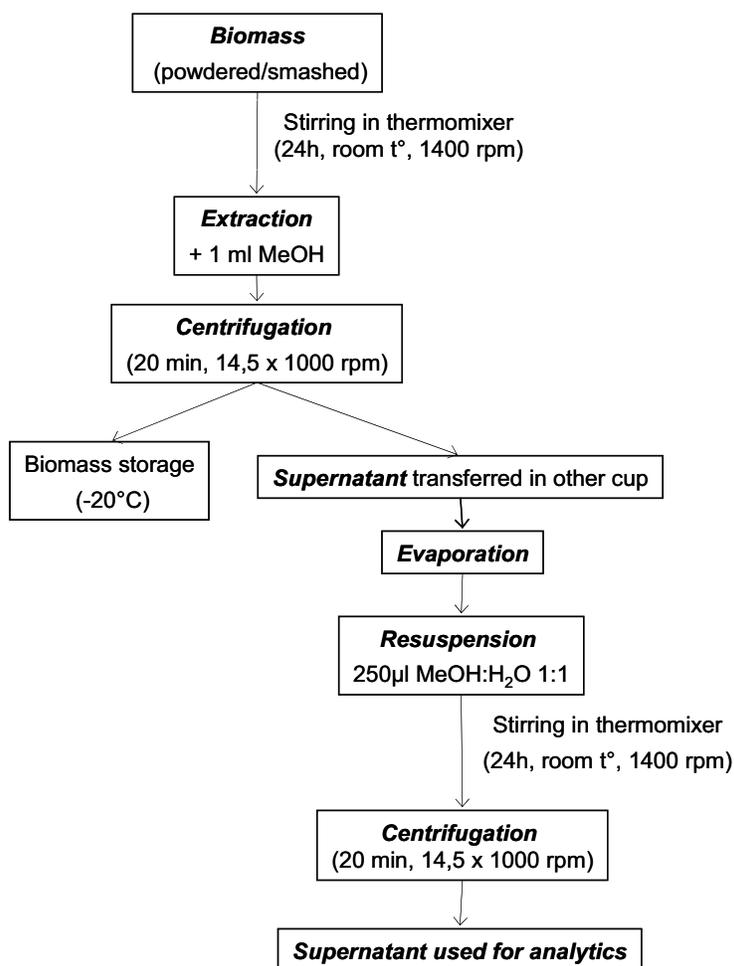


Figure 6. Flow-chart of the used extraction method

3.2. Results and Discussion

3.2.1. Adsorption studies

3.2.1.1. Adsorption tests

The adsorption experiments between the selected ionic liquids and the surface of the AquaHab[®] consisting materials (*i.e.* polycarbonate, polyurethane, silicone, polyester, polypropylene, aluminium, polyoxymethylene, teflon, stainless steel, filter materials) revealed as presented in Figure 9 that filter stones (zeolite) adsorb over 90% of the tested ILs at concentrations of 250 μ M. All the other materials from the AquaHab[®] system (*see* Figures 7 and 8 as well as Figures 29 to 36 from *Appendix*) do not adsorb the ILs of interest.

Figure 7. Adsorption of the selected ILs on polycarbonate (component in the tubes from AquaHab[®])

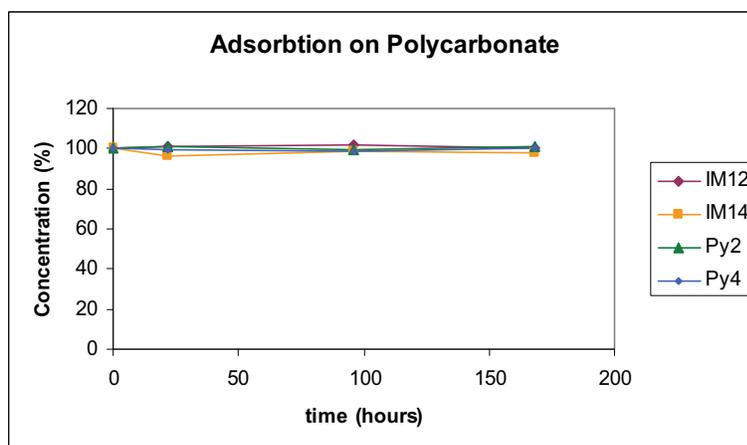


Figure 8. Adsorption of the selected ILs on polyurethane (component in the tubes from AquaHab[®])

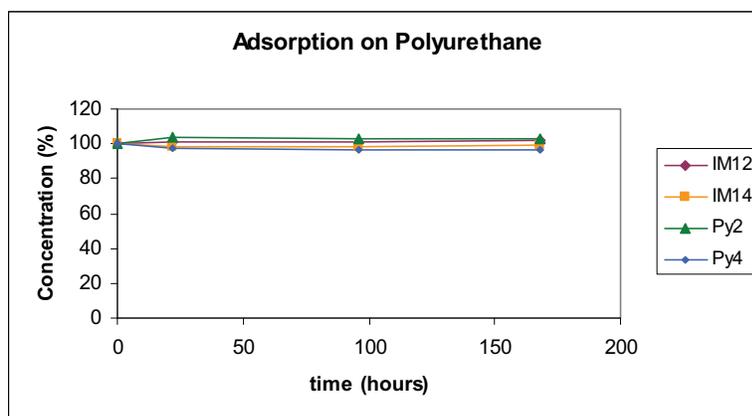
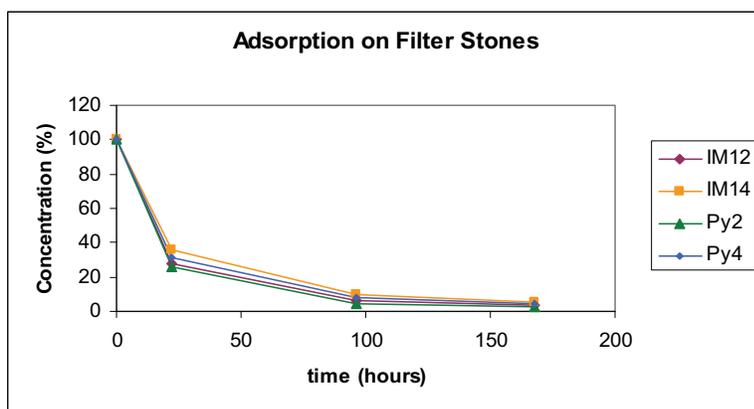
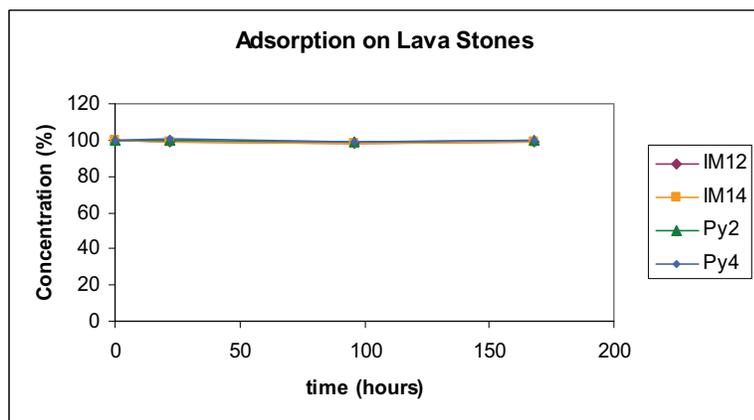


Figure 9. Adsorption of the selected ILs on filter stones (Zeolite)



Consequently adsorption studies were performed with lava filter stones which shown no adsorption for the selected ionic liquids as presented in Figure 10.

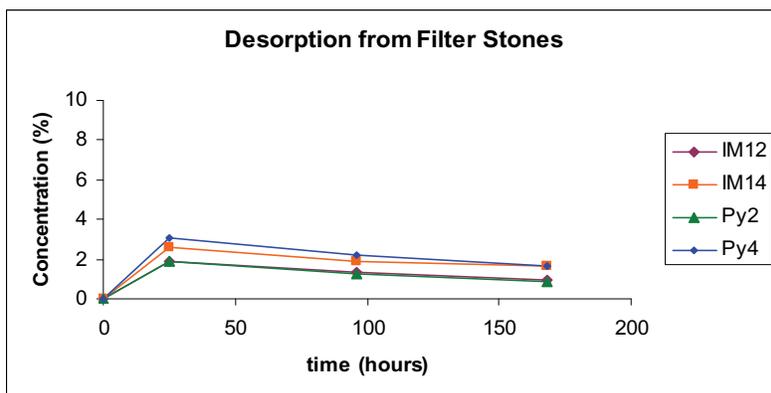
Figure 10. Adsorption of the selected ILs on lava stones (volcanic stones)



3.2.1.2. Desorption tests

As shown in Figure 11 the tests performed with the filter stones (zeolite) previously exposed to IM12Cl, IM14Cl, Py2Cl and Py4Cl indicated that only 1,5 to 3 % of the adsorbed IL's are also desorbed.

Figure 11. Desorption of the selected ILs from filter stones (Zeolite)



3.2.1.3. Saturation limit tests

Zeolite was tested for the saturation limit towards selected ILs. Figure 12 describes the limit of saturation for the filter stones as being over 10 mM for the ILs of interest.

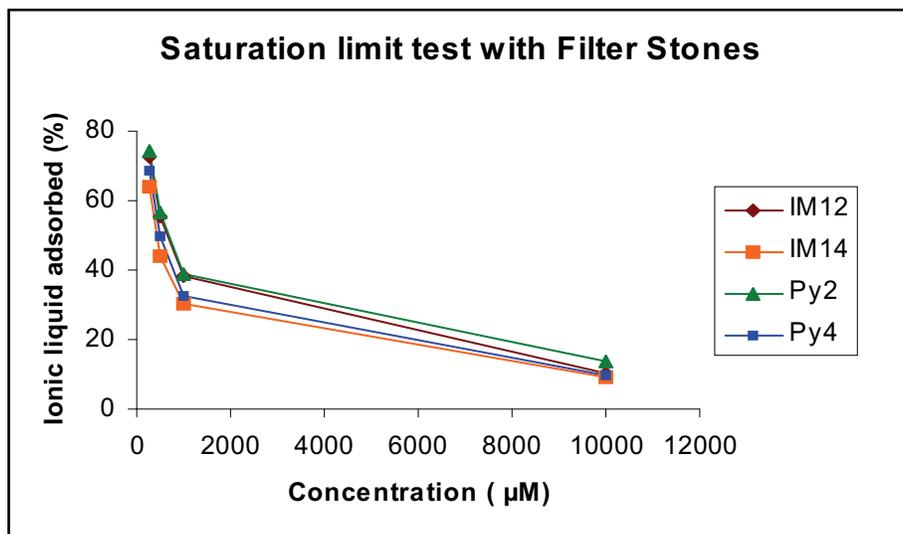


Figure 12. Saturation limit test for the adsorption of ILs on filter stones (Zeolite)

The adsorption/desorption/saturation limit tests revealed that none of the consisting materials from the AquaHab[®] system adsorb the ionic liquids of interest excepting the filter stones (zeolite). The percentage of selected ILs adsorbed by zeolite is very high (over 90%) and this process is not reversible. This phenomenon might be a consequence of zeolite's microporous structure that can bind by ion-ion interaction a wide variety of cations. Even if positive ions are rather not restrained by zeolite and can easily be exchanged for others in a contact solution the desorption tests with the IL's of interest proved zeolite to act as a "molecular sieve" (having ability to retain molecules based on ion-ion interactions and a size exclusion process) for IM12⁺, IM14⁺, Py2⁺ and Py4⁺. According to the saturation limit test a decrease in the amount of selected ILs in water takes place even at very high concentration (10mM) – which are less probably to ever occur in nature.

For a reliable test design it is very important that the concentration of IL added in the aquatic ecosystem remain constant in the absence of the organisms meaning there is no interaction/adsorption of the chemicals with/by the system. In this way, in the presence of organisms any decrease of chemicals concentration can be considered as an uptake of ILs by the organisms and/or plants.

Therefore after having tested another type of volcanic material and proving that this caused no decrease in the concentration of the ionic liquids studied in this thesis, lava filter stones were chosen to be further used in the AquaHab[®] testsystem.

3.2.2. Monospecies tests

3.2.2.1. Range finding tests

The concentration range which can be used in the optimized aquatic ecosystem AquaHab[®] was established by performing preliminary acute toxicity tests with each specie.

3.2.2.1.1. Cation effect

Ceratophyllum demersum (plants)

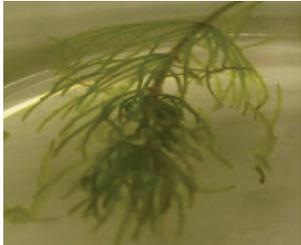
According to the visual evaluation no clear effect of the tested ILs could be observed at concentrations up to 500µM inclusive. The scions developed substantial morphological transformations when the test solution contained 2500µM and 5000µM ILs as shown in Table XIII and XIV while the six control scions remained unchanged. In the case of the tested imidazolium compounds the morphological changes appeared after 168 hours exposure to IM12Cl / 2500µM / 5000 µM and 96 hours exposure to IM14Cl / 2500µM / 5000µM (indication for *side chain effect*). There are slight differences in the morphology of plants exposed for 7 days to IM12Cl and IM14Cl (both 5000µM). In contrast, exposition to pyridinium ILs determined alteration in plants morphology already after 24 hours exposure to Py2Cl / 2500µM and Py4Cl / 5000µM (suggestion for *head group effect* but not a clear side chain effect).

The endpoints “*length*” and “*fresh weight*” couldn’t be analysed statistically because of the high variability displayed by each of the tested scions.

The sensitivity of *Ceratophyllum demersum* towards tested ILs increases as follows:

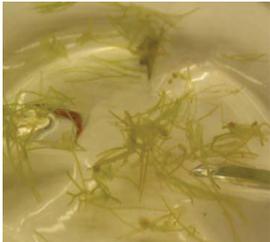
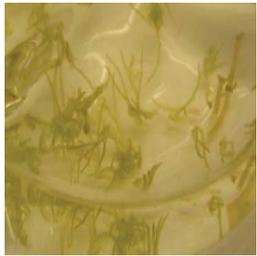
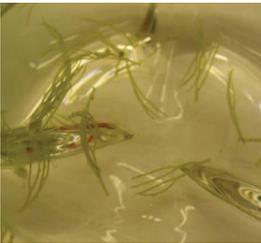
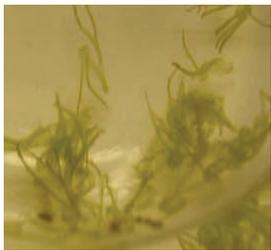


Table XIII. Acute toxicity tests: morphological changes on *Ceratophyllum demersum* exposed to high concentrations (2500 and 5000 μ M) of IM2Cl and IM14Cl

Tested IL	Conc. [μ M]	Exposure time		
		t= 0	t= 96 hours	t= 7 days
IM12Cl	2500		 + + +/- (*)	 + +/- -
	5000		 + + -	 + - -
IM14Cl	2500		 + + -	 + +/- -
	5000		 + - -	 - - -

(*) per each IL and concentration three replicates were tested; under each picture it is summarized how many scions: (a) did not exhibit any morphological transformations (+), (b) were only partially transformed (+/-), (c) shown drastic morphological changes (-).

Table XIV. Acute toxicity tests: morphological changes on *Ceratophyllum demersum* exposed to high concentrations (2500 and 5000 μ M) of Py2Cl and Py4Cl

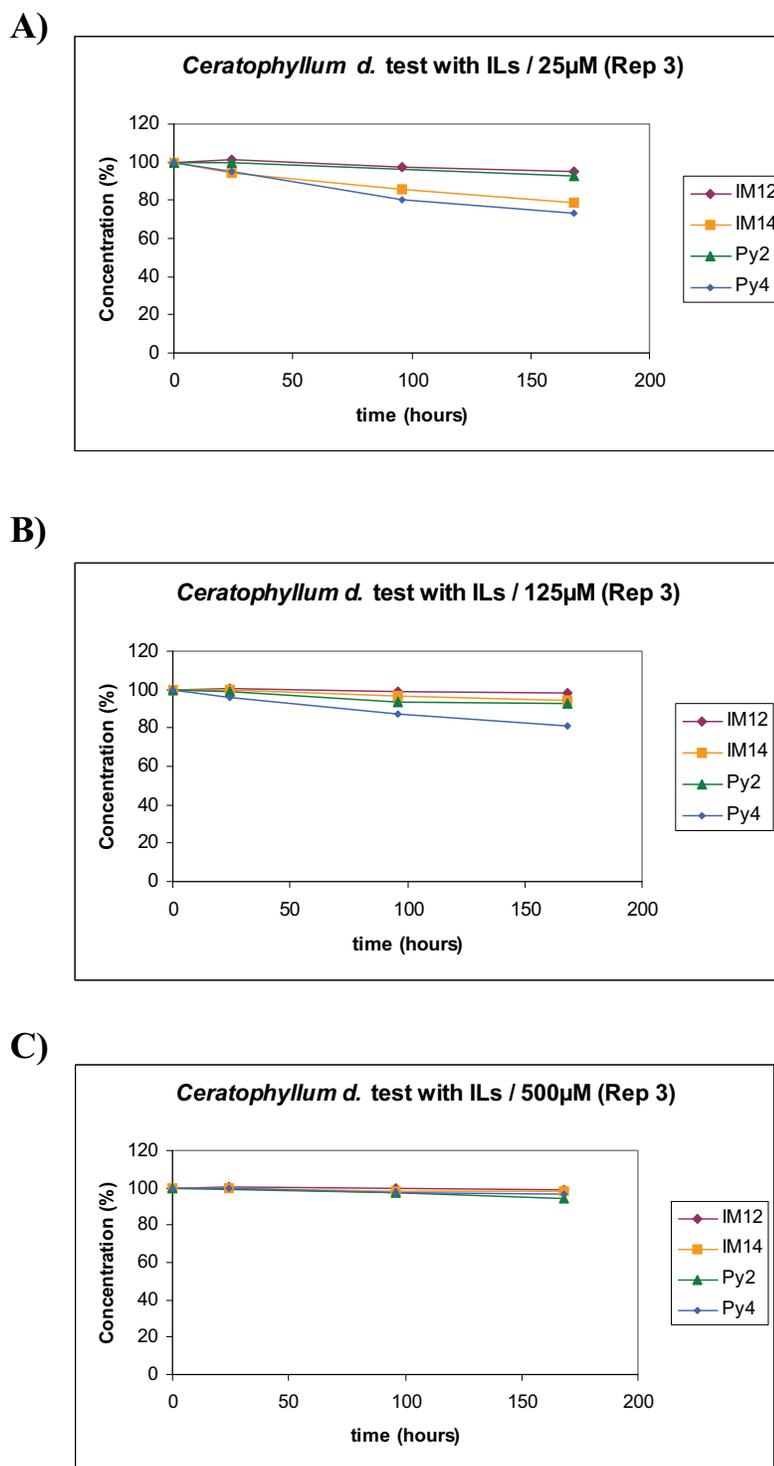
Tested IL	Conc. [μ M]	Exposure time		
		t= 0	t= 96 hours	t= 7 days
Py2Cl	2500		24 hours ^a  + +/- - ^b	 + - -
	5000		 - - -	 - - -
Py4Cl	2500		 - - -	 - - -
	5000		24 hours ^a  + - -	 - - -

^a ample morphological changes could be already observed after 24 hours exposure to ILs ;

^b per each IL and concentration three replicates were tested; under each picture it is summarized how many scions: (a) did not present any morphological transformations (+), (b) were only partially transformed (+/-), (c) shown drastic morphological changes (-).

