Universität Bremen*

Zentrum für Umweltforschung & nachhaltige Technologien

Degradability of ionic liquids

Systematic investigations on aerobic and anaerobic biodegradability of ionic liquid cations and anions as well as on the hydrolytical stability of ionic liquid anions - contributions to a sound hazard assessment

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Declaration:
Herewith I declare that this thesis is the result of my independent work. All sources and auxiliary materials used by me in this thesis are cited completely.

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Bremen, 31 October 2013

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Bremen, 31. Oktober 2013

ERKLÄRUNG

Hiermit erkläre ich, dass ich die Doktorarbeit mit dem Titel:

Degradability of ionic liquids
Systematic investigations on aerobic and anaerobic biodegradability of ionic liquid cations and anions as well as on the hydrolytical stability of ionic liquid anions - contributions to a sound hazard assessment

selbständig verfasst und geschrieben habe und außer den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

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(Unterschrift)

To all those we love

“Let us seize the day,

Even though the sun is setting, nothing will be lost, …

Looking at the horizon, I see the one who never gives up.“

Freely translated from Münchhausen (Stuttgart, 2012)

Summary

The presented studies deal with the degradability of ionic liquids under conditions that occur in biological waste water treatment and within the environment. Degradation studies are relevant for the assessment of the technological applicability of chemicals and of their hazard potential, including their environmental fate. In former studies some ionic liquids have shown to remain stable against abiotic and biotic degradation processes. The systematic investigation of chemical structures which are more easier subject to degradation processes shall help to design ionic liquids that, although stable in technical applications, avoid the risk of environmental persistency and is the aim of the presented studies. It is not only the chemical structure that influences the (bio)degradability of a substance; environmental conditions also play a major role. For instance, the availability of oxygen affects the composition of the microbial community and the activity of enzymes that are involved in the degradation process. In previous biodegradation studies the focus was only laid on aerobic conditions, showing a tendency towards low biodegradation potential for most of the investigated ionic liquids. Therefore, the question arose whether the (bio)degradability of ionic liquids can be enhanced, (1) by changing the environmental redox conditions for bacterial growth from aerobic to nitrate-reducing ones, (2) by changing the structural composition of the ionic liquid, namely the anion and the cationic head group, and (3) by specifically selected bacteria for an enhanced biodegradation process. Moreover, the question of whether cyano-based ionic liquid anions that were not biodegradable can be degraded abiotically at different pH values or enzymatically hydrolysed in vitro has been discussed. The investigations showed that the biodegradability of the tested ionic liquid cations with alkyl side chains are even worse under denitrifying conditions than under aerobic ones. However, the one with a hydroxylated side chain could still be biodegraded under the anaerobic conditions. Under aerobic conditions, the change of the cationic head group can enhance the biodegradability of ionic liquids when combined with an appropriate side chain. None of the investigated fluoroorganic and cyano-based ionic liquid anions could be biodegraded neither under aerobic nor denitrifying conditions, nor by the cyanide-degrading bacteria strain *Cupriavidus spp.* All of these anions were further stable under neutral and slightly basic conditions in the hydrolytical test procedure. However, in strong acidic and basic solutions, $N(CN)_2^-$ and $C(CN)_3^-$ showed hydrolytical degradation potential without the formation of hydrogen cyanide. The hydrolytical transformation could further be enhanced enzymatically in vitro at pH 7 by nitrile hydratase leading to the hydrolysis of all of the cyano-based anions. The data concerning the (bio)degradability contribute to the hazard assessment of ionic liquids and support the proactive substance design for inherently safer chemicals.