The HPLC analysis of the test solution showed a slight concentration decrease in the tests with 25, 125 and 250 μ M ILs (*see* Figure 13). For tests with 500, 2500 and 5000 μ M IL the concentration of chemicals remained constant.

Figure 13. ILs concentration in the test solution while performing an acute toxicity test with *Ceratophyllum demersum*



***Hyaella azteca* (crustaceans)**

As indicated in Table XV the crustaceans started to die when they were exposed to IM12Cl at 250µM, to IM14Cl at 25µM and to Py2Cl and Py4Cl at 50 µM. After 96 hours exposure to IM12Cl at 250µM only 20% of the tested crustaceans survived and all of the 10 organisms treated with IM14Cl / 250µM were dead after 48 hours (evidence for a *side chain effect*). After 24 hours exposure to Py2Cl and Py4Cl (both 250µM) all the tested organisms were dead. The side chain effect for the tested pyridium compounds is suggested by the 50% surviving rate when organisms were treated with Py2Cl / 125µM compared with only 20% surviving rate in test with Py4Cl / 125µM.

When comparing effects of pyridium and imidazolium compounds with a side chain of four carbon atoms at 125µM the imidazolium compound indicated a higher toxicity (only 10% crustaceans survived after 24hours exposure to IM14Cl while 80% crustaceans exposed to Py4Cl survived after 24hours). For imidazolium and pyridinium compounds with a side chain of two carbon atoms the observed effects indicate a higher toxicity towards crustaceans for the pyridinium compounds (after 24 hours exposure to Py2Cl / 250µM all the crustaceans were dead while 30% of the organisms treated with IM12Cl survived). These observations are not an indication for a clear head group effect.

The sensitivity of *Hyaella azteca* towards selected ILs increases as follows:



Table XV. *Hyaella azteca* - number of surviving organisms during 96 hours acute toxicity tests (total organisms tested per IL and concentration were 10).

Conc. (µM)	Number of surviving organisms																
	IM12Cl				IM14Cl				Py2Cl				Py4Cl				
	Hours	24	48	72	96	24	48	72	96	24	48	72	96	24	48	72	96
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
25	10	10	10	10	10	8	8	6	10	10	10	10	10	10	10	10	10
50	10	10	10	10	10	9	9	9	10	9	9	8	10	10	9	9	
125	10	10	10	10	7	1	-	-	10	10	9	5	10	8	7	2	
250	9	7	5	2	5	-	-	-	10	-	-	-	9	-	-	-	
500	8	3	3	2	6	-	-	-	10	6	-	-	9	-	-	-	

* 20 organisms were used as controls and all of the survived until the end of test

***Biomphalaria glabrata* (snails)**

Throughout the 48 hours acute toxicity tests even at 5000 μ M concentration of any IL tested no effect on the snails could be observed. The 8 days acute toxicity test with 5000 μ M ILs revealed that the snails resist to such high IL concentrations for 4 days (shown in Table XVI). On the fifth day the snails started to show changes in their activity (*i.e.* very slow reactions when touched with a stick or lying down on the bottom of the test vessel). At the end of test 50% of the snails treated with IM12Cl /5000 μ M showed no visible effects. All organisms treated with IM14Cl / 5000 μ M developed inactivity while 17% of the snails exposed to Py2Cl and Py4Cl (both 5000 μ M) were not affected by the chemicals.

When exposed to 10.000 μ M ILs in case of IM14Cl, Py2Cl and Py4Cl the snails died already after 12 hours while 50% of the snails exposed to IM12Cl survived for 48 hours. After 120 hours all of them died.

The snails proved to be very resistant towards the IL entities tested. An attempt to compare their sensibility towards the tested compounds their acute toxicity increases as follows:

$$\text{IM12Cl} < \text{Py2Cl} \approx \text{Py4Cl} < \text{IM14Cl}$$

Table XVI. *Biomphalaria glabrata* - number of surviving organisms during 8 days (192 hours) acute toxicity tests (total organisms tested per IL and concentration were 6).

Hours Conc (μ M)	Number of surviving organisms							
	IM12Cl		IM14Cl		Py2Cl		Py4Cl	
	5000	10.000	5000	10.000	5000	10.000	5000	10.000
12	6	3	6	-	6	-	6	-
48	6	3	6	-	6	-	6	-
72	6	1	6	-	6	-	6	-
96	6	1	6	-	6	-	5	-
120	5	1	4	-	5	-	5	-
144	5	-	4	-	2	-	4	-
168	5	-	3	-	1	-	3	-
192	3	-	-	-	1	-	1	-

* 6 organisms were used as controls; all of them survived until the end of tests;

Oryzias latipes (fishes)

The 96 hours acute toxicity tests with 250 μM and 500 μM ILs exhibited no visible effects of the selected ionic liquids on fishes. They all remained fit until the end of the tests. During the 192 hours (8 days) acute toxicity test at a concentration of 5000 μM ILs it was observed that after 24 hours the organisms exposed to IM14Cl died. The same effect for Py4Cl could be observed after 72 hours. The organisms resisted to IM12Cl for 6 days, no lethal effect of Py2Cl was observed until the end of the test.

The sensitivity of *Oryzias latipes* towards high concentration of selected ILs increases as follows:

$$\text{IM12Cl} \approx \text{Py2Cl} < \text{Py4Cl} < \text{IM14Cl}$$

General discussion of the cation effect

An overview of the results obtained for different IL cation *via* monospecies tests with the four species inhabiting the AquaHab[®] system are presented in Table XVII.

Table XVII. The cation effect in 8 days monospecies tests - acute effects (time and concentration at which all or 50% of the organisms died or at which the plants developed ample morphological transformations); h = hours; d = days; n.t.= not tested

Organisms tested	Tested concentration [μM]															
	IM12Cl				IM14Cl				Py2Cl				Py4Cl			
	10.000	5000	2500	250	10.000	5000	2500	125	10.000	5000	2500	250	10.000	5000	2500	250
<i>Oryzias latipes</i> (fishes)	n.t.	50% in 6d	n.t.		n.t.	24 h	n.t.		n.t.		n.t.		n.t.	3-4 days	n.t.	
<i>Biomphalaria glabrata</i> (snails)	24 h	50% in 8d	n.t.		24 h	50% in 7d	n.t.		24 h	50% in 6d	n.t.		24 h	50% in 7d	n.t.	
<i>Hyalella azteca</i> (crustaceans)	n.t.	n.t.	n.t.	50% in 48 h	n.t.	n.t.	n.t.	48 h	n.t.	n.t.	n.t.	48 h	n.t.	n.t.	n.t.	48 h
<i>Ceratophyllum demersum</i> (plants)	n.t.	24 h	7 d		n.t.	96h	96h		n.t.	24h	24h		n.t.	24h	96h	

* No effect at concentrations were no specification is written

The results obtained within the monospecies test permitted the identification of those ionic liquids concentrations which produce a clear effect for a distinct IL cation.

While the crustaceans (*Hyalella azteca*) die after 24 hours at very low ILs concentrations (under 250µM for all the four tested ionic liquids), the snails (*Biomphalaria glabrata*) proved to be very resistant towards even high ILs concentrations and long exposure time (*i.e.* 5000 µM of a selected IL / 8 days test). For plants (*Ceratophyllum demersum*) and fishes (*Oryzias latipes*) effects could be observed only at very high concentrations (*i.e.* 2500 – 5000 µM selected ILs).

Putting together the sensitivity of the four species from AquaHab[®] testsystem towards the selected ionic liquids could be expressed as decreasing in the following order:

Crustaceans >>> Plants >> Fishes > Snails

only in the case of Py2Cl the fishes seems to be slightly more resistant than the snails (Py2Cl: Crustaceans >>> Plants >> Snails > Fishes).

The fact that for a defined concentration of a selected IL no effect occurs on species like *Biomphalaria glabrata* or *Oryzias latipes* but all the crustaceans (*Hyalella azteca*) are already dead made it difficult to decide on the start concentration to be tested in the AquaHab[®] testsystem. We choose IM12Cl, which exhibited in the monospecies tests the lowest toxicity to be the first IL entity to be tested in the closed aquatic ecosystem at a concentration of 250 µM.

While performing the monospecies tests the concentration of ILs in the test solution was controlled after each 24 hours. For the plant tests a slight decrease of the concentration could be observed up to 250µM. During the testing times of the crustaceans, snails and fishes the concentration of the ILs in test solution remained constant. This might be due to the very small amount of biomass present in these tests. An uptake of ILs by the organisms was under the HPLC detection limit.

In the case of *Biomphalaria glabrata* the acute toxicity test method adapted from Giovanelli *et al.*, 2002 (24 hours exposure to chemicals plus 24 hours recovery period) may have been too short as to permit observations of relevant effects on this organism. The resistance limit of the snails was tested performing 8 days tests at the very high IL's concentration, 5000 µM and 10.000 µM. The results proved that even when exposed for longer time the snails are capable to resist. One can assume that such concentrations will not be reached in real ecosystems.

Up to date there exist no studies concerning the toxicity of the selected ionic liquids towards those species as used in the AquaHab[®] tests.

For comparison the following literature data are of interest.

As already presented in Chapter 2.3.- *see* Table V and included references , IM14Cl was

tested on the algae species *Selenastum capricornutum*. They proved to be around ten times less resistant towards this compound as *Ceratophyllum demersum*.

The sensitivity of *Daphnia magna* (another crustacean species) towards IM14Cl is similar as the one we have found for *Hyaella azteca* (below 100 μ M).

In our investigation the snail *Biomphalaria glabrata* was found to be five times more resistant towards IM14 cation then the data published for the snail species *Physa acuta*.

When testing Py4Cl the algae *Selenastum capricornutum* proved to be around five times more sensitive than *Ceratophyllum demersum* and again *Daphnia magna*'s sensitivity was lying in the same range as the one of *Hyaella azteca* (below 125 μ M).

Residue analysis monospecies tests – cation effect

Ceratophyllum demersum

Figure 14 shows the ILs' content in the plant after 168 hours of exposure. The IL content in the plants' extracts increases up an exposure concentration of 500 μ M and remains in the same range at higher exposure concentrations.

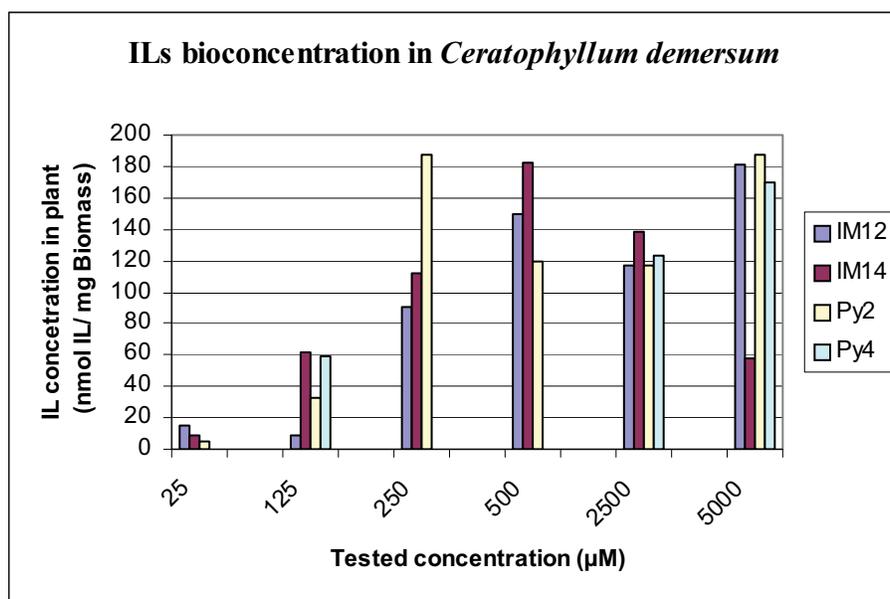


Figure 14. Bioconcentration of selected ionic liquids in *Ceratophyllum demersum*

It is not clear whether the measured IL amount is only the internal content. Since the plants' scions were harvested from the test solution and gently dabbed off with a tissue paper it might well be that IL still remained attached to the plant's surface. Thus the hypothesis of a saturated plant surface at concentrations higher than 500 μ M IL would explain the residue

analysis results. The same hypothesis might justify the results obtained *via* HPLC analysis of the test solution (*see* chapter 3.2.2.1.1 – *Ceratophyllum demersum*).

Hyalella azteca

The residue analysis of the crustaceans revealed a dose-effect curve as showed in Figure 15. The IL internal concentration increases continuously while increasing the exposure concentration.

When treated with IM12Cl 80% of the crustaceans died at 0,6 nmol IL/mg biomass internal IM12⁺ concentration. Already four times less (ca. 0,15 nmol IL/mg biomass) internal concentrations of IM14⁺, Py2⁺, Py4⁺ ions generated a similar effect.

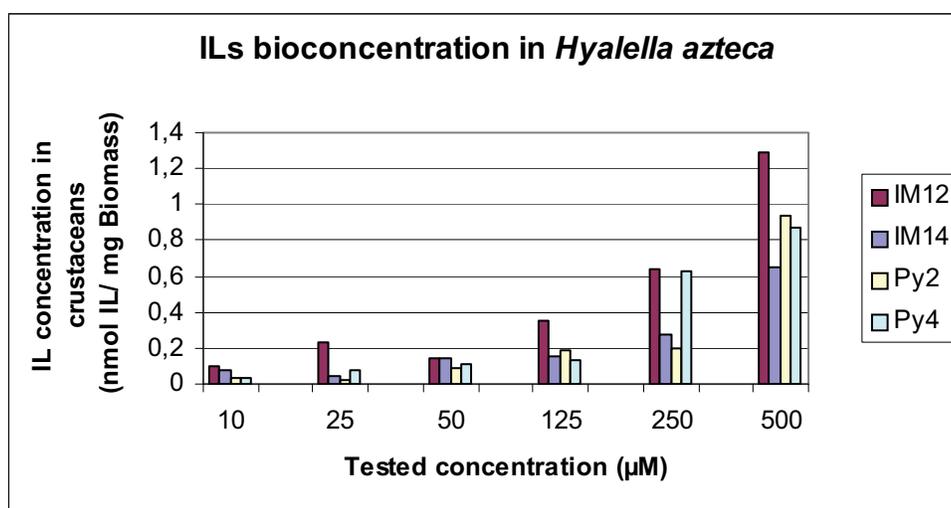


Figure 15. Bioconcentration of selected ionic liquids in *Hyalella azteca*

Biomphalaria glabrata

The snails' flesh and shell were independently analyzed. The residue analyses indicate time-effect curves as presented in Figure 16 and Figure 17. The content of IM12Cl, Py2Cl and Py4Cl in snails exposed in 48 hours acute toxicity tests to high exposure concentrations (2500 or 5000 µM) was very low - frequently under the HPLC detection limit.

The extracts of the snails exposed to 500µM IM14Cl for 192 hours contained about three times more IL than the snails treated with the same test solution for 24 hours. The snails treated with 5000µM Py2Cl and Py4Cl for 192 hours contained nearly 15 times more Py2⁺ and Py4⁺ in their flesh than the snails treated with the same test solution for 24 hours. The Py2⁺ and Py4⁺ contents in the shell were only eight times (for Py2Cl) and two times (for Py4Cl) higher in the snails exposed for 192 hours compared with the snails exposed for 24 hours to 5000µM IL.

For the 192 hours test with 5000 μ M IM12Cl and IM14Cl the IL content in snails' flesh was 60 (for IM12Cl) and 40 (for IM14Cl) times higher than the IM12⁺ and IM14⁺ amount in the snails' flesh after 24 hours exposure to the same test solutions. The snails' shell contained only 30 times more IM14⁺ after 192 hours compared with 24 hours exposure results.

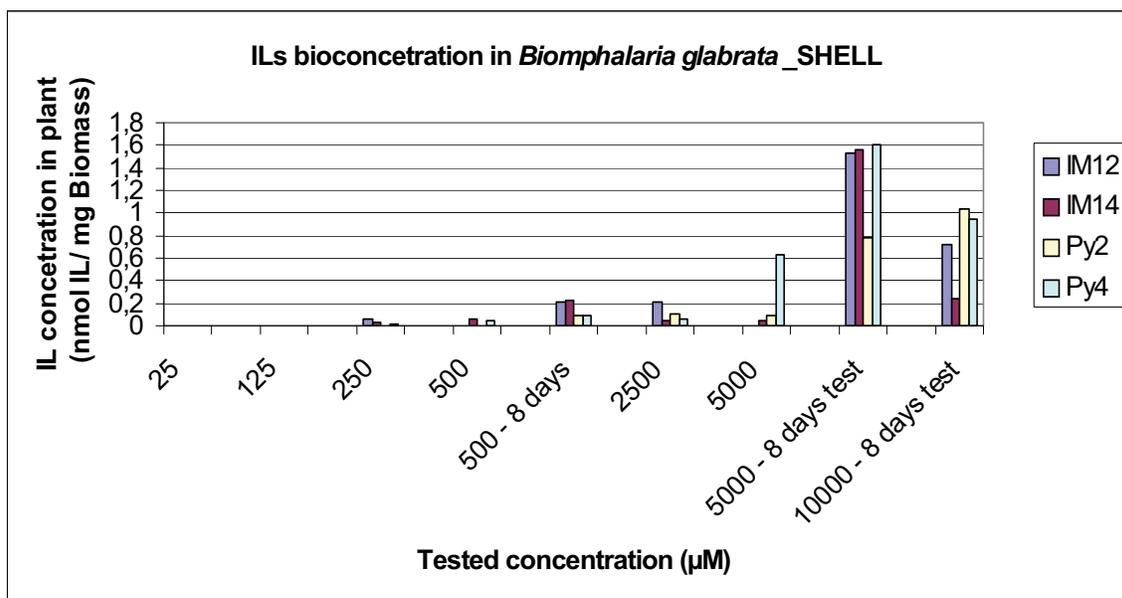


Figure 16. Bioconcentration of selected ionic liquids in the shell of *Biomphalaria glabrata*

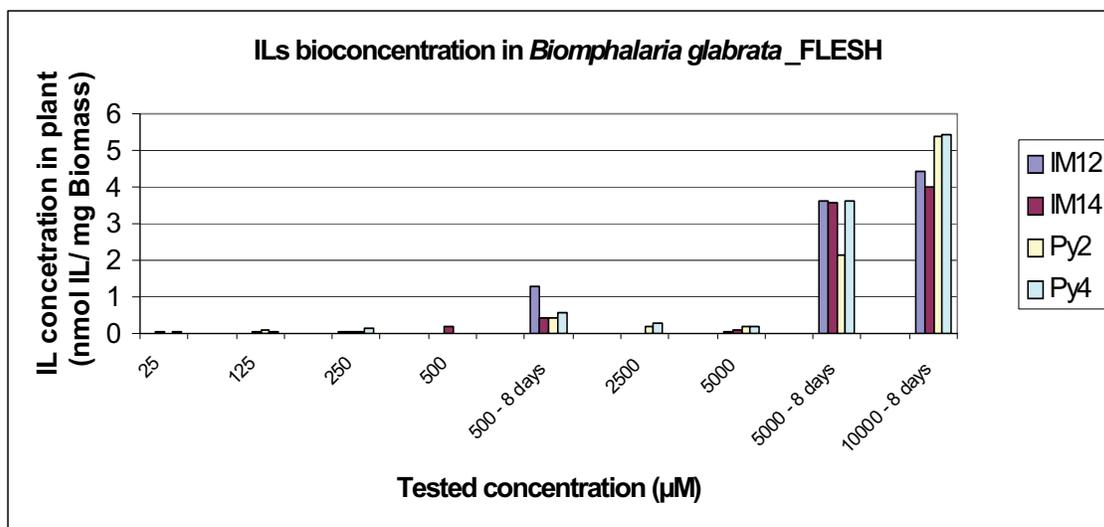


Figure 17. Bioconcentration of selected ionic liquids in the flesh of *Biomphalaria glabrata*

For the same exposure time (192 hours) a dose-effect curve - constantly increasing internal IL concentration - in snails' flesh was indicated (*see* Figure 17). The amount of IL in the snails' flesh proved to be two times higher than in the snails' shell when they were exposed for 192 hours to 5000 μ M IL. For the same exposure time to 500 μ M and 10.000 μ M IL the IL content in the snails' flesh was about five times higher than in the snails' shell. Only in the case of 10.000 μ M IM14Cl – 192 hours exposure - the flesh contained 20 times more IM14⁺ than the shell.

Half of the snails died when the internal IM12⁺ and IM14⁺ concentration was around 3,5 nmol IL/mg biomass in the snails' flesh and ca. 1,5 nmol IL/mg biomass in the snails' shell. The same Py4⁺ content caused the death of 90% of the tested snails.

Oryzias latipes

The residue analysis results for the fishes are shown in Figure 18. The organisms were exposed for 96 hours to 250 and 500 μ M ILs. None of them died and the IL content was determined after the 96 days. To 5000 μ M test solution the fishes were exposed for 8 days. They died after different exposure times (*see* chapter 3.2.2.1.1- *Oryzias latipes*). Their internal IL content was measured after their death. Only the fishes exposed to 5000 μ M Py2Cl survived after 8 days.

The internal IL content in the dead fishes was comparable - between 2,3 and 3,5 nmol IL/mg biomass (ca. $0,5 \cdot 10^{-3}$ mg IL/mg biomass), even if they died after different exposure times.

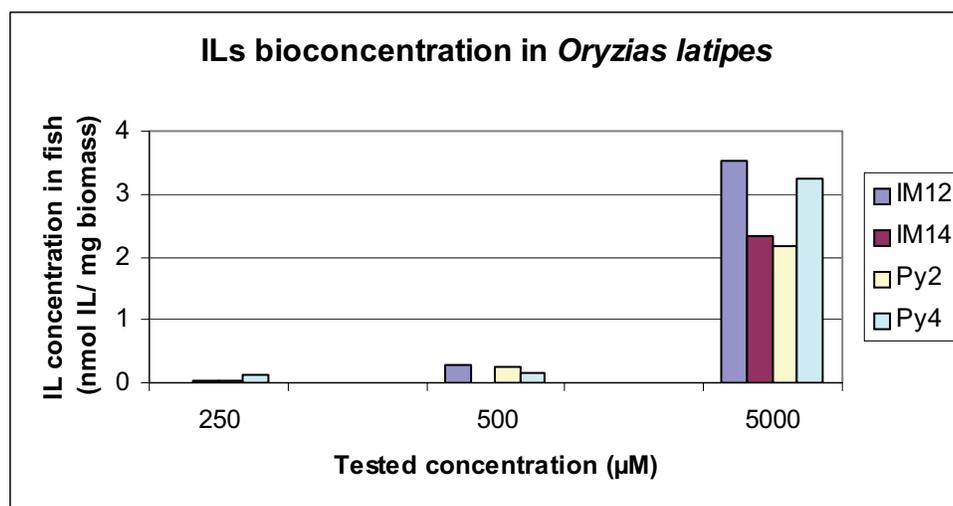


Figure 18. Bioconcentration of selected ionic liquids in *Oryzias latipes*

3.2.2.1.2. Anion effect

Ceratophyllum demersum (plants)

No visible effects could be observed when *Ceratophyllum demersum* scions were exposed for 8 days to LiBTA (500 μ M / 2500 μ M / 5000 μ M) or LiCl (500 μ M / 5000 μ M).

Hence the plants evidenced high resistance towards BTA anion.

Hyaella azteca (crustaceans)

When treated with 50 μ M / 100 μ M / 250 μ M LiBTA the crustaceans did not undergo relevant changes (*see* Table XVIII). They did not die even after 8 days of exposure. If compared with the positive control LiCl it might be that the Li⁺ ions may have a lethal effect on the *Hyaella azteca*.

Thus it was shown that *Hyaella azteca* have the ability to resist to a concentration of 250 μ M LiBTA.

Table VIII. *Hyaella azteca* - number of surviving organisms in 8 days acute toxicity tests with LiBTA (total organisms tested per compound and concentration were 10).

Hours Conc. (μ M)	Number of surviving organisms				
	LiBTA			LiCl (positive control)	
	250	100	50	250	50
24	10	10	10	10	10
48	10	9	10	10	10
72	10	9	10	10	10
96	10	9	10	10	10
120	10	9	10	10	10
144	10	9	10	10	10
168	10	9	10	10	10
192	10	9	10	9	10

* 10 organisms were used as controls; all of them survived until the end of tests;

***Biomphalaria glabrata* (snails)**

As shown in Table XIX the snails were not affected by 500 μ M LiBTA in the 8 days acute toxicity test but 50% died after 48 hours and 100% after 5 days exposure to 2500 μ M LiBTA. When treated with 5000 μ M LiBTA the snails did not survive the first 24 hours. The positive control (LiCl 5000 μ M) indicated a 50% survival rate after 48 hours and 0% survival one day later. These observations suggest a clear toxic effect manifested by Li⁺ ions on snails.

The observations made on snails as they were treated with LiBTA revealed some changes in behavior prior to their death: first the organisms retracted into their shells followed the next days by an uncommon, excessive extension out of the shell. After their death the snails presented a rigid appearance.

Thus it was shown that *Biomphalaria glabrata* can resist an exposure to LiBTA treatment only up to 500 μ M concentration.

Table XIX. *Biomphalaria glabrata* - number of surviving organisms in 8 days acute toxicity tests with LiBTA (total organisms tested per compound and concentration were 6).

Hours Conc. (μ M)	Number of surviving organisms				
	LiBTA			LiCl (positive control)	
	5000	2500	500	5000	500
24	-	3	6	6	6
48	-	3	6	3	6
72	-	2	6	-	6
96	-	2	6	-	6
120	-	-	6	-	6
144	-	-	6	-	6
168	-	-	6	-	6
192	-	-	6	-	6

* 6 organisms were used as controls; all of them survived until the end of tests;

***Oryzias latipes* (fishes)**

Table XX presents the results obtained when fishes were treated with LiBTA and LiCl. They survived for 8 days to 500 μM LiBTA without any notable changes in behavior while one fish from the positive control (500 μM LiCl) manifested severe behavior alterations (continuously rotating around the own caudal fin). When exposed to 2500 μM LiBTA the first fish died already after 18 hours and the second one within the first 24 hours. Exposure to 5000 μM LiBTA killed the fishes after 12 (one of them) and 18 (the second one) hours respectively while the same concentration of LiCl caused the death after 72 and 96 hours.

Our results denote a clear toxic effect induced by the lithium cation (which is well known as posing neurological effects in the human body) as well as by the LiBTA molecule. Comparing the effects of 500 μM treatment with Li and LiCl it becomes clear that the alteration in the fish behavior it is a consequence of exposure to Li^+ ions and is not determined by the BTA anion. In the same time the 5000 μM treatment with LiBTA and LiCl reveals the contribution of the BTA anion to the endpoint, because in the presence of the BTA anion the fishes died six times faster as the fishes exposed to the Li^+ ions only.

Hence *Oryzias latipes* proved to resist to 500 μM but not to 2500 μM LiBTA.

Table XX. *Oryzias latipes* - number of surviving organisms in 8 days acute toxicity tests with LiBTA (total organisms tested per compound and concentration were 2).

Hours Conc. (μM)	Number of surviving organisms				
	LiBTA			LiCl (positive control)	
	5000	2500	500	5000	500
24	- **	-***	2	2	2
48	-	-	2	2	2
72	-	-	2	1	2
96	-	-	2	-	2
120	-	-	2	-	2
144	-	-	2	-	2
168	-	-	2	-	1****
192	-	-	2	-	1****

* 2 organisms were used as controls; all of them survived until the end of tests

** one fish died already in the first 12 hours; the second fish died after 18 hours

*** one fish died already after 18 hours

**** one fish started to develop a strange behavior

General discussion of the anion effect

Bis(trifluoromethyl-sulfonyl)imide (BTA) is a commonly used anion which revealed only a moderate cytotoxicity ($EC_{50} = 2200\mu\text{M}$) (Stolte *et al.*, 2006). The chemical structure is presented in Table VII from Chapter 2.3. The low viscosity and high thermal, electrochemical and hydrolytic stability of BTA ionic liquids lead to a broad applicability in different fields of industry. From (eco)toxicological point of view the BTA anion is precarious because of its undoubtedly intrinsic toxicity towards different species. So it was found to manifest a moderate toxicity for the duckweed *Lemna minor* - a fresh water plant – ($EC_{50} = 6300\mu\text{M}$) and a much stronger effect to the limnic green algae *Scenedesmus vacuolatus* ($EC_{50} = 125\mu\text{M}$) (Matzke *et al.*, 2007). Nevertheless the question arises if these toxic effects can be verified also in higher level test systems (e.g. plants, animals) and thus a need for additional investigations was emphasised (Stolte *et al.*, 2007b).

Therefore the BTA anion was chosen to be tested on the four AquaHab[®] species in form of its lithium salt. LiCl was chosen as a positive control for a potential toxicity of the lithium cation. In previous studies neither NaCl nor LiCl exhibited a cytotoxicity in concentration up to $5000\mu\text{M}$. Thus it was concluded that neither the sodium nor the lithium cation has an intrinsic cation effect, and furthermore, that the chloride anion does not exhibit an intrinsic anion effect (Stolte *et al.*, 2006). The relevance of a cytotoxicity assay for aquatic organisms was indicated (Stolte *et al.*, 2007a).

If an anion effect has been revealed, an ionic liquid which is formed by BTA and one of the cations tested above (IM12^+ , IM14^+ , Py2^+ , Py4^+) has to be tested in the complex microcosm AquaHab[®].

The plant species *Ceratophyllum demersum* – a submerge fresh water plant – used in AquaHab[®] showed a high resistance towards LiBTA (no visible effects even at $5000\mu\text{M}$ exposure for 8 days – see Table XXI). This moderate toxicity of the BTA anion is consistent with the effects observed by Matzke *et al.* (2007) on floating fresh water plant *Lemna minor*.

As compared to the findings on the sensitivity of the crustaceans *Daphnia magna* towards the BTA anion (48h $EC_{50} = 270\mu\text{M}$, Wells and Combe, 2006), *Hyalella azteca* showed a higher resistance towards BTA anion. None of the organisms died after 8 days exposure to $250\mu\text{M}$ LiBTA.

So far no results concerning a BTA anion effect on snails have been published. In this study we found that the snails were sensitive towards BTA anion. They died very fast (after 24 hours) when treated with $5000\mu\text{M}$ LiBTA. Furthermore an intrinsic lithium cation effect could be observed. Snails died only 3 times slower (after 72 hours) if exposed to $5000\mu\text{M}$ LiCl.

The only data on BTA ionic liquids effects on fish was published by Pretti *et al.*, (2005). Among other ILs they investigated the toxicity of IM14BTA ($LC_{50} \cong 240\mu\text{M}$) and Py4BTA ($LC_{50} > 240\mu\text{M}$) if exposed to zebrafishes (*Danio rario*). The design of that study did not allow to identify which part of the ion pairs was responsible for the detrimental effect on

zebrafishes. However a suggestion has been made, that the modification of the cationic counterpart is responsible for the difference in toxic behaviour of these two BTA ionic liquids.

Our observations originating from *Oryzias latipes* (fishes) acute toxicity tests with LiBTA and LiCl substantiated an intrinsic cation effect of lithium towards fishes. When exposed to 5000 μ M LiCl the fishes died within 96 hours. When treated with 5000 μ M LiBTA the fishes died five times faster (within 18 hours) – based on a combined effect of both anion and cation. Additionally changes in fish behavior after 168 hours exposure time to 500 μ M LiCl established a toxic intrinsic effect of lithium cations.

All these results stand for a clear indication of a mixture toxicity posed by the lithium cation and BTA anion towards snails and fishes.

Not at all negligible is also the suggestion that the relevance of cytotoxicity assay for aquatic organisms - previously indicated by [Stolte et al. \(2007a\)](#) - might be not valid at higher level test systems (*i.e.* animals). LiCl did not show cytotoxicity in the tested concentration range up to 5000 μ M (48 hours test - [Stolte et al., 2006](#)) but proved its intrinsic cation effect on snails (after 48 hours) and fishes (after 96 hours) – *see* Table XXI.

Table XXI. The anion effect in 8 days monospecies tests - acute effects (time and concentration at which all or 50% of the organisms died or at which the plants decomposed); h = hours; d = days; n.t.= not tested

Organisms tested	Tested concentration [μ M]									
	LiBTA						LiCl (positive control)			
	5000	2500	500	250	100	50	5000	500	250	50
<i>Oryzias latipes</i> (fishes)	12 h	18 h	-*	n.t.	n.t.	n.t.	3-4 d	7d ^(a)	-	-
<i>Biomphalaria glabrata</i> (snails)	24 h	50% in 48 h	-	n.t.	n.t.	n.t.	48 h	-	-	-
<i>Hyalella azteca</i> (crustaceans)	n.t.	n.t.	n.t.	-	-	-	n.t.	n.t.	-	-
<i>Ceratophyllum demersum</i> (plants)	-	-	-	-	-	-	-	-	-	-

^(a) changes in behaviour

* No effect at concentrations were no specification is written

3.2.2.2. Influence of excretion on the IM12Cl bioconcentration in fish

The HPLC analysis of the fishes extracts obtained after the fish monospecies test with IM12Cl clearly indicates an increase of IM12⁺ content in the biomass (*see* Figure 19): i) 0,5 nmol IL/mg biomass (after 2 days); ii) 2,3 nmol IL/mg biomass (after 4 days); iii) ca. 3,7 nmol IL/mg biomass (after 6 and 8 days); iv) 5,4 nmol IL/mg biomass (after 10 days). The fishes used for residue analysis after 4, 6 and 8 days exposure to IM12Cl were already dead.

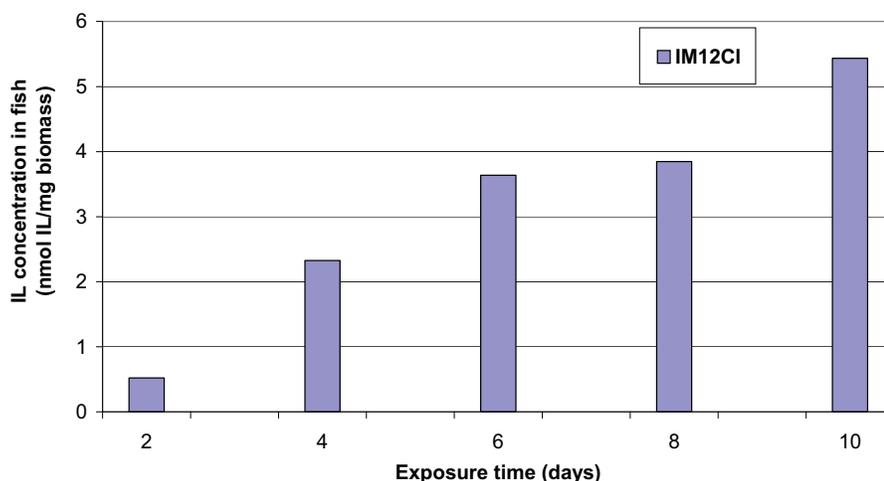


Figure 19. Influence of excretion process on IM12Cl / 5000 μ M bioconcentration in *Oryzias latipes*

Discussion

IM12Cl was chosen to be the first IL to be tested in the AquaHab[®] microcosm due to results in previous monospecies tests. It turned out to be the least toxic IL tested on the species inhabiting AquaHab[®] as well as in other previous studies at molecular and cellular level (Stolte *et al.*, 2006, 2007a).

Prior to the complex AquaHab[®] tests the influence of the excretion process on the bioconcentration of IL in *Oryzias latipes* was determined. The IM12Cl biotest concentration of 5000 μ M was selected based on the results obtained in the range finding tests (*see* 3.2.2.1.1.): i) fishes' good survival rate in 8 days acute toxicity test; ii) a good detectability in the residue analysis *via* HPLC.

For further experiments in the complex aquatic microcosm it was very important to find out if the concentration in fishes remains constant after 2 / 4 / 6 / 8 or 10 days exposure to chemicals. If so the results could be interpreted that the same amount of IL which is continuously taken up by the fishes is consequently eliminated by excretion.

In contrast to this working hypothesis the HPLC analysis of the fish extracts clearly

demonstrated an increase in the IL concentration. The fish which died in the 2nd day contained 0,5 nmol IL/mg biomass while the one which died in the 10th day contained 5,4 nmol IL/mg biomass. Thus the IL bioconcentration process is not influenced by the excretion process which means that the IM12⁺ ion remains in fishes. The reduced number of organisms used in our test has to be considered.