Zusammenfassung

Die vorliegenden Studien behandeln die Abbaubarkeit von ionischen Flüssigkeiten unter Bedingungen, die in der biologischen Abwasserreinigungen und in der Umwelt auftreten. Abbaustudien sind relevant für die Bewertung der technologischen Anwendbarkeit von Chemikalien einerseits und ihres Gefährdungspotenzials, einschließlich des Umweltverhaltens, andererseits. Vorherige Studien haben gezeigt, dass sich einige ionische Flüssigkeiten weder abiotisch noch biotisch abbauen lassen. Die systematische Untersuchung auf chemische Strukturen, die abgebaut werden können, soll dabei helfen solche ionischen Flüssigkeiten zu entwerfen, die, obwohl stabil bei technischer Anwendung, ein geringeres Risiko von Langzeitstabilität in der Umwelt haben. Das ist das Ziel der vorliegenden Studien. Dabei ist es nicht nur die chemische Struktur, die den (biologischen) Abbau einer Substanz beeinflusst; Umweltbedingungen spielen zusätzlich eine große Rolle. Zum Beispiel beeinflusst die Verfügbarkeit von Sauerstoff, ebenso wie die Zusammensetzung der mikrobiellen Gemeinschaft und die Aktivität von Enzymen den Abbauprozess. In früheren Untersuchungen zum biologischen Abbau lag der Fokus ausschließlich auf aeroben Bedingungen. Das Ergebnis war eine tendenziell niedrige biologische Abbaubarkeit von den meisten der untersuchten ionischen Flüssigkeiten. Daher stellte sich die Frage, ob die (biologische) Abbaubarkeit von ILs gesteigert werden kann, (1) indem die Redoxbedingung für das Bakterienwachstum von aerob zu nitratreduzierend geändert wird, (2) indem die strukturelle Zusammensetzung der ionischen Flüssigkeiten bezogen auf ihr Anion und die kationische Kopfgruppe verändert werden und (3) durch speziell ausgewählte Bakterien für einen gesteigerten biologischen Abbauprozess. Darüber hinaus wurde die Frage, ob cyanobasierte Anionen, die nicht biologisch abbaubar sind, abiotisch bei verschiedenen pH-Werten oder enzymatisch in vitro hydrolysiert werden können, diskutiert. Die Untersuchungen zeigten, dass die biologische Abbaubarkeit der getesteten Kationen der ionischen Flüssigkeiten mit Alkylseitenketten unter denitrifizierenden Bedingungen noch geringer ist als unter aeroben Bedingungen. Allerdings konnte das untersuchte Kation mit einer hydroxylierten Seitenkette auch noch unter diesen anaeroben Bedingungen biologisch abgebaut werden. Unter aeroben Bedingungen kann mit einer anderen kationischen Kopfgruppe eine Verbesserung der biologischen Abbaubarkeit von ionischen Flüssigkeiten erreicht werden, wenn diese mit einer angemessenen Seitenkette kombiniert wird. Keiner der untersuchten fluororganischen und cyanobasierten Anionen von ionischen Flüssigkeiten konnte unter aeroben oder denitrifizierenden Bedingungen biologisch abgebaut werden. Selbst durch die cyanidabbauenden Bakterien *Cupriavidus spp*. konnte der Abbau nicht verbessert werden. Zwar blieben alle untersuchten Anionen bei Untersuchungen zur Hydrolyse unter neutralen und leicht basischen Bedingungen weiterhin stabil, jedoch wurden in stark sauren oder basischen Lösungen $N(CN)_2^-$ und $C(CN)_3^-$ schrittweise hydrolysiert, auch ohne die Bildung von Blausäure. Durch die Verwendung von Nitril-Hydratase konnte die hydrolytische Umwandlung im neutralen pH-Bereich weiter verbessert werden. Das führte schließlich zur Hydrolyse aller cyanobasierten Anionen. Die Daten über die (biologische) Abbaubarkeit tragen zu einer Beurteilung des Gefährdungspotenzials von ionischen Flüssigkeiten bei und unterstützen die proaktive Gestaltung inhärent sicherer Chemikalien.