3.2.2.3. Influence of light on the IM12Cl bioconcentration in plants

The influence of light on the IM12Cl bioconcentration in *Ceratophyllum demersum* was tested (see Figure 20). In the absence of light only 5% of the tested IL amount was taken up by the plants within 336 hours while in the presence of light 20% of IL was removed from the test solution.

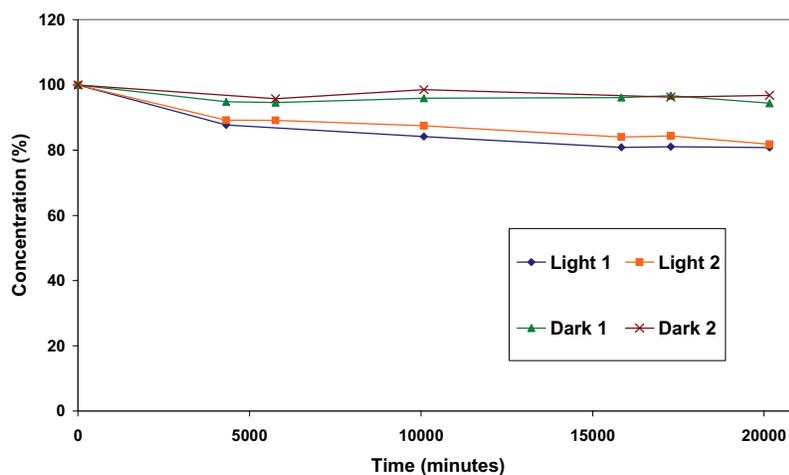


Figure 20. Influence of light on the reduction of IM12Cl (300µM) concentration in the test solution by *Ceratophyllum demersum*

The residue analysis of the scions of *Ceratophyllum demersum* in this experiment revealed ca. 53 nmol IL/mg dried biomass for each of the 2 scions grown under the light conditions and ca. 3 nmol IL/mg dried biomass for each of the 2 plant scions grown in the dark.

Discussion

These studies were performed in a parallel experiment, because it was observed that in the plant chamber of the AquaHab[®] in test 1 (*see* 3.2.3.1) the water tank is most of the time dark, while in the animal chamber is constant light. Both chambers houses plants, but the one with constant light contains 5 times less than the other one.

The results indicated a four times stronger decrease in the IL's biotest solution concentration by those plants placed under light conditions as compared to the plants placed in the dark. The internal IL concentration was nearly 20 times higher for the plants exposed to light.

These observations represent a hint that photosynthesis might play a crucial role in the uptake of ILs by the plants. Further tests are needed to understand this mechanism of action and to identify in which compartment of the plant the ILs are retained.

3.2.3. AquaHab[®] studies

3.2.3.1. Cation effects

AquaHab[®] test 1: 250 μ M IM12 Cl / 5 weeks (species were added one after the other)

As presented in Figure 21 the determination of the IM12Cl concentration during AquaHab[®] test 1 indicated that: (i) after the first week (without any organisms) the IL concentration remained constant; (ii) after the 2nd week (the plants were added) - the IL concentration decreased by ca. 2 % - probably as a consequence of the tap water added in order to substitute the lost of test solution caused by water sampling; (iii) after the 3rd week (the crustaceans were added) – the IL concentration decreased by ca. 7 % (compared to (ii)); (iv) after the 4th week (the snails were added) – the IL concentration did not change (compared to (iii)); (v) after the 5th week (the fishes were added) - IL concentration did not change (compared to (iii)).

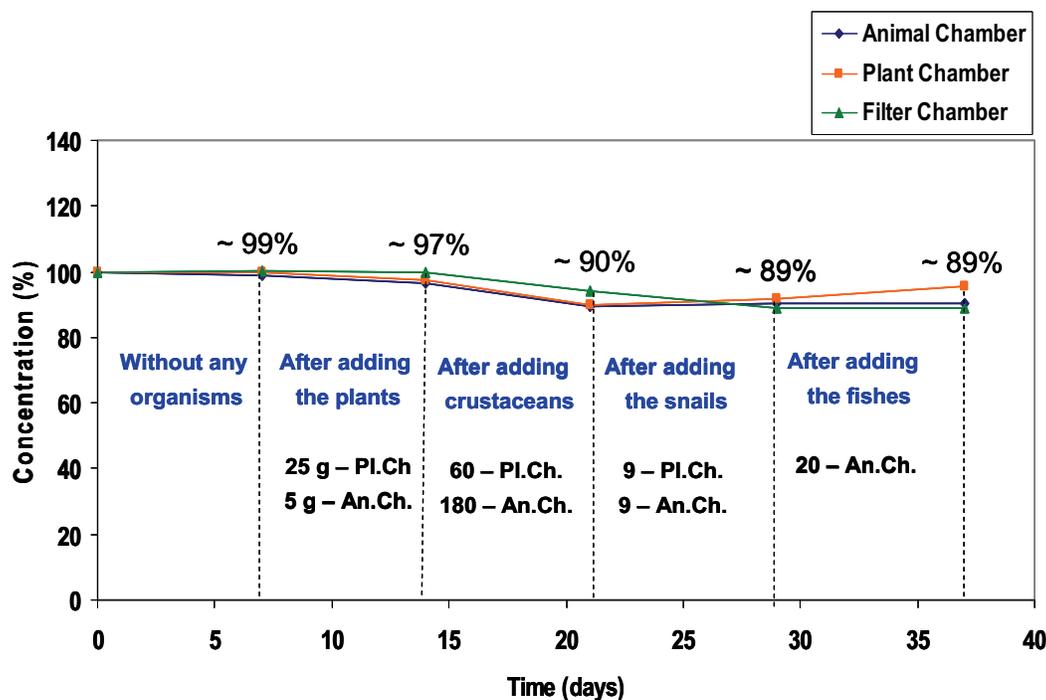


Figure 21. IM12Cl concentration in the testsystem during the five weeks of AquaHab[®] test 1

The desorption test from filter materials (at the end of test) showed that the filter cotton (20 g dried weight) adsorbed ca. 2 % of IL within five weeks, while the lava stones (160 g wet weight) only adsorbed 0,8 % IL.

The quality control of the sensors included in the control unit of the AquaHab[®] system at the end of test validated a perfectly functioning system.

In conclusion a hypothetically possible IM12Cl adsorption by the system materials could not be observed.

The nitrite content in the first two days was around 0,3 mg/L, in the next four days 0 mg/L and after seven days again around 0,3 mg/L.

During the five week experiment the following further observations have been made.

Most of the *crustaceans* died within four days after they were added to the system. They were lying on the bottom of the AquaHab[®] water tank. After seven days all organisms were dead. The dead crustaceans disappeared within one day after the snails were added to the system.

At the end of the experiment the *plants* showed no morphological alterations but color and weight changes. The plants from the plant chamber were bright green (it was mostly dark there). In the animal chamber (constant light conditions, 2 $\mu\text{mol}/\text{m}^2/\text{sec}$) - the plant

weight was doubled in five weeks and their color was unchanged. In the dark of the plant chamber (mostly dark) the plant weight remained the same.

When opening the water tank at the end of the five weeks it was observed that one *snail* had died in the plant chamber (after two weeks of exposure).

The *fishes* survived the one week exposure during the AquaHab[®] test 1. Two fishes subsequently died during the “recovery experiment”.

At the end of AquaHab[®] test 1 the IM12 cation was found in plants, snails and fishes. The crustaceans could not be used for residue analysis because they disappeared from the system.

After 30 days of exposure the plants contained 10 nmol or 26 nmol IL/mg biomass in plant chamber or animal chamber respectively.

After 16 days of exposure the surviving snails contained ca. 0,1 nmol IL/mg biomass in the shell and 0,28 nmol IL/mg biomass in the flesh. The same amount was found in the living snails originating from both animal and plant chamber. The residue analysis of the dead snail revealed ca. 0,1 nmol IL/mg biomass in the shell and 0,82 nmol IL/mg biomass in the flesh.

Fishes from the “recovery” aquarium

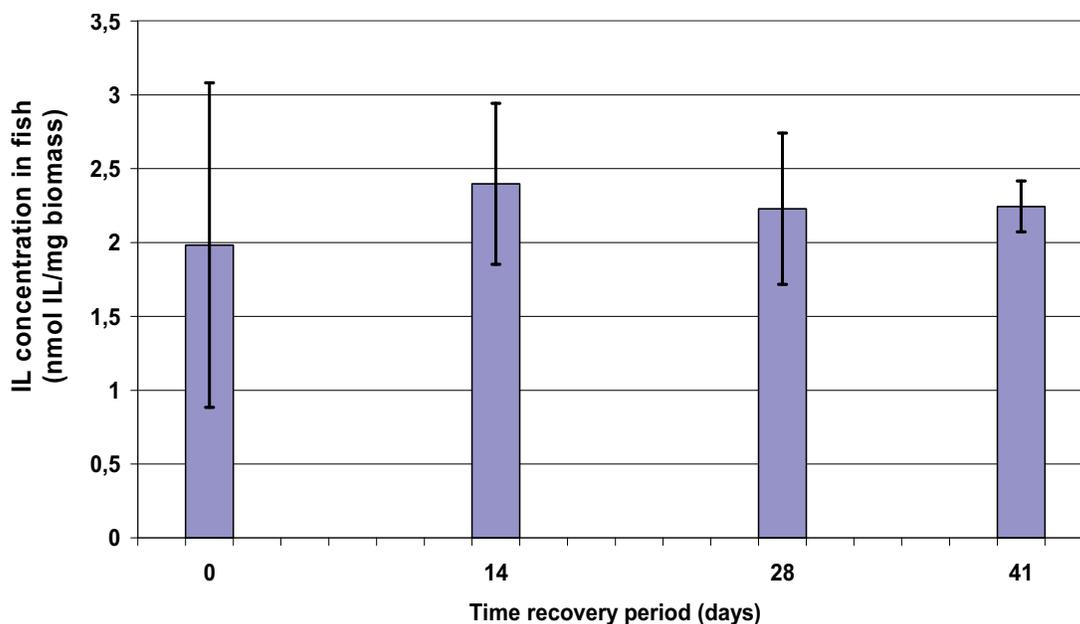


Figure 22. Residue analysis of the fishes at the end of AquaHab[®] test 1: 0 days “recovery” period and after 14, 28, 41 days “recovery” period in an external aquarium (number of fishes: for the 0 and 14 days “recovery” period n=5 and for 28 and 41 days of “recovery” period n=3)

After four weeks “recovery” period two fishes died. They disappeared from aquarium because they were consumed by snails. After six weeks “recovery” period another fish died. The IM12⁺ ion was found in all fishes (see Figure 22): no “recovery” period yielded 1,98 nmol IL/mg biomass, after 2 weeks of “recovery” period 2,4 nmol IL/mg biomass were found, after 4 weeks 2,22 nmol IL/mg biomass and even after 6 weeks “recovery” period the concentration was 2,24 nmol IL/mg biomass.

The snails from the “recovery” aquarium were analyzed and IM12⁺ could be found in snails shell (0,25 nmol IL/mg biomass) and as well as in snails flesh (0,52 nmol IL/mg biomass).

Discussion

The AquaHab[®] test 1 protocol was designed and performed in order to determine the influence of the system consisting materials and of each species on the IM12⁺ ion concentration.

Thus in the first week the test was running without any organism. Afterwards each week one species was added to the system. In this way it was shown that the system consisting materials do not adsorb IM12⁺ cation as was proposed due to the pretests results with each consisting material of AquaHab[®]. A detectable decrease in the tested IL concentration was caused by the plants and the crustaceans.

The plants did not show any morphological or color changes. Only the weight appeared to be influenced by the luminosity conditions.

For the long exposure time of 250µM IM12Cl used in AquaHab[®] test 1 this concentration proved to be too high for the crustaceans species because most of them died after four days.

The role of the snails in the closed aquatic ecosystem as saprophagous was confirmed by this experiment one more time. After the snails were added to the system the dead crustaceans disappeared within one day.

All organism samples measured contained the IM12 cation (crustaceans could not be analyzed because they disappeared). The plants from the animal chamber contained two times more IM12⁺ than the one from plant chamber. This result substantiated the hypothesis phrased in chapter 3.2.2.3.: luminosity conditions and thus photosynthesis may play a crucial role in ILs’ bioconcentration in *Ceratophyllum demersum*.

The IL content in surviving snails originating from two chambers of the water tank was the same. There was no difference in the IM12⁺ content in the shell between dead and living snails after 16 days of exposure but the amount of the IM12 - cation in the dead snail’s flesh was about 3 times higher than in the living snails’ flesh.

The capacity of the system to metabolize the excretion products was certified by constant nitrite measurements.

In the “recovery experiment” the amount of IM12 cation found in the fishes remained nearly constant (around 2 nmol IL/mg biomass) after 0, 2, 4 and 6 weeks of “recovery” suggesting a “steady state” effect. Thus our working hypothesis was validated. If the IL binds to the biological structures of fish it will remain in the organism and can be found there for long time even after removing the exposure to the chemical entity.

Additionally a transfer of the IM12 cation from contaminated fish to the so far not exposed snails was found in the “recovery experiment”. Residue analysis indicated the presence of the IL cation in both snail shell and snail flesh. This is the first indication that IM12Cl may have a potential for biomagnification.

The estimated bioconcentration factor (BCF = partitioning of the chemical between the biota and water) for the IM12 cation according to [Ropel *et al.* \(2005\)](#) is 10 L/kg and is remarkably consistent with the 1-*n*-octanol/water partition coefficient value from literature ($\log P_{o/w} < -2$) ([Choua *et al.*, 2003](#)). This result indicates that the potential of IM12Cl to bioaccumulate is very low. According to [Ropel *et al.*, 2005](#) substances with a BCF < 250 are classified as having “low potential for bioaccumulation”.

AquaHab[®] test 2 and 3: 250 μ M IM18Cl and 200 μ M Py8Cl / 2 weeks (no organisms)

As shown in Figure 23 the IM18⁺ concentration in the test solution remained approximately constant after two weeks while the Py8Cl concentration decreased with ca. 10% (*see* Figure 24).

The desorption test from filter materials (at the end of Aqua Hab[®] test 2 and test 3) showed that the filter cotton (20 g dried weight) adsorbed 0,5 % IL in two weeks and the lava stones (160 g wet weight) adsorbed 0,35 % IL in two weeks.

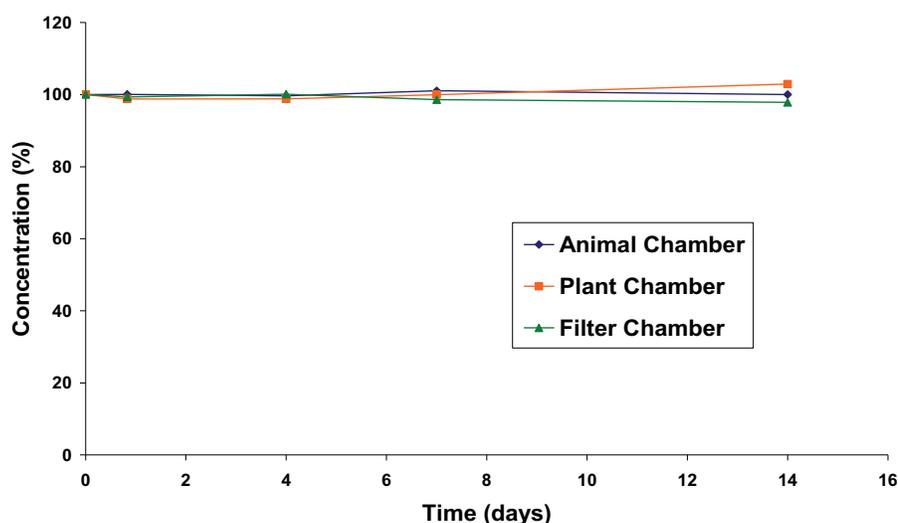


Figure 23. IM18⁺ ion concentration in Aqua Hab[®] test 2

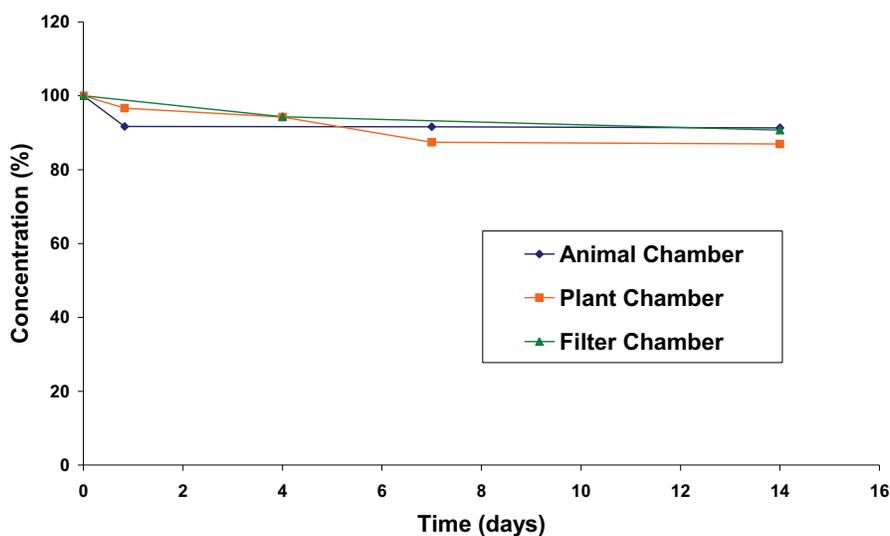


Figure 24. Py8⁺ ion concentration in Aqua Hab[®] test 3

Discussion

Because in the AquaHab[®] test 1 it was proven that IM12Cl is not adsorbed by the system in the absence of organisms raised the question if the whole class of imidazolium and/or pyridinium ionic liquids could be tested in AquaHab[®] without adsorption interferences from the system consisting materials.

Therefore AquaHab[®] test 2 and test 3 were accomplished with IM18Cl and Py8Cl - being the ionic liquids with the longest side chain which possess no inherent hazard potential from ecotoxicological point of view - in a two weeks test without any organisms. The results revealed a *head group effect* – only Py8Cl was adsorbed on the system components.

AquaHab[®] test 4: 100 μ M IM12Cl / 6 weeks (all organisms added at the beginning)

As presented in Figure 25 the evolution of IM12Cl concentration during the six weeks AquaHab[®] test 4 indicated a 20% decrease of the initial IL concentration in the biotest solution.

The desorption test from filter materials (at the end of test) showed that the filter cotton (20 g dried weight) adsorbed 0,27 %, the lava stones (160 g wet weight) adsorbed 0,16 % IL within six weeks.

The perfect performance of the system was confirmed at the end of test by the quality

control of the sensors included in the control unit of the AquaHab[®] system.

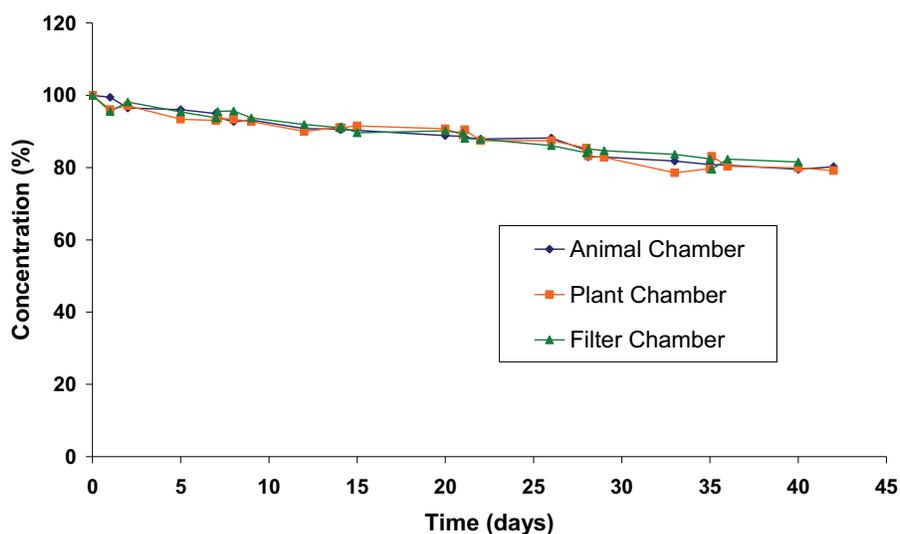


Figure 25. IM12⁺ ion concentration in AquaHab[®] test 4

Organisms monitoring revealed for the *plants* no morphological alterations. The color of the plants from the plant chamber changed to bright green, since it was mostly dark there. The following weight modifications were recorded: i) the plant weight was doubled within the six weeks in the animal chamber (constant light conditions, 12 $\mu\text{mol}/\text{m}^2/\text{sec}$) and ii) no weight changes was observed in the plant chamber (mostly dark).

The *crustaceans* were hiding at the corners in the first week. After the first week they disappeared from aquarium. This might be explained as they have been consumed by the fishes.

Nine *snails* out of 18 (four of nine in animal chamber, five of nine in plant chamber) died within the following time scale: i) after two weeks – two snails died in both of the chambers; ii) after three weeks – one snail more died in the animal chamber; iii) after four weeks – two snails more died in the plant chamber; iv) after six weeks (end of test) – one snail more died in each chamber (animal and plant chamber). At the end of test in the plant chamber *eight snail progenies* were found on the plants.

Within the first three weeks every week one *fish* died. Each of them was quite fast decomposed because they disappeared within two days from the aquarium. In the last week one fish died and was just partially consumed - only the head till the end of test.

The nitrite content increased up to 0,2 mg/L in the 2nd week and then decreased to 0 mg /L in the 4th week.

The residue analysis of the fishes (see Figure 26) revealed that the IM12⁺ amount (ca. 0,11 nmol IL/mg biomass of the fish) was the same after 7 days and after 28 days of exposure to IM12Cl. The concentration within the fishes slightly increased 0,14 nmol IL/mg biomass after 35 and 42 days respectively. The fish which died in the last week (after 42 days) and was just partially consumed by the snails (it remained without a head) contained only 0,014 nmol IL/mg biomass.

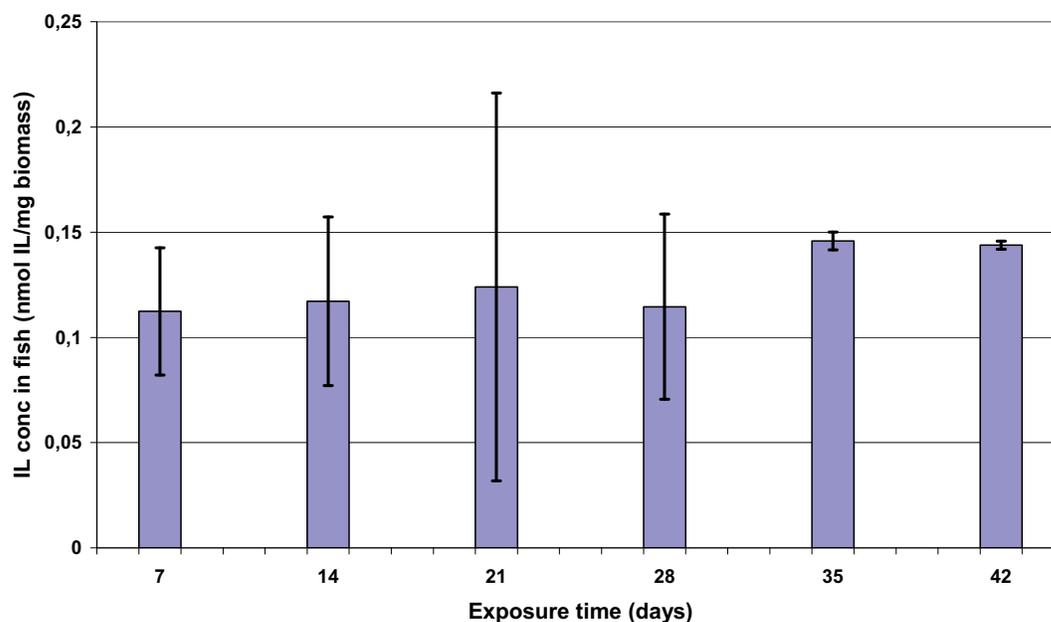


Figure 26. Residue analysis of the fishes originating from the Aqua Hab[®] test 4 (n=3 for 7, 14, 21, 28, 35 days of exposure and n=2 for 42 days of exposure)

Analyzing the snails' extracts after 42 days of exposure the following results were obtained: i) the living snails from the plant chamber contained ca. 0,1 nmol IL/mg biomass in the shell and 0,85 nmol IL/mg biomass in the flesh; ii) the living snails from the animal chamber contained ca. 0,06 nmol IL/mg biomass in the shell and 0,42 nmol IL/mg biomass in the flesh; iii) the dead snails from both plant and animal chamber contained 0,005 nmol IL/mg biomass in their shells. The flesh could not be measured because it was not in the shell anymore.

Discussion

In order to avoid the quick disappearance of one of the main species from the ecosystem as observed for the crustaceans during the AquaHab[®] 1 test a second test protocol was designed. The IL concentration was thus reduced to 100 μM IM12Cl.

Furthermore AquaHab[®] test 4 was performed i) to investigate a possible bioaccumulation potential of IM12Cl in more detail, ii) to observe possible changes in the species behavior and iii) to investigate the tendency of IM12Cl to exert subchronic effects. Therefore all test organisms were added to the system from the beginning and the exposure time was extended to six weeks.

The decrease of the IL concentration observed during the six weeks (*see* Figure 25) might be due to three main cumulated reasons: (a) adsorption by the filter materials (ca. 2%) – proved by the desorption test; (b) dilution due to the tap water added in order to substitute the lost of biotest solution caused by sampling; (c) adsorption by the organisms.

As in the AquaHab[®] test 1 the plants were not affected by the chemical (no morphological or color changes in the animal chamber). Only the weight seemed to be influenced by the luminosity conditions.

In this test the crustaceans survived the first week but disappeared completely within the second week from the system. This might be explained as they have been consumed by the fishes.

The role of the snails as primary consumers and saprophagous in the closed aquatic ecosystem was confirmed again.

For the presence of eight snail progenies in the system at the end of test there are two possible reasons: (a) the eggs were already on the plant when adding it to the system or (b) the snails in plant chamber produced new progenies – young snails appear in 7-11 days after eggs exposure (Pimentel, 1957). Such an effect could not be observed in animal chamber because of the fishes which consume the snail eggs and even their own ones.

Once again the nitrite measurements demonstrated the capacity of the system to metabolize the excretion products.

In addition to AquaHab[®] test 1 a subchronic effect of IM12Cl on the snails and fishes could be observed:

- i) for the snails: 50% of them died after six weeks of exposure to the lower IM12Cl concentration of 100 μM in contrast to only 5% dead snails after two weeks of exposure at the higher IM12Cl concentration of 250 μM ;
- ii) for the fishes : 20% died after six weeks exposure to 100 μM IL in contrast to none of the fishes died after one week of exposure to 250 μM IL.

The subchronic effect of IM12Cl is confirmed also by the results gained *via* monospecies tests: in an eight days test with 5000 μM IM12Cl all the snails survived for

four days and half of them for eight days and the fishes started to die only after six days of exposure to this concentration of IM12Cl.

As in the AquaHab[®] test 1 all organisms contained the IM12 cation. Once again the concentration in the crustaceans could not be determined.

The extract analysis of that fish which died in the last week and was only partially consumed by snails – lost only its head - proved to contain 10 times less IM12⁺ as those fishes which were exposed for the same period to the chemicals but were still alive. This observation might lead to the working hypothesis that the IL enters the fish body mainly through the gills, binds and remains preferentially there. Further research is needed to verify this hypothesis.

While the amount of IM12⁺ ion in living snails' shell was almost the same for organisms originating from the plant and the animal chamber, the IL quantity in the snails' flesh was two times higher within those snails which lived in the plant chamber - even if the snails in animal chamber consumed the dead fishes containing IM12⁺ ion. In the plant chamber there are no fishes meaning that the IM12⁺ ion bioaccumulation in snails is a consequence of ingesting plants containing IM12⁺ ions and IM12⁺ bioconcentration. These results suggest that an IL transfer between species (biomagnification) may be more efficient *via* plants ingestion than *via* dead organic matter (fishes) consumption. This hypothesis is still to be confirmed in further studies.

The residue analysis in fish confirmed our hypothesis of a “steady state” effect of around 0,12 nmol IL/mg biomass after one to six weeks exposure to IL and substantiated the tendency for a low bioaccumulation potential (BCF \approx 1,5 L/kg) of the IM12 cation detected in the first AquaHab[®] test.

3.2.3.2. Anion effect

AquaHab[®] test 5: 100 μ M IM12 BTA / 5 weeks (all organisms added from the beginning)

As presented in Figure 27 the changes in the IM12BTA concentration during the five weeks AquaHab[®] test 5 indicated a 25% decrease of the initial IL concentration in the biotest solution.

A desorption test from filter materials showed that the filter cotton (20 g dried weight) adsorbed 2,5 % IM12 cation within five weeks while the lava stones (160 g wet weight) did not adsorb any IM12⁺ ions.

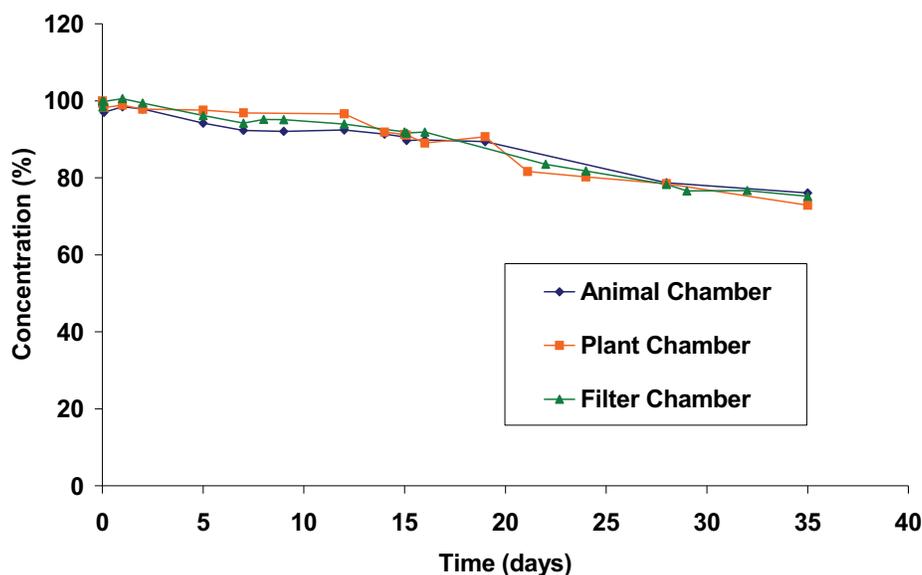


Figure 27. IM12BTA concentration during five weeks in AquaHab[®] test 5

The quality control of the sensors included in the control unit of the AquaHab[®] system certified the perfect performance of the system.

Organisms monitoring revealed no morphological alterations for the *plants*. Only color and weight changes could be observed. Again plants from the plant chamber were bright green since it was mostly dark there. The weight of the plant in animal chamber was doubled within five weeks at constant light conditions ($2\mu\text{mol}/\text{m}^2/\text{sec}$). In the plant chamber the plant weight did not increase.

The *crustaceans* used to hide in the corners and could be observed in the system for four weeks. They were consumed by the fishes while they were still alive. A reduction in their number was recorded from the second until the fourth week (*i.e.* drastic decrease in the second week: from day 12 until day 19).

Three *snails* out of 18 (two of nine in the animal chamber, one of nine in the plant chamber) died as follows: i) two in the animal chamber at day 25; ii) one snail more was dead in the plant chamber at the end of test (after 35 days). The snails in the animal chamber died after four dead fishes disappeared from animal chamber – two of those fishes manifested strange behavior prior to their death.

Five *fishes* out of 20 died during the test. One fish died within the first five days and was rapidly consumed by the snails. One more fish died in the second week (after 13 days) and was consumed by the snails. In the third week (after 20 days) two fishes exhibited an abnormal behavior – one was swimming on one side at reduced pace while the other one kept its head down sporadically moving the caudal fin. Within the next day both of them died. In the fourth week one more fish died and was later consumed by the snails too.

The nitrite content increased until 0,05 mg/L in the second week (after 9 days) and decreased afterwards until 0 mg /L in the fifth week (after 26 days).

An increase of the oxygen consumption could be observed only within the first week (day 7).

Residue analysis

After 35 days of exposure to IL the plant in the animal chamber contained ca. 23 nmol IL/ mg biomass. The plant taken from plant chamber contained ca.10 nmol IL/mg biomass.

At the end of AquaHab[®] test 5 the snails in the animal chamber contained 0,05 nmol IL/ mg biomass in the shell and 0,16 nmol IL/ mg biomass in the flesh. The snails from the plant chamber contained 0,12 nmol IL/ mg biomass in the shell and 0,38 nmol IL/ mg biomass in the flesh.

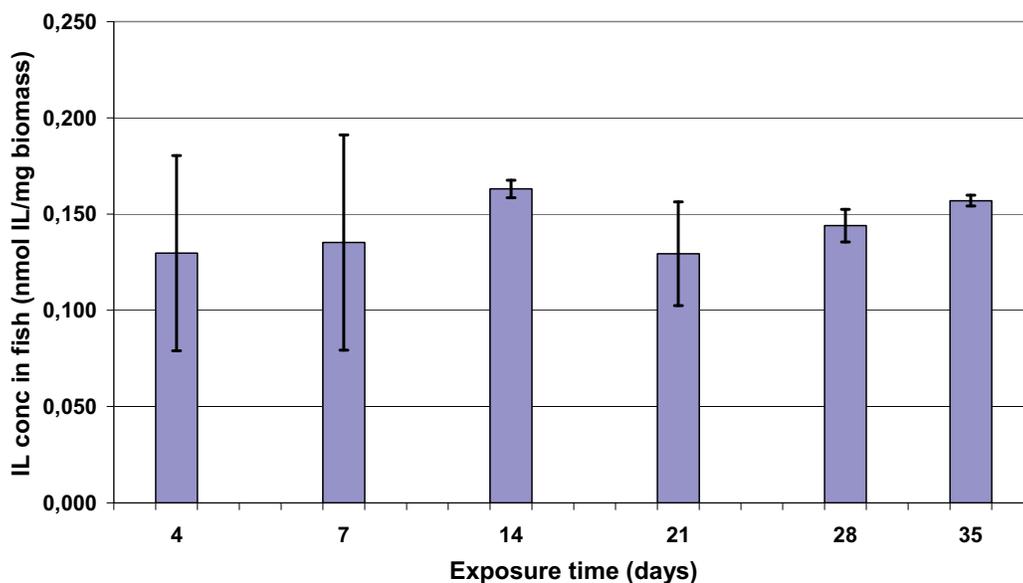


Figure 28. Residue analysis of the fishes originating from the AquaHab[®] test 5 (n= 3 for 7 and 21 days of exposure and n= 2 for 4, 14, 28, 35 days of exposure)

As shown in Figure 28 the amount of IM12⁺ ions found in the fishes originating from Aqua Hab[®] test 5 was practically the same after 7, 14, 21, 28 and 35 days of exposure to IL (ca.0,15 nmol IL/mg biomass). For the fishes exposed for 28 and 35 days the IM12⁺ content was determined in extracts prepared from fishes' head and fishes' body. The amount of the IM12 cation proved to be around 0,15 nmol IL/mg biomass in both extracts.

Discussion

AquaHab[®] test 5 was performed to investigate the influence of an unnatural anion as part of an IM12 ionic liquid on the toxicity, biocentration, biomagnification and bioaccumulation of the IM12 cation in the four organisms.

As already mentioned in chapter 3.2.2.1.2 the BTA anion was selected to be tested at microcosm level due to i) its presence in multiple applications and ii) its moderate toxicity. In order to enable a comparison of the results with the data gained in the previous AquaHab[®] tests the five weeks exposure time, the 100 μ M exposure concentration and the same test design as for AquaHab[®] test 4 were chosen.

Like in the AquaHab[®] test 4 the decrease of the IM12⁺ concentration during the five weeks might be caused by three main cumulated factors: (a) adsorption by the filter materials (ca. 2%) – proved by the desorption test; (b) dilution due to the tap water added in order to substitute the loss of biotest solution caused by sampling; (c) adsorption by the organisms.

The observations made on the *plants* in AquaHab[®] test 1 and 4 proved to be valid one more time.

Crustaceans' presence in the system for four weeks validated their resistance towards the BTA anion and thus the results obtained in monospecies tests.

Three times less *snails* died in AquaHab[®] test 5 (three dead snails) as compared to AquaHab[®] test 4 (nine dead snails). A lethal effect of IM12BTA (100 μ M) was recorded only after 4 weeks exposure while the snails exposed to IM12Cl (100 μ M) started to die already after 2 weeks. This observation indicates the BTA anion as a reducer of lethal effect of the IM12 cation. An explanation for this effect could be that IM12BTA is not totally dissociated and thus the actual concentration of the IM12 cation is lower in AquaHab[®] test 5. This different dissociation constant of IM12Cl and IM12BTA might also explain the survival of crustaceans in AquaHab[®] test 5.

More *fishes* died in AquaHab[®] test 5 (five fishes within the first 4 weeks) than in AquaHab[®] test 4 (three fishes within the first 4 weeks). Furthermore, two of the fishes from AquaHab[®] test 5 developed a strange behavior prior to their death. The strange movements and abnormal position of their bodies stand for a clear indication of equilibrium's disturbance. Thus the BTA anion seems to potentiate the IM12 cation effect on fishes.