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Content

Table of abbreviations

A-	Anion
biodegr.	biodegradable
C^+	Cation
C6	Hexyl group
CAS	Chemical Abstracts Service
CLEAs	Cross-linked enzymes aggregates
CMR	Carcinogenic, Mutagenic and toxic for Reproduction
cP	Centipoise (in dynamic viscosity; 1 cP = 1 g m^{-1} s^{-1} = 0.1 Pa*s)
d	Day or smaller diameter of ellipse
DDT	1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane
Dep.	Department
doi	Digital object identifier
Dr. h. c.	Doctor honoris causa, honorary doctorate
Dr.-Ing.	Doktor der Ingenieurwissenschaften, German engineering doctorate
Dr. rer. nat.	Doctor rerum naturalium, doctor of natural sciences
e.g.	Exempli gratia, for example
ECHA	European Chemical Agency
edn.	Edition
eq.	Equation
ESI-MS	Electronspray ionisation – mass spectrometry
et al.	Et alii, and others
etc.	Et cetera, and so on
Fig.	Figure
h	Hour(s)
HPLC	High-performance liquid chromatography
ID code	Identification code
IL	Ionic liquid
ILs	Ionic liquids
Inh.	Inhabitants
IPC-81	Rat leukaemia cells
ISWA	Institut für Siedlungswasserbau, Wassergüte- und Abfallwirtschaft, Institute for Sanitary Engineering, Water Quality and Solid Waste Management
IUPAC	International Union of Pure and Applied Chemistry
KS-7D	Bacterial mixture of *Cupriavidus basilensis* and *Cupriavidus eutrophus*
LC-MS	High - Pressure Liquid Chromatography - Mass Spectrometry

Ltd.	Private company limited by shares
m^3	Cubic metre
M.Sc.	Master of Science
mp	Melting point
MPa	Megapascale (1 MPa = 10^6 kg/ms^2)
NAILs	Naphthenic acid ionic liquids
NHase	Nitrile hydratase
NLase	Nitrilase
No.	Numero, number
OECD	Organisation for Economic Co-operation and Development
PBT	Persistent, Bioaccumulative and Toxic
POPs	Persistent Organic Pollutants
pp.	Paginae, pages
Prof.	Professor
PVC	Polyvinyl chloride
R	Rest of a chemical structure
$R_{1,2,3,4}$	Chemical side chain No. 1, 2, 3 or 4
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SAE	Society of Automotive Engineers
SM	Service Mark
sp.	Species (singular, in biology)
spp.	Species (plural, in biology)
SVHC	Substances of very high concern
Tab.	Table
T-SAR	Thinking in terms of structure-activity relationship
UBA	Umweltbundesamt, German Federal Ministry of Health
UFT	Zentrum für Umweltforschung und nachhaltige Technologien; Centre for Environmental Research and Sustainable Technology
UN	United Nations
UNCED	United Nations Conference on Environment and Development
UNCHE	United Nations Conference on the Human Environment
UNEP	United Nations Environment Programme
UV-Vis	Ultraviolet–visible
Verl.	Verlag, Publisher
vPvB	Very Persistent and very Bioaccumulative
WHO	World Health Organisation

Table of symbols

®	Registered trademark
β	beta
ω	omega
%	Percentage
#	Any number
*	Wildcard character (in internet search)
~	Approximately
>	Larger than
≥	Larger than or equal as
°C	Degree Celsius (in temperature measurement)
1st	First
1980s	The Nineteen-Eighties; here: years 1980-1989