These results represent an evidence of a mixture toxicity posed by the IM12 cation and the BTA anion towards fishes. It has to be investigated whether this holds true also for the snails and crustaceans.

As in the AquaHab[®] test 1 and test 4 all organisms contained the IM12 cation. Once again for crustaceans this could not be measured.

The plant in the animal chamber (constant light) contained twice as much IM12⁺ ion than the plant from the plant chamber (mostly dark conditions). This finding substantiates one more time the hypothesis formulated in chapter 3.2.2.3: photosynthesis might play a

crucial role in the ILs bioconcentration.

Similar to AquaHab[®] test 4 the snails from plant chamber contained two times more IM12⁺ than the snails from the animal chamber. This result substantiates the hypothesis phrased after AquaHab[®] test 4: an IL transfer between species (biomagnification) may be more efficient *via* plants ingestion than *via* dead organic matter (fishes) consumption.

The differences between the IM12⁺ content in snails' shell and flesh proved to be in the same range as in AquaHab[®] test 4.

The residue analysis in fish confirmed again our hypothesis of a “steady state” effect yielding in a concentration around 0,15 nmol IL/mg biomass after one to five weeks of exposure to the IL. The tendency for a low bioaccumulation potential (BCF=1,5 L/kg) of the IM12 cation detected in the AquaHab[®] test 1 and 4 was validated again.

Analyzing one of the dead fishes from AquaHab[®] test 5 the IM12⁺ internal concentration was not considerably different compared with the IM12⁺ content in living fishes. Thus there is no clear indication at which internal IM12⁺ ion content a lethal effect is generated.

An IL bioaccumulation in fishes' gills was not confirmed in AquaHab[®] test 5. The extracts prepared from fishes' head and body contained similar amounts of IM12 cation. Thus the hypothesis formulated in AquaHab[®] test 4 could not be confirmed in this study.

The BTA anion bioaccumulation in organisms has to be investigated in future studies.

4. Conclusions and outlook

The present work serves as a contribution to the development of a flexible ecotoxicological test battery as main component of a strategy which aims at an environmental hazard and risk assessment already within the design process of new sustainable chemicals.

The strong need of more relevant standardized systems on laboratory scale for the prospective risk assessment of chemicals and drugs in general is a very important issue especially in the context of the new European Guideline (REACH) demands. They claim the elucidation of environmental fate pathways and (bio)transformation data as well as bioaccumulation potential of chemicals in order to reduce the uncertainties in their hazard assessment.

The literature screening showed that up to day no data on standardized microcosm studies on industrial chemicals were published. Even if microcosm studies are not specifically addressed in regulatory demands it is clear that such tests are necessary in order to obtain a risk assessment of the industrial chemicals as close to reality as possible and as early as possible.

A standardized closed aquatic ecosystem at laboratory scale termed AquaHab[®] designed by the company *OHB-System AG* (Bremen, Germany) is proposed as a tool for (eco)-toxicity investigations at microcosm level filling out the levels of a flexible biological test battery. The system was implemented within the present work for the investigation of ionic liquids - model compounds with high ecotoxicological relevance for the future.

To analyse whether the selected ILs will be adsorbed onto the different consisting materials of the AquaHab[®] system adsorption pre-tests were performed with each material the system consists of. No significant adsorption was found.

Additionally, range finding single-species tests were performed to define an appropriate concentration for the - in contrast to monospecies tests - more complex and time consuming AquaHab[®] tests. The cation (IM12⁺, IM14⁺, Py2⁺, Py4⁺) and the anion (Cl and BTA) effects were investigated. Data concerning the acute toxicity of the selected ionic liquids towards four species - *Ceratophyllum demersum* (plants), *Hyalella azteca* (crustaceans), *Biomphalaria glabrata* (snails), *Oryzias latipes* (fish) - were provided.

For the cation effect studies the most important observation was the difference in sensitivity of these organisms against the investigated ILs. The crustaceans (*Hyalella azteca*) proved to be the far most sensitive organism. They already started to die after 24 hours of exposure to very low ILs concentrations (below 250µM for all the four tested ILs). In contrast, the snails (*Biomphalaria glabrata*) demonstrated resistance even towards very high concentrations during short and long time exposure to the test chemical (*i.e.* 5000µM in an 8 days test).

Furthermore for the first time bioconcentration studies with the selected ILs have been carried out. Performing residue analysis with the tested organism new data concerning the

internal IL concentration which causes the death of the crustaceans, snails and fishes were stated. Fishes died after different exposure time to the selected ILs but at the same internal IL concentration. Further studies are needed to confirm the hypothesis that distinct ILs have different bioconcentration times.

Further single-species tests indicated that the IM12⁺ ion bioconcentration process is not influenced by the excretion process. The IM12⁺ ion remains in fishes. More studies are required to confirm these findings. Concerning the species physiology future research is required to find out where in the organism the ILs are retained.

Monospecies test with plants (*Ceratophyllum demersum*) led to the suggestion that photosynthesis might play a crucial role in the uptake of ILs by the plants. Further tests are needed to understand this mechanism of action and to identify in which compartment of the plant the ILs are stored.

The anion effects were tested in single species tests with the BTA anion as lithium salt. Its moderate toxicity toward *Ceratophyllum demersum* was stated. *Hyalella azteca* showed a higher resistance towards the BTA anion as compared to previous published results on *Daphnia magna*. A higher sensitivity of the snails towards the BTA anion in contrast to their high resistance towards the tested cations (IM12⁺, IM14⁺, Py2⁺, Py4⁺) was emphasized.

The anion effects data stand for a clear indication of a mixture toxicity posed by the lithium cation and BTA anion towards snails and fishes.

Based on monospecies tests results the IM12Cl was chosen to be tested in the AquaHab[®] system. This was the first hazard assessment of an ionic liquid performed at the microcosm level in a standardized closed aquatic multispecies system.

The IM12⁺ cation remained stable within a period of six weeks in the aquatic medium and did not bind to sinks like sediments, plants or organisms. This suggests a hazard potential “persistence” and a high bioavailability of the IM12⁺ cation in the aquatic environment. Acute and subchronic effects were observed within the test period of six weeks.

The “Recovery”-experiments with fish indicate a low excretion or biotransformation. This is of importance for the observed subchronic effects. The IM12 cation can be transferred from one organism to another within the food chain, which is the first indication for a biomagnification potential of an ionic liquid. But the bioconcentration factors calculated from fish (BCF of 1.5 – 10) suggest a low bioaccumulation potential. This is in accordance with the low lipophilicity ($\log P_{o/w} < -2$) of IM12Cl.

The persistence observed together with the subchronic effects indicate a hazard potential that has to be studied in additional long-termed investigations with other more lipophilic ionic liquids too.

The influence of the BTA anion being the counterpart of an IM12 ionic liquid on the toxicity, bioconcentration and bioaccumulation of the IM12 cation at microcosm level was studied. Our results represent an evidence for a mixture toxicity posed by the IM12 cation and the BTA anion if exposed to fishes. It has to be investigated whether this holds true also for the snails and crustaceans. The BTA anion fate in the organisms has to be investigated in future studies.

AquaHab[®] proved to be a useful tool to investigate acute and subchronic ecotoxicological effects, bioconcentration, biomagnification, bioaccumulation and persistence of imidazolium ionic liquids in an aquatic ecosystem.

The results obtained in this work further reduce the uncertainties within the ecotoxicological risk profile of the ionic liquids selected here. New studies to confirm a predictive potential of our results and thus an extrapolation of the obtained ecotoxicological data to another species have to be designed.

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Detailed information for the toxicity studies at microcosm level presented in subchapter 2.2.1.

A) Indoor microcosms

Pesticides

An aquatic indoor microcosm was used to study effects of the pesticides parathion-methyl and prometryn on phototrophic flagellates (*Cryptomonas sp.*) and predatory ciliates (*Urotricha furcata*). The effects caused by prometryn on the flagellates resulted in a two times higher NOEC in the multi-species microcosm compared to the single-species tests. For ciliates the NOEC decreased by factor 145 in the multi-species test compared to single-species test when exposed to prometryn. The lower NOEC for ciliates exposed to prometryn in the microcosm was most likely caused by an indirect effect due to reduced availability of flagellates as food. The measurement of nutrient concentrations in the test media and organisms facilitated the modelling of effects. The presented aquatic indoor microcosm was considered as a tool which could be standardised and applied as an instrument to provide data for higher tier risk assessment (Liebig *et al.*, 2008).

To validate the use of small indoor microcosms for the risk assessment of pesticides, the fate and effects of chlorpyrifos, carbendazim, and linuron were studied in 8.5-liter indoor freshwater microcosms. Functional and structural responses to selected concentrations were evaluated and compared with responses observed in larger-scale model ecosystem studies. Overall, the microcosms adequately displayed the chain of effects resulting from the application, although they did not always predict the exact fate and responses that were observed in larger semifield studies. Because closed systems were used that did not contain sediment and macrophytes, pesticides were relatively persistent in the present study. However, the simple design offers the potential to perform experiments under more controlled conditions than larger and, consequently, more complex model ecosystems, while maintaining relatively high ecologic realism compared with standard laboratory tests (Daam, Van den Brink, 2007).

Microcosm studies were undertaken to relate biomarker responses to the insecticide chlorpyrifos (organophosphate) with biomass changes. Microcosms were filled with soil from the field of interest and earthworms of the species *A. caliginosa* were introduced. The microcosms were treated with a series of concentrations of chlorpyrifos in the laboratory under controlled conditions. The biomass of the worms was determined regularly for a period of 5 weeks. Earthworms were removed from the microcosms for biomarker tests: for cholinesterase (ChE) inhibition assays every week and for a neutral red retention determination 2 weeks after the exposures started. Inhibition of ChE increased with higher exposure concentrations and with time but there was no clear dose-related response. A clear dose-related response with exposure concentration was established for the neutral red retention assay. A correlation between ChE inhibition and biomass change existed directly after the second application of chlorpyrifos (Reinecke, S., Reinecke, A., 2007).

Three related amides (diuron, 2-(octyloxy) acetanilide, and salicylanilide) were evaluated for toxicity to aquatic microcosm communities. Effects were measured at the ecosystem level using changes in pH, redox potential, and dissolved oxygen as indicators of toxicity. These values were used to calculate the resistance, flexibility, and relative instability of the microecosystems to each compound at comparable dose levels. While microcosm toxicity tests were slightly less sensitive than some single species tests, they provided important additional information on the extent of perturbations and the rate of ecosystem recovery (Flum, Shannon, 1987).

The fungicide fluazinam, the insecticide lambda-cyhalothrin, and the herbicides asulam and metamitron were applied to indoor freshwater microcosms (water volume around 0.6 m³). The treatment regime was based on a realistic application scenario in tulip cultivation. The fate of the compounds in the water, and responses of phytoplankton, zooplankton, periphyton, macroinvertebrates, macrophytes, decomposition, and water quality were followed for 13 weeks (van Wijngaarden *et al.*, 2004).

Carbendazim as a representative pesticide was used by Burrows and Edwards (2004) for testing an integrated soil microcosm (ISM) test protocol. Microcosms, set up in a greenhouse, consisted of cylinders made from high-density polyethylene (HDPE) pipe, 7.5 cm (i.d.) x 15 cm high. A fine nylon mesh was placed across the bottom of each microcosm for leachate collection. Field soil, (silty clay loam), collected from Flörsheim, Germany, was sieved through a 5 mm screen and mixed thoroughly. Earthworms, enchytraeids, and microarthropods were added to each microcosm. Each microcosm contained five wheat seedlings, and was maintained at a 12 h-12 h light-dark cycle. Artificial rainwater was used to water microcosms as required. Soil microcosms were treated with carbendazim at concentrations 1, 3, 9, 27, and 81 times higher than the predicted environmental concentration (PEC). A water-only control treatment was also used. The key soil processes used as endpoints were microbial activity, nitrogen mineralization, soil enzymatic activity, ammonium and nitrate leaching, organic matter decomposition and biological feeding activity. Key structural parameters measured were microbial biomass, nematode communities, microarthropod populations and diversity, enchytraeid and earthworm populations and plant growth. Pesticide degradation, leaching and uptake into plants and earthworms were also assessed. A comparison of the obtained results with results from single-species tests, small microcosms, large terrestrial model ecosystems, and field tests indicated that the ISM protocol may adequately predict environmental effects.

Experimental results were reported by Leeuwangh *et al.*(1994) on four types of freshwater model ecosystem after administration of a single dose of chlorpyrifos. The fate, and primary and secondary effects of chlorpyrifos were compared between the model ecosystems. 'Slootbox', a fate model used in the ecotoxicological risk assessment of pesticides in the Netherlands, overestimated chlorpyrifos concentrations. The primary effects of chlorpyrifos can be predicted accurately on the basis of single species laboratory toxicity data. The population effects observed in the microecosystems, microcosms, and mesocosms were consistent between all experiments and with the single species tests. Community metabolism, as a functional endpoint, was less sensitive than the structural parameters measured. Secondary effects, both for structural and functional endpoints, varied between the micro- and mesocosm experiments. Recovery of populations affected by insecticide stress was found to depend on factors such as life cycle characteristics and

ecological infrastructure, in addition to the toxicant concentration. The onset of (potential) recovery is likely to start at an approximate concentration of the EC₁₀ (48 h).

Herbicides

Effects of the bipyridylium herbicide diquat and tank-mix adjuvant Agral90 were investigated on various life history traits of the freshwater pulmonate snail *Lymnaea stagnalis*. Trait expression was measured in simple laboratory bioassays on small size groups of snails, and under more complex, indoor microcosm conditions, on larger groups of snails. Microcosms were provided with sediment, plants, and fish, thus allowing a more complex level of intra and inter-specific interactions to develop. Treatments were performed with substances alone or in mixture. Adult growth, fecundity, progeny development, hatching and chemical analysis in water were followed. Some of these parameters expressed differently in bioassays and microcosms probably influenced by the level of intraspecific interaction, which differed among the systems. Antagonistic interaction between diquat and Agral 90 were indicated (Coutellec *et al.*, 2008).

The sensitivity of 12 aquatic plant species (*Lemna minor*, *Lemna trisulca*, *Spirodela polyrrhiza*, *Elodea canadensis*, *Ceratophyllum demersum*, *Ceratophyllum submersum*, *Myriophyllum spicatum*, *Potamogeton crispus*, *Batrachium trichophyllum*, *Callitriche platycarpa*, *Sparganium emersum*, *Berula erecta*) to the herbicide metsulfuron-methyl was tested in microcosm experiments for 14 days under two growth conditions. As reference species, barley (*Hordeum vulgare*) and oil-seed rape (*Brassica napus*) were grown with their roots submerged in the microcosms. Two response variables were chosen: relative growth rate and specific leaf area (Cedergreen *et al.*, 2004).

The effects of the herbicide terbutryn on a simple lotic food web were investigated during a 72 days exposure period in five artificial indoor streams in a greenhouse. The model compound terbutryn - inhibitor of photosynthesis - was applied once in each stream at various nominal concentrations. Terbutryn concentrations in the water were analyzed by gas chromatography/mass spectrometry, and an overall time to 50% dissipation of 28 days was calculated. The development of aufwuchs and the population growth and development of the oligochaete *Lumbriculus variegatus* were investigated. The effect of terbutryn on the aufwuchs was a direct effect of decreases in the periphyton. However, the effects on *L. variegatus* were an indirect effect of terbutryn as a consequence of decrease in the aufwuchs food source and occurred at three-orders-of-magnitude-lower concentrations of terbutryn than the acute toxicity effects. This study demonstrated the utility of indoor lotic microcosm studies for evaluating both direct and indirect effects of contaminants on aquatic ecosystems (Brust *et al.*, 2001).

Insecticides

The role of pH in determining the environmental fate of ¹⁴C-aldicarb was examined in an aquatic microcosm. Aldicarb sulfoxide was found as the major metabolite and aldicarb was completely lacking at all three pH levels tested. Since aldicarb sulfoxide is biologically active, disappearance of the parent compound does not eliminate hazard (Soursa, Fisher, 1986).

Effects of chronic application of a mixture of the insecticides chlorpyrifos and lindane were studied in indoor freshwater microcosms containing natural sediment, periphyton, zooplankton, macroinvertebrates. The exposure concentrations (based on 0, 0.005, 0.01, 0.05, 0.1 and 0.5 times the LC_{50} of the most sensitive standard test organism for each compound) were kept at a constant level for four weeks. The observed effects could be explained from the individual toxicity of the insecticides to the invertebrates, and did not indicate synergistic effects (Cuppen *et al.*, 2002).

Metals

A microcosm study was undertaken to examine the effects of dissolved cadmium at various concentrations on biofilm accumulation and diatom assemblages. A natural biofilm sampled from river was inoculated into three experimental systems, where biofilm settled on glass slides. Samples collected after 1, 2, 4, and 6 weeks of colonization were analyzed for metal accumulation (total metal content and intracellular metal content in the biofilm), biomass (as measured through dry weight and ash-free dry matter), and quantitative as well as qualitative analysis of diatom assemblages. There was a positive correlation between cadmium accumulation and dissolved cadmium concentrations and duration of exposure (Moris *et al.*, 2008).

The response of meiofauna and nematode communities to increased levels of contaminants (organic carbon, Zn, Cu, Pb, Fe) was assessed in a laboratory microcosm experiment. The setup was allowed to mature for 32 days. Nematode identification was done to the genus level. The results indicate that higher concentrations of heavy metals and organic carbon had an impact on the nematode density, diversity, and community structure. Nematode genera such as *Axonolaimus*, *Theristus*, and *Paramonohystera* were found to be tolerant to metal pollution. This study also observed that nematode communities react differently to metal and organic pollution (Gyedu-Ababio, Baird, 2006).

The influence of ionic copper on the fitness of *Ceriodaphnia dubia* was assessed by Gagneten and Vila (2001) in microcosm experiments under different conditions of Cu^{+2} and pH. Two groups of experiments were conducted: effects on survival and fecundity, and effects on population dynamics. Hedfi *et al.* (2007) studied the effects of nickel on offshore nematode communities in microcosm experiments for 30 days.

Microcosm experiments addressed the impact of a mixture of Cu, Cr, Cd, Pb, and Hg at three concentrations after 36 h, 12 days, and 30 days on a meiofauna-dominated salt marsh community. In addition to analyzing effects on meiofaunal abundances, the study quantified the sediment metal concentrations of all five metals and pore-water concentrations, speciation, and ligand complexation of Cu (Millward *et al.*, 2001).

Aiming to investigate the influence of acid volatile sulfides (AVS) and simultaneously extracted metals in sediments on the bioavailability and toxicity of Cd, Ni, and Zn in sediments to polychaete worms *Neanthes arenaceodentata* laboratory microcosm experiments lasting for 20 days were conducted by Lee J.S. and Lee J.H. (2005). Mortality, growth rate, and metal bioaccumulation were registered. Overall, the bioavailability and toxicity of metals in sediments was not well predicted by sediment metal concentrations only, but considering the influence of geochemical factors (AVS) on the metal

bioavailability improved the prediction of toxicity.

Fuma *et al.* (2003) evaluated the effect levels of various toxic agents compared with acute doses of ionizing radiation for the experimental model ecosystem, *i.e.*, microcosm mimicking aquatic microbial communities. For this purpose, the authors used the microcosm consisting of populations of the flagellate alga *Euglena gracilis* as a producer, the ciliate protozoan *Tetrahymena thermophila* as a consumer and the bacterium *Escherichia coli* as a decomposer. Effects of aluminum and copper on the microcosm investigated in this study were compared with effects of gamma-rays, ultraviolet radiation, acidification, manganese, nickel and gadolinium reported in previous experiments. The microcosm could detect not only the direct effects of these agents but also the community-level effects due to the interspecies interactions or the interactions between organisms and toxic agents. The resulting effects data contributed to an ecological risk assessment of the toxic agents compared with acute doses of ionizing radiation.

Hydrocarbons

Traditional single species toxicity tests and multiple component laboratory-scaled microcosm assays were combined by Johnson and Romanenko (1989) to assess the toxicological hazard of diesel oil, a model complex mixture, to a model aquatic environment. A multicomponent laboratory microcosm was designed to monitor the biological effects of diesel oil on four components: water, sediment (soil + microbiota), plants (aquatic macrophytes and algae), and animals (zooplanktonic and zoobenthic invertebrates). To determine the sensitivity of each part of the community to diesel oil contamination and how this model community recovered when the oil dissipated, limnological, toxicological, and microbiological variables were considered. This experimental design emphasized monitoring toxicological responses in aquatic microcosm.

Toxicity and temporal changes in toxicity of freshwater-marsh-microcosms containing diesel fuel and treated with a cleaner or dispersant, were investigated using *Chironomus tentans*, *Daphnia pulex*, and *Oryzias latipes*. Bioassays used microcosm water (for *D. pulex* and *O. latipes*) or soil slurry (for *C. tentans*) (Bhattacharyya *et al.*, 2003).

Naphthalene biodegradation capability of *P. chrysosporium* and *T. harzianum* in soil microcosms was investigated by Mollea *et al.* (2005). Considering the high naphthalene volatility, a suitable soil microcosm was set-up and used. During experimental time course naphthalene concentration, CO₂ evolution as well as phytotoxicity tests were performed as monitoring parameters. The results shown that *T. harzianum*, differently than in liquid culture, is not able to biodegrade naphthalene directly in soil microcosm, while *P. chrysosporium* in the same conditions biodegrades the polycyclic aromatic hydrocarbon till a certain concentration.

Pharmaceuticals

Because microorganisms provide important ecosystem services (*e.g.* nutrient recycling, organic matter mineralization and degradation of pollutants) in a microcosm study, natural marine sediment hosting indigenous microorganisms and spiked with ¹⁴C-labelled pyrene was exposed to five concentrations of antibiotic ciprofloxacin in a single dose to the

overlying water. The complete mineralization of pyrene (*i.e.* $^{14}\text{CO}_2$ production) was measured during 11 weeks. Sediment samples for bacterial community structure analysis were taken after 7 weeks. Results showed a significant dose-dependent inhibition of pyrene mineralization measured as the total $^{14}\text{CO}_2$ production (Näslund *et al.*, 2008).

Folsomia candida (springtails) was exposed to five different concentrations of the widespread anthelmintic veterinary medicine ivermectin in replicated microcosm experiments lasting 49 days. At high density populations were able to compensate for the effects of the chemical. This result demonstrated that regulatory protocols carried out at low density (as in most past experiments) might seriously overestimate effects in the field, where densities are locally high (Noël *et al.*, 2006).

Toxins

A microcosm experiment was conducted to examine the depuration of microcystins (hepatotoxic nonribosomal peptides produced by cyanobacteria) in tilapia fish (*Oreochromis niloticus*). Fish were grown in a 100 L aerated recirculation tank containing dechlorinated water at room temperature (25+/-2 degrees C) for 96 h. Concentrations in livers, intestines, and gallbladders of each daily sacrificed fish were determined by both enzyme-linked immunosorbent assay (ELISA) and protein phosphatase inhibition assay (PIIA). Microcystins concentrations in the surrounding water were also determined by the same methods. This study revealed that tilapia fish can depurate and excrete microcystins into the bile and surrounding water as a way to avoid toxicity from such a potent hepatotoxin (Mohamed, Hussein, 2006).

Volatile organic compounds

Methyl *tert*-butyl ether (MTBE) is a widespread recalcitrant contaminant frequently detected in the environment being a major volatile organic compound in oxygenated fuel (10-15% by volume) or reformulated gasoline (11-15% by volume) (Herrick, 2000). MTBE was classified by the U.S. Environmental Agency as a potential human carcinogen. Assessing the soil ecotoxicity of MTBE using earthworm bioassay An (2005) demonstrated that ecotoxicity of such volatile organic compounds can be evaluated in closed soil microcosm with short-term exposure duration.

Antifouling biocides

Blue mussels, *Mytilus edulis*, were exposed to two different concentrations of tributyltin (TBT) in seawater, for 4 days, in order to evaluate the bioaccumulation of TBT by mussels *Mytilus edulis* in microcosms. Analyses of dissected organs and/or tissues demonstrated that TBT accumulated to the greatest extent in gills in the experiment with higher concentration tested and in the digestive gland in the experiment with low concentration tested. The bioconcentration factors were determined. The biomarkers used in this work were acetylcholinesterase, glutathione S-transferase, catalase activities, and thiobarbituric acid-reactive substances (TBARS) contents. No significant changes were observed in the measured enzyme activities or in TBARS concentration after the 4 days TBT exposure (Devier *et al.*, 2003).

B) Outdoor microcosms

Pesticides

The effects of nonylphenol (NP)- ingredient and adjuvant added by the pesticides users - on phytoplankton (including free floating aquatic plants) and periphyton (comprising sessile organisms *i.e.* algae, small crustaceans) were studied in 230 L outdoor microcosms. Phytoplankton cell density and biomass, phytoplankton and periphyton diversity, and assemblage composition were analyzed during a four-week preapplication period, followed by six weeks of NP treatment via controlled release and a six weeks postapplication period (Hense *et al.*, 2003).

The responses of a wide array of freshwater taxa (including invertebrates *e.g.* Mollusca, Annelida, Turbellaria, phytoplankton and macrophytes) from acute laboratory single species tests with the fungicide triphenyltin acetate (TPT) were compared with the concentration-response relationships of aquatic populations in two types of freshwater microcosms. Representatives of several taxonomic groups of invertebrates, and several phytoplankton and vascular plant species proved to be sensitive to TPT, illustrating its diverse modes of toxic action. Responses observed in the microcosms did not differ between system types and sampling dates, indicating that ecological threshold levels are not affected by different community structures. Possible explanations for the more sensitive field response are delayed effects and/or additional chronic exposure via the food chain in the microcosms (Roessink *et al.*, 2006a). The impact of TPT on test systems with clean or polluted sediments (10 microcosms each) was also investigated. Differences in sediment quality affected the structure of the aquatic communities that developed in the microcosms (Roessink *et al.*, 2006b).

Herbicides

The effects of the herbicide Roundup (glyphosate) on natural marine microbial communities were assessed in a 7 day field experiment using microcosms. Bottles were maintained underwater at 6m depth, and 10% of their water content was changed every other day. The comparison of control microcosms and surrounding surface water showed that the microcosm system tested here can be considered as representative of the natural surrounding environment (Stachowski-Haberkorn *et al.*, 2008a).

Daam and co-workers compared the fate and effects of herbicide linuron in an outdoor plankton-dominated microcosm study carried out in Thailand with those reported in temperate model ecosystem studies. Lower linuron concentrations disappeared slightly faster from the water compartment compared to temperate conditions, which appeared to be related with the experimental design rather than differences in climatic conditions. Sensitivity of primary producers and zooplankton were similar for the climatic regions, whereas effects on ecosystem functioning were less pronounced in tropical microcosms. Recovery potential of affected endpoints looked higher for tropical ecosystems compared to their temperate counterparts. These findings supported the use of toxicity data generated in temperate countries in the tropics (Daam *et al.*, 2009).

Effects of the herbicide Basamaïs (bentazon) and the fungicide Opus (epoxiconazole) on oyster spat (*Crassostrea gigas*) were assessed using in-situ microcosms in a field experiment lasting 13 days. Laboratory controls were not significantly different from in-situ microcosm controls in the field, for organic weight content or growth. It was concluded that such microcosm systems can also be run in a laboratory water bath instead of more technically difficult immersed field experiments (Stachowski-Haberkorn *et al.*, 2008b).

Insecticides

The sensitivity of a range of freshwater invertebrates to insecticide gamma-cyhalothrin (GCH), a single enantiomer of the synthetic pyrethroid lambda-cyhalothrin, was assessed by van Wijngaarden *et al.* (2009) in single species laboratory tests and in an outdoor multi-species ecosystem test. The most sensitive species in the laboratory single species tests with GCH was *Chaoborus obscuripes* and these results proved to be consistent with the one obtained in the outdoor microcosm tests.

The fate and effects of the pyrethroid insecticide lambda-cyhalothrin were compared in mesotrophic (macrophyte-dominated) and eutrophic (phytoplankton-dominated) ditch microcosms of approximately 0.5 m³ by Roessink *et al.* (2005).

An outdoor microcosm study was also conducted by Rand (2004) to determine the fate of the insecticide-miticide chlorfenapyr and its effects on zooplankton, macroinvertebrates, phytoplankton, and fish in a freshwater system under exposure conditions representing simulated surface runoff and/or spray drift. Chlorfenapyr was more toxic via spray to the water than via an exposure simulating surface runoff. The toxicity data from laboratory and microcosm studies along with water exposure data indicate low hazard to zooplankton species in the water column.

Twenty-four outdoor microcosms (24 m³ each) were used for 11 months to investigate the biological effects on phytoplankton and periphyton of the insecticide-miticide, pyridaben. The effects on phytoplankton populations were associated with the decline of zooplankton populations as a result of a direct effect of pyridaben exposure. There were no effects of pyridaben on periphyton communities or on functional endpoints (Rand *et al.*, 2001).

Scott and Kaushik (2000) conducted microcosm trials with the botanical insecticide Margosan-O(R) to assess the potential hazards of the product to aquatic organisms. Laboratory chronic bioassays with water from the treated microcosms were conducted. Results from chronic tests showed Margosan-O toxicity to be greater in the laboratory exposures than in situ with Culicidae larvae (*Culex pipiens* and *Culex restuans*) exposed to the same concentrations. Other invertebrates seeded to the system were *Daphnia magna* and *Chironomus riparius*.

Metals

Field and stream microcosm experiments were conducted by Clark and Clements (2006) to assess population-level (density, size distribution) and community-level (species richness metrics, multivariate analysis of community composition) responses of macroinvertebrates

to heavy metals. They concluded that phenology and developmental stage are important factors influencing responses of some aquatic macroinvertebrates to metals. Thus, timing bioassessments to coincide with the presence of the sensitive life stages can improve the ability to detect subtle contaminant effects.

A microcosm approach was used to test chironomids (Diptera) for adaptation and species differences in heavy metal tolerance. In one experiment, microcosms containing different levels of contaminants were placed in polluted and reference locations. In a second experiment, microcosms with different contaminant levels were placed at two polluted and two unpolluted sites. It was concluded that adaptation to heavy metals may be uncommon and species specific (Bahrndorff *et al.*, 2006).

The effects of total organic carbon (TOC) and UV-B radiation on Zn toxicity and bioaccumulation in a stream community were assessed in a 10 days microcosm experiment. The results indicated that UV-B and TOC affect Zn bioavailability and toxicity by impacting species abundance, behavior, and ecosystem processes (Kashian *et al.*, 2004).

Two microcosm-scale wetlands (570-liter containers) were designed and constructed to investigate transfers and transformations of zinc associated with an aqueous matrix, and to provide future design parameters for pilot-scale constructed wetlands. The fundamental design was based on biogeochemical principles regulating fate and transformations of zinc (pH, redox etc.). Each wetland consisted of a 45cm hydrosol depth inundated with 25 cm of water, and planted with *Scirpus californicus*. Zinc as ZnCl₂ was amended to each wetland for 62 days. Total recoverable zinc was measured daily in microcosm inflow and outflows, and zinc concentrations in hydrosol and *S. californicus* tissue were measured pre- and post-treatment. *Ceriodaphnia dubia* and *Pimephales promelas* 7 day aqueous toxicity tests were performed on wetland inflows and outflows, and *Hyaella azteca* whole sediment toxicity tests (10 day) were performed pre- and post-treatment. Data illustrated the ability of designed wetlands to transfer and sequester zinc from the water column while concomitantly decreasing associated toxicity (Gillespie *et al.*, 1999).

Hydrocarbons

Mahmoudi *et al.* (2005) carried out a microcosm experiment to study the influence of diesel on a free living nematode community of a Tunisian lagoon. Sediments were contaminated by increasing diesel concentrations and effects were examined after 90 days. Gradual changes in community structure were revealed depending on the quantity of diesel administered. Results from multivariate analyses of the species abundance data demonstrated that responses of nematode species to the diesel treatments were varied: some of them were "sensitive" species (intolerant or significantly affected), others appeared to be "opportunistic" species (significantly increased at certain concentrations of diesel) and others proved to be "diesel-resistant" species (increased at all high doses).

If the response of benthic communities to diesel contamination is altered by the historical exposure to hydrocarbon pollution was investigated by Carman *et al.* (2000) using a microcosm experiment employing two benthic salt-marsh communities (dominated by the cord grass *Spartina alterniflora*, *Juncus roemerianus* and including nematode, ostracod, naupli, copepod, algal biomass), one chronically exposed to petroleum hydrocarbons for decades (LA) and the other relatively uncontaminated (MS). It was concluded that the MS

community was more sensitive to diesel contamination than was the LA community.

A field-based microcosm experiment was conducted to determine whether natural and other types of high-molecular weight petroleum hydrocarbons produce similar effects on indigenous benthic macroinvertebrates as was induced by synthetic motor oils (Anson *et al.*, 2008)

The response of zooplankton and phytoplankton communities to creosote-impregnated Douglas fir pilings (as a common source of polycyclic aromatic hydrocarbons) was investigated in freshwater microcosms for 83 days. It was proven that creosote leached from impregnated pilings deployed under typical conditions (*e.g.* wharves) may cause transient toxicity to benthic or limnetic communities shortly after deployment, but this likely poses few long-term risks to aquatic freshwater plankton communities (Sibley *et al.*, 2001; 2004). Another outdoor microcosm study evaluated the kinetics of immunomodulation in rainbow trout (*Oncorhynchus mykiss*) exposed to liquid creosote. Fish were sampled from microcosms dosed initially with creosote. Pronephros leukocytes were monitored for phagocytic activity, oxidative burst, and surface immunoglobulin-positive (SIg+) B-cell counts. The overall results confirmed that creosote has the potential to alter certain immune parameters, and emphasize the importance of monitoring changes in the immune system during exposure. Polycyclic aromatic hydrocarbons, a major constituent of liquid creosote, were the suspected immune-altering agents (Karrow *et al.*, 2001).

Mixtures

A microcosm experiment was carried out to study the influence of cadmium and diesel, individually and in a mixture, on a free living nematode community. Sediments were contaminated with cadmium, by diesel, by a cadmium-diesel mixture and effects were examined after 90 days. All the species, "cadmium-diesel sensitive" or "cadmium-diesel resistant", were not affected by either cadmium or diesel alone (Beyrem *et al.*, 2007).

Surfactants

The toxicity of perfluorooctane sulfonic acid (PFOS) – anthropogenic contaminant renowned for its persistence - to the aquatic macrophytes *Myriophyllum sibiricum* and *M. spicatum* was investigated using 12 m³ outdoor microcosms. Replicate microcosms (n = 3) were treated with four concentrations of PFOS as the potassium salt and assessed at regular intervals during a period of 42 days. *M. sibiricum* was more sensitive to PFOS under simulated field conditions than *M. spicatum*. A risk assessment for these plants estimated a negligible probability of toxicity from PFOS exposure at current environmental concentrations (Hanson *et al.*, 2005). Furthermore the persistence of PFOS was investigated over 285 days in microcosms under natural conditions. PFOS concentration showed no drastic reduction in any treatment microcosm over the entire study period, confirming that this compound undergoes little degradation in aquatic systems (Boudreau *et al.*, 2003).

Oakes and his team investigated reproductive impairment and biochemical changes in fathead minnow (*Pimephales promelas*) exposed for 39 days to varying concentrations of perfluorooctanoic acid (PFOA) under microcosm conditions. While the concentrations tested were much higher than those normally found in the environment, no mortality was

associated with PFOA exposure. Perfluorooctanoic acid appears to be relatively nontoxic at environmentally relevant concentrations but may impact biochemical and reproductive endpoints under conditions associated with environmental spills (Oakes *et al.*, 2004).

Halogenated organic compounds

The fate and toxicity of monochloroacetic acid, chlorodifluoroacetic acid and a binary mixture of trichloroacetic acid and trifluoroacetic acid to the same macrophytes species as presented above for surfactants was assessed by Hanson and the team using 12m³ outdoor microcosms (semi-natural field conditions) for 49 days. The plants were sampled at regular intervals and assessed for the somatic endpoints of plant length, root growth, number of nodes and wet and dry mass and the biochemical endpoints of chlorophyll-a, chlorophyll-b, carotenoid content and citric acid levels (Hanson *et al.*, 2001; 2002a; 2002b).

Pharmaceuticals

In order to investigate the effects of a realistic pharmaceutical mixture on an ecosystem, studies utilizing 12m³ aquatic microcosms under semi-field conditions was carried out by Brain and co-workers (2004; 2005). The microcosms were treated with eight common pharmaceuticals (atorvastatin, acetaminophen, caffeine, sulfamethoxazole, carbamazepine, levofloxacin, sertraline, and trimethoprim). Phytotoxicity was assessed on a variety of somatic and pigment endpoints in rooted (*Myriophyllum sibiricum*) and floating (*Lemna gibba*) macrophytes over a 35 day period. EC₁₀, EC₂₅ and EC₅₀ values were calculated for each endpoint exhibiting a concentration-dependent response. Through single compound 7 day daily static renewal toxicity tests with *L. gibba*, the sulfonamide antibiotic sulfamethoxazole, the fluoroquinolone antibiotic levofloxacin and the blood lipid regulator atorvastatin were found to be the only compounds to elicit phytotoxic effects in the concentration range utilized. The microcosm data suggested that biologically significant risks are low for *L. gibba* and *M. sibiricum* exposed to similar mixtures of pharmaceutical compounds.