List of tables

List of figures

Nomenclature of ionic liquids

The concept of naming a chemical according to the International Union of Pure and Applied Chemistry (IUPAC) is standard and understood all around the chemical world, but it leads to one specific problem in the case of ionic liquids (ILs): the name is too long to be easily read and quickly understood in its chemical composition, e.g. 1-butyl-3-methyl-3H-imidazolium bis(trifluoromethylsulfonyl)amide(1-). Therefore, different working groups have developed several abbreviations for ILs. One of the most widely used abbreviations for the aforementioned compound is “Bmim Ntf2”. The cation name “Bmim” is derived from the name components butyl (B), methyl (m) and imidazolium (im). Looking at the anion name “Ntf2”, the abbreviation is given in reverse order and starts from the back of the IUPAC name: the amide comes first (N), then the two trifluorinated methylsulfonyl groups (tf2). In other studies this anion is abbreviated as BTA, which is derived from the IUPAC name in the reading order. In addition to the confusing abbreviations in different reading orders, abbreviations that are derived from the name can also be misleading in the case of ILs - 1-methyl-3-propyl-3H-imidazolium bromide and 1-methyl-3-pentyl-3H-imidazolium chloride can both be abbreviated as Mpim. To combat this confusion, the working group around Prof. Jastorff developed an “ID code” that is not based on the IUPAC name, but on the chemical structure of the ILs, e.g. IM14 for Bmim and IM13 or IM15 for Mpim, respectively. The cationic core structure comes first (IM) and then the number of carbon atoms in the alkyl side chains (e.g. 1 and 4). Functional groups can then be easily added, e.g. IM14OH for 1-(4-hydroxybutyl)-3-methyl-3H-imidazolium. Anions are usually abbreviated according to their structural formula. Ntf2, for example, is $(CF_3SO_2)_2N^-$. In the presented studies ILs have been investigated according to their chemical structure guided by a “thinking in terms of structure-activity relationships” (T-SAR). The ID code based on the chemical structure of the IL as in IM14 $(CF_3SO_2)_2N$ is therefore used throughout this thesis. A list of abbreviations of the selected ILs can also be found below in: “ID code of ionic liquid cations - A short overview of ID codes used in this thesis”.

For further information and a more detailed description see:

The UFT / Merck Ionic Liquids Biological Effects Database
http://www.il-eco.uft.uni-bremen.de/index.php?page=home&chent_id=&view=ilcode&lang=en#intro

All molecular structures used in this thesis have been taken from the abovementioned web page, except for naphthenic acid and those structures containing “R”. They were drawn using Symyx Draw 4.0.

ID code of ionic liquid cations - A short overview of ID codes used in this thesis

Cationic head groups

IM1R	1-Alkyl-3-methylimidazolium
IM1R-2Me	1-Alkyl-1,2-dimethylimidazolium
PyR	1-Alkylpyridinium
PyR-4NMe2	1-Alkyl-4-(dimethylamino)pyridinium
PyR-3COOBu	1-Alkyl-3-(butoxycarbonyl)pyridinium
Pyr1R	1-Alkyl-1methylpyrrolidinium
Pip1R	1-Alkyl-1-methylpiperidinium
$PR_1R_2R_3R_4$	Tetraalkylphosphonium
$NR_1R_2R_3R_4$	Tetraalkylammonium
$SR_1R_2R_3$	Trialkylsulfonium

Side chains

R = 2	Ethyl
R = 3	Propyl
R = 4	Butyl
R = 8	Octyl
R = 1CO02	2-Ethoxy-2-oxoethyl
R = 1CO04	2-Butoxy-2-oxoethyl
R = 1CONHBu	2-(Butylamino)-2-oxoethyl
R = 2OH	2-Hydroxyethyl
R = 3OH	2-Hydroxypropyl
R = 8OH	2-Hydroxyoctyl
R = 1O2	Ethoxymethyl
R = 2O1	Methoxyethyl
R = 2O2	Ethoxyethyl
R = 1CN	Cyanomethyl

Anions

Molecular formulas are used as abbreviations.

1 Introduction

"I see trees of green, red roses too. I see them bloom for me and you, and I think to myself: What a wonderful world."