Antifouling biocides

A microcosm approach was designed to study the combined effects of tributyltin (TBT) from antifouling paints and ultraviolet-B radiation (UVBR: 280-320 nm), on a natural planktonic assemblage (<150µm). Microcosms (9 L, cylindrical Teflon bags, 75 cm height x 25 cm width) were immersed in the water column of mesocosms (1800 L, polyethylene bags, 2.3 m depth) and exposed to two different UVBR regimes: natural ambient UVBR (NUVBR), and enhanced level of UVBR (HUVBR). During consecutive 5 days, effects of a certain TBT concentration and enhanced UVBR were monitored. The results clearly demonstrate that the combination of TBT and UVBR stresses have synergistic effects affecting the first trophic levels of the marine food web (Sargian *et al.*, 2005).

C) Combined microcosm and field approach

The validity and effectiveness of the PERA-TE (probabilistic ecological risk assessment-toxic equivalent) combination approach has been tested in field microcosm studies using pesticide mixtures. The related laboratory studies, using *Daphnia magna*, were conducted to verify the conclusions made regarding the toxicity and interaction of the mixtures tested in the microcosms. Two types of pesticide mixture were assessed: the first consisted of pesticides with similar modes of action (chlorpyrifos, diazinon, and azinphosmethyl), and the second consisted of pesticides with different modes of action (chlorpyrifos, endosulfan, and trifluralin). The results supported the conclusions drawn from the field studies (George, Liber, 2007).

The potential effects of the organophosphate insecticide azinphosmethyl (AZP) in a combined microcosm and field approach were evaluated. Stones free of contamination were transferred from the river to outdoor microcosms (1.5 x 0.2 x 0.2 m), providing 12 core species and approximately 350 individuals per microcosm. Microcosms were contaminated with AZP and acute effects on survival were evaluated 6 days following exposure. In parallel, a quantitative macroinvertebrate survey was conducted at the control site and the contaminated site of the river after the seasonal pesticide application period. Five of the eight species that were affected by AZP in the microcosm study occurred in the field at significantly lower densities at the contaminated than at the control site or were absent at the contaminated site. All of the four species that were unaffected in the microcosm occurred at significantly higher densities at the contaminated field site. Only 3 of the 12 species reacted differently in the microcosm and the field study. Therefore it was concluded that microcosm studies employing a field-relevant design could be linked successfully to field studies (Schulz *et al.*, 2002).

Thiere and Schulz (2004) investigated the acute (5 days) effects of particle-associated azinphosmethyl (pesticide, acetylcholine esterase inhibitor) in multispecies microcosms and assessed the results in the context to data obtained from a parallel field study. They concluded that particle-associated azinphosmethyl has the potential to affect the invertebrate community structure in the field site and that microcosm studies employing field-relevant exposure scenarios may be valuable for a local risk assessment of pesticide-related community disruptions.

Table XXII. Acute toxicity of different ILs tested on aquatic plants, invertebrates and vertebrates

Organism Group	Specie	Ionic liquids tested ^a	Method	Endpoint ^b	Reference
Plants	<i>Oocystis submarina</i> (green alga)	Imidazolium IM12 BF ₄ IM14 BF ₄ IM16 BF ₄ IM1-1Ph BF ₄	Growth inhibition test (11 days)	500 µM → I _n ~ 80 % → I _n ~ 40 % → I _n ~ 70 % → I _n ~ 50 %	Latała <i>et al.</i> , 2005
	<i>Cyclotella meneghiniana</i> (diatom)		Growth inhibition test (10 days)	500 µM → I _n ~ 75 % → I _n ~ 65 % → I _n ~ 70 % → I _n ~ 75 %	
	<i>Selenastrum capricornutum</i> (algae)	Imidazolium IM14 PF ₆ IM1-12 Cl IM1-16 Cl IM1-18 Cl Phosphonium P444-2 (EtO) ₂ PO ₂ P666-14 Cl Ammonium N888-1 N(SO ₂ CF ₃) ₂ Ecoeng 500 Anion effect NaPF ₆ Na MeSO ₄ HN(SO ₂ CF ₃) ₂	Algal growth inhibition (72h)	48h EC ₅₀ = 45 mg/L = 158 µM = 0,0011 mg/L = 0,003µM = 0,0041 mg/L = 0,011µM = 0,0129 mg/L = 0,034µM = 6,2 mg/L = 16,14 µM = 0,042 mg/L = 0,08 µM = 0,058 mg/L = 0,09 µM = 0,088 mg/L = 0,109µM = 42 mg/L = 250 µM > 100 mg/L ~ > 746 µM = 61 mg/L = 217 µM	Wells and Combe, 2006
				<i>Lemna minor</i> (aquatic plant)	Imidazolium IM14 BF ₄ IM18 BF ₄
Crustaceans	<i>Daphnia magna</i>	Imidazolium IM14 Br IM16 Cl IM18 Cl IM14 BF ₄ IM16 BF ₄ IM18 BF ₄ IM14 PF ₆ IM16 PF ₆ IM18 PF ₆	Immobilisation test (24h)	IC ₅₀ = 60,25 µM = 12,3 µM = 3,47 µM = 61,65 µM = 13,43 µM = 4,57 µM = 89,12 µM N.A. N.A.	Garcia <i>et al.</i> , 2005

^b I_n = percentage inhibition of the cell growth; N.A. = not available

Table XXII. (continued)

Organism group	Specie	Ionic liquid tested ^a	Method	Endpoint	Reference
Crustaceans	<i>Daphnia magna</i>	Imidazolium IM14 PF ₆ IM1-12 Cl IM1-16 Cl IM1-18 Cl Phosphonium P444-2 (EtO) ₂ PO ₂ P666-14 Cl Ammonium N888-1 N(SO ₂ CF ₃) ₂ EcoEng 500 Anion effect NaPF ₆ Na MeSO ₄ HN(SO ₂ CF ₃) ₂	Acute toxicity (48h)	48h EC₅₀ = 24 mg/L = 84 μM = 0,0043 mg/L = 0,015 μM = 0,0034 mg/L = 0,01 μM = 0,0017 mg/L = 0,004 μM = 11 mg/L = 28,6 μM = 0,072 mg/L = 0,14 μM = 0,2 mg/L = 0,31 μM = 1 mg/L = 1,24 μM >100 mg/L ~> 595 μM > 100 mg/L ~> 746 μM = 76 mg/L = 270 μM	Wells and Combe, 2006
		Imidazolium IM14 Br IM14 BF ₄ IM14 PF ₆ Anion effect NaBF ₄ NaPF ₆	Acute toxicity (48h)	LC₅₀ = 8,03 mg/L = 36,63 μM = 10,68 mg/L = 47,2 μM = 19,91 mg/L = 70 μM LC₅₀ = 4765,75 mg/L = 43,3 * 10 ³ μM = 9344,81 mg/L = 55,6 * 10 ³ μM	Bernot <i>et al.</i> , 2005a
		Imidazolium IM16 Br IM18 Br IM16-2Me Br Pyridinium Py4 - 3 Me Br Py6 - 3 Me Br Py8 - 3 Me Br Py4 -3Me-5Me Br Py6 Br Py6-4(Me2N) Br Py6-3-Me- 4(Me2N) Br Py6-4Pip Br Phosphonium P4444 Br Ammonium N4444 Br	Acute toxicity (48h)	LC₅₀ = 6 μM = 0,04 μM = 6,45 μM = 57,5 μM = 3,9 μM = 2,51 μM = 97,7 μM = 11,7 μM = 0,52 μM = 1,62 μM = 0,22 μM = 8,91 μM = 29,5 μM	Couling <i>et al.</i> , 2006

Table XXII. (continued)

Organism group	Specie	Ionic liquid tested ^a	Method	Endpoint	Reference
Gastropodes (Snails)	<i>Physa acuta</i>	Imidazolium IM14 Br IM16 Cl IM18 Cl IM14 PF ₆ Pyridinium Py4-3Me Br Py6-3Me Br Py8-3Me Br Phosphonium P4444 Br Ammonium N4444 Br	Acute toxicity (96h -static)	LC₅₀ = 229 mg/L = 1044,8 μM = 56,2 mg/L = 227,4 μM = 8,2 mg/L = 29,8 μM = 123,3 mg/L = 434 μM = 325,2 mg/L = 1413 μM = 226,7 mg/L = 878 μM = 1 mg/L = 3,49 μM = 208 mg/L = 613,6 μM = 580,2 mg/L = 1802 μM	Bernot <i>et al.</i> , 2005b
Fish	<i>Danio rerio</i> (Zebra fish)	Imidazolium IM12 Me-4-C ₆ H ₄ SO ₃ IM14 PF ₆ IM14 BF ₄ IM14 NO ₃ IM14 N(SO ₂ CF ₃) ₂ IM14 CF ₃ SO ₃ IM14 N(CN) ₂ IM14 -2Me PF ₆ Pyridinium Py4 N(SO ₂ CF ₃) ₂ Pyrrolidinium Pyrr4 N(SO ₂ CF ₃) ₂ Ammonium AMMOENG 110 AMMOENG 112 AMMOENG 100 AMMOENG 130	Acute toxicity (96h -static)	LC₅₀ > 100 mg/L > 355 μM > 252 μM > 442 μM > 497 μM > 238 μM > 347 μM > 653 μM > 335 μM > 240 μM > 237 μM LC₅₀ > 100 mg/L LC₅₀ < 100 mg/L	Pretti <i>et al.</i> , 2005

^a the names and structures of the tested ionic liquids cations and anions are given in Table VI and Table VII from Chapter 2.3.

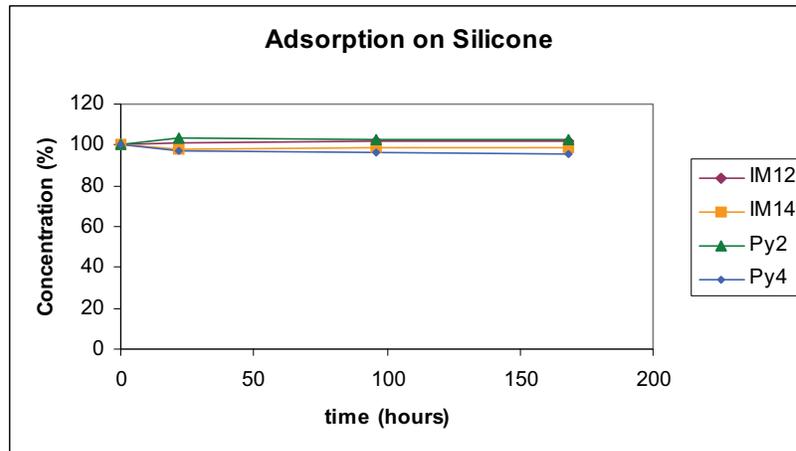


Figure 29. Adsorption of the selected ILs on silicone (component in the walls and lattice adhesive from AquaHab[®])

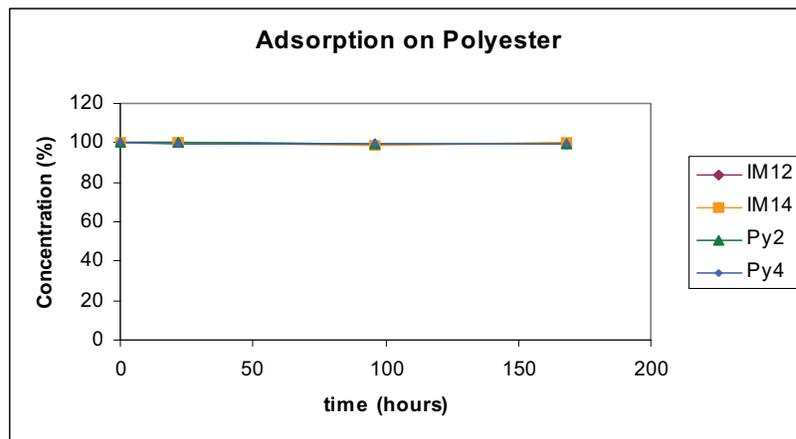


Figure 30. Adsorption of the selected ILs on polyester (component in the filter cotton from AquaHab[®])

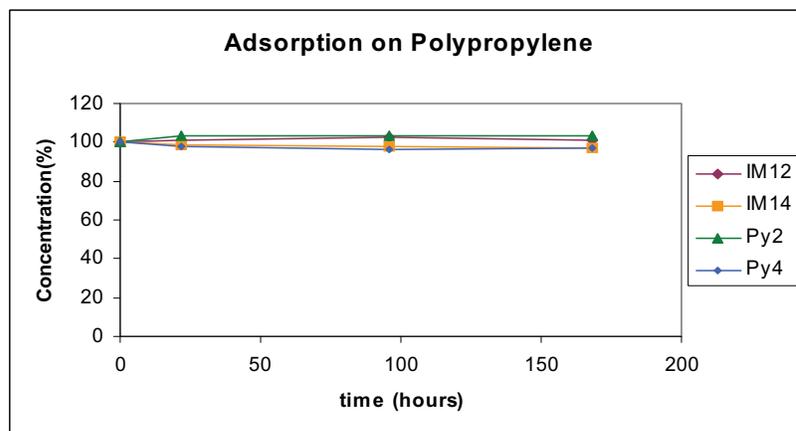


Figure 31. Adsorption of the selected ILs on polypropylene (component in the lattice filter from AquaHab[®])

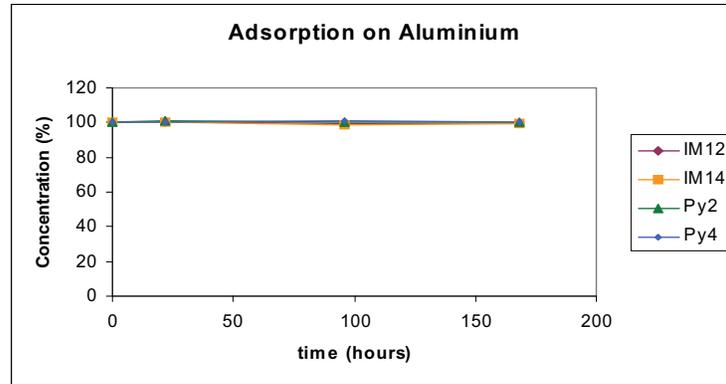


Figure 32. Adsorption of the selected ILs on aluminium (component in the separation wall from AquaHab[®])

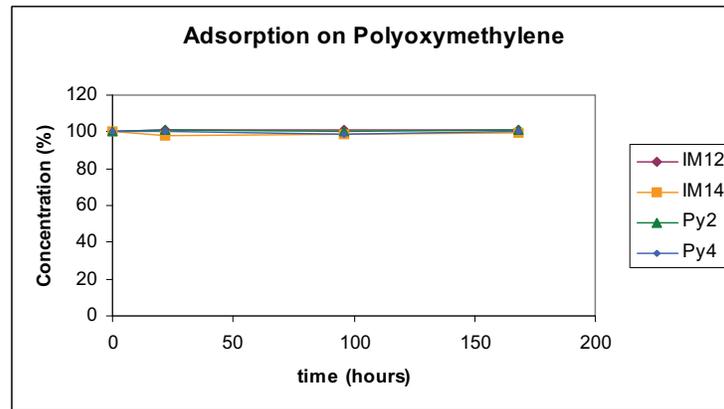


Figure 33. Adsorption of the selected ILs on polyoxymethylene (component in the connectors and sensors from AquaHab[®])

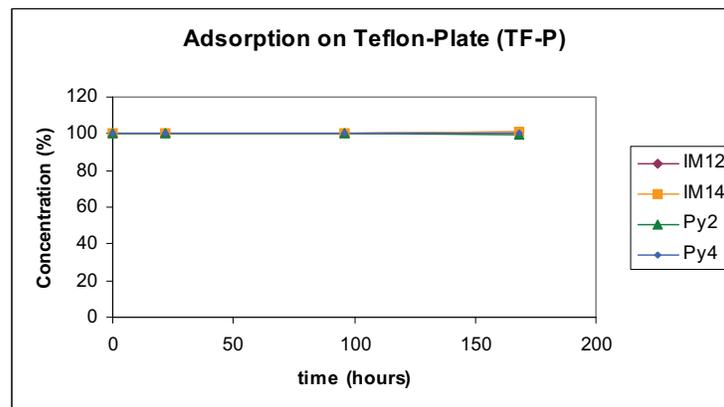


Figure 34. Adsorption of the selected ILs on polytetrafluoroethylene (component in the water pump from AquaHab[®])

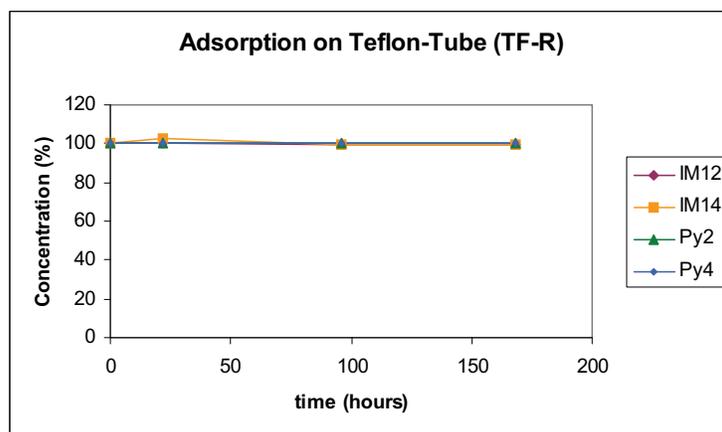


Figure 35. Adsorption of the selected ILs on polytetrafluoroethylene (component in the closures from AquaHab[®])

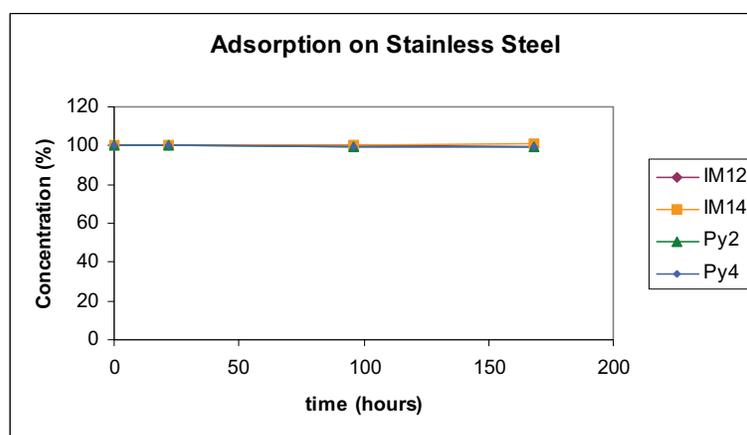


Figure 36. Adsorption of the selected ILs on stainless steel (component in the water pump/flow-through/cooling unit from AquaHab[®])

Curriculum Vitae

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