Bob Thiele and George David Weiss

In 1968, the famous American jazz singer and trumpeter Louis Armstrong sang the lines above – the first words of the song "What a Wonderful World". In my understanding, this song describes the ideal perfect world: one that contains human beings and their environment in perfect harmony. However, at that time the balance between human life and natural habitat had already been threatened. Only six years prior to the song, the biologist Rachel Carson had published the book "Silent Spring" (Fig. 1) as a warning to the chemical industry to reduce the use of pesticides, in order to prevent negative effects on the ecological system. From observations on wildlife, she feared that especially 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane, DDT for short, and other chlorinated organic compounds may lead to the death of animals, mainly birds, resulting in a literally silent springtime.[1] As a consequence, the use and misuse of chemical pesticides has largely been debated.[2]

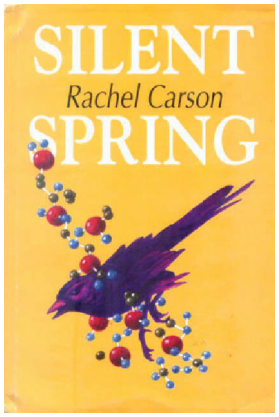


Fig. 1: Silent Spring. Cover of the Indian edition.[1,12]

Finally, in 1972 the first environmental world conference, the United Nations Conference on the Human Environment (UNCHE) was held in Stockholm. It was then officially declared that the human environment affects not only the life of people, but also the economy throughout the world. Therefore, it was decided that the participating states needed to work together on the protection and improvement of the state of the world.[3] As a result, the United Nations Environment Programme (UNEP) was founded to support and strengthen the results from Stockholm transnationally over the following years. In this context, science and technology both played a key role as they were seen as a provider of solutions for the environmental problems when used for the "identification, avoidance and control of environmental risks".[4] However, the evaluation of the benefits and risks of environmental hazards often leads to a huge controversy, of which the abovementioned DDT is one of the best examples.

DDT is the cheapest and most effective agent for vector control against *Anopheles* mosquitoes, which can transmit malaria parasites. By using DDT, it is therefore possible to protect millions of people, especially children, in developing countries against contracting the often-fatal malaria. However, it is also a severe hazard to the environment, has a drastic impact on wildlife population and is persistent, accumulative and magnifying within the biosphere and human beings.[5]

So, even 50 years after the publication of "Silent Spring", the debate regarding the usefulness and risks of DDT still goes on.[6,7] Humankind lives in the dilemma of providing adequate living conditions for a world population that is still growing without exhausting the environmental capacities for maintaining and developing a world worth living in.[8]

1.1 Sustainable development and Green Chemistry

"Human beings are at the centre of concerns for sustainable development. They are entitled to a healthy and productive life in harmony with nature."

Principle 1 of the Rio declaration [9]

The best way to maintain the balance between the benefits and risks of human development was largely debated during and after the first environmental world conference UNCHE in 1972.[10,11] One idea then finally gained popularity in Rio de Janeiro in 1992 at the UN conference on Environment and Development (UNCED)[9]: "sustainability". This term was already used in 1713 to refer to responsible forestry operations,[12] and was redefined for modern society in *Our Common Future* in 1987 "to meet the needs of present generations without compromising the ability of future generation to meet their own needs".[11] This aim should be accomplished by applying a set of priorities in every area of our lives. In the course of the following years, sustainability became the key concept for a safe development of the future in balance with our natural habitat.

These debates and the necessity for a sustainable development laid the foundations for further political action, so that at the Stockholm Convention in 2001, almost 40 years after the publication of Rachel Carson's "Silent Spring", the use of DDT was finally restricted, together with further selected chemicals whose use was limited or even completely banned. The criteria for a chemical's restriction or exclusion from the global market had been set up based upon the most relevant impacting factors that influence health and environment: persistency, bioaccumulation, long-range transport within the environment and toxic effects in wildlife and humans. Since the abovementioned restricted and banned chemicals are negatively related to these attributes, they have gained notoriety as the "Dirty Dozen".[13] Now, after more than a decade, the Stockholm Convention is still working on the identification, avoidance and minimisation of environmental hazards. Within this process, the "Dirty Dozen" did not remain the only chemicals of concern. At this point, 22 popular chemicals are categorised as Persistent Organic Pollutants (POPs) that need to be restricted or banned for the protection of human health and the environment.[14] Restriction and elimination could be even tighter in the next few years, as there are between 190 to 1200 potential POPs estimated, among them high-volume production chemicals with high industrial relevance.[15]

Within the European Union, the production and usage of chemicals is controlled by the European regulation "Registration, Evaluation, Authorisation and Restriction of Chemicals" (REACH), which

came into force in 2007. Since then, chemicals that are produced at a rate of more than one tonne per year have had to be registered by the European Chemical Agency (ECHA) for legal use. The license for use can only be given when the producers have tested their chemicals in respect to environmental and operational safety, and the ECHA has evaluated the data concerning their environmental impact with special attention on substances of very high concern (SVHCs). SVHCs can then only be authorised with time limitations, and their use can be restricted or even prohibited if the risks are higher than the social-economical benefit. Those measures are applied for chemicals that fulfil at least one of the following environmentally relevant criteria: being (1) carcinogenic, mutagenic, or toxic for reproduction (CMR), (2) persistent in the environment, accumulating in organisms and toxic (PBT), (3) very persistent in the environment and strongly accumulating in organisms (vPvB) or (4) possessing similar worrisome properties, e.g. causing endocrine disruption.[16] Although REACH is regarded as one of the most modern and strict chemical regulations in the world,[16] it is arguable that this is necessary to ensure a high protection level for human health and the environment in respect to the amount and impact of chemicals in our everyday life.

Consequently, the potential negative environmental impact and the process of eliminating well-used chemicals from the global market put pressure on the chemical industry to re-think the design and production processes of their chemicals. Along with the debate on the environmental impact of chemicals, the search for chemicals that are safe and do not harm human beings and the environment during their production, application or disposal became a major aim, resulting in a new philosophy known as "Green Chemistry". The thinking with respect to greener chemistry has become the strategic guideline of chemists that strive to use less hazardous substances, less energy and overall safer chemicals and processes in order to provide the basis for a sustainable future around the world. The corresponding strategic guidelines, The Twelve Principles of Green Chemistry, were finally formulated in 1998 by Paul T. Anastas and John C. Warner, and helped to identify and create greener processes.[17] They were later abbreviated as the mnemonic PRODUCTIVELY (see box above).[18]

P - Prevent wastes
R - Renewable materials
O - Omit derivatization steps
D - Degradable chemical products
U - Use safe synthetic methods
C - Catalytic reagents
T - Temperature, pressure ambient
I - In-process monitoring
V - Very few auxiliary substances
E - E-factor maximize feed in product
L - Low toxicity of chemical products
Y - Yes, it's safe

Mnemonic for the Twelve Principles of Green Chemistry[18]

Similar ideas have been applied to create Green Engineering, abbreviated as IMPROVEMENTS, as the twin philosophy for a sustainable development in engineering. The evaluation of the "greenness" of the overall technological process includes life cycle assessment, heat integration

and heat recovery within the process, amongst others.[19] The combination of Green Chemistry and Green Engineering has recently been modified for the application in African countries, and has been abbreviated as GREENER AFRICA. The natural prerequisites of the African continent and the low economically developed status have been taken into account.[20] These measures show that the ways to develop sustainably and care for social equality, economic growth and environmental health are very complex and may differ with respect to the discipline and in the parts of the world where they are applied. As a relatively young discipline in science, this field is still learning and defining what sustainable development is, what best promotes it and how to achieve it. The founders of Green Chemistry hoped for a situation in which to integrate the ideas for a sustainable development into the general practices of chemistry, so that the term Green Chemistry will one day be unnecessary, since all of the chemistry would be green.[21,22]

1.2 Eco-design for a sustainable development

> *"Green Chemistry is ensuring that all of that creative ability that is the long tradition of the field of chemistry is practised in a way that builds in impact on people and the planet as a design criterion."*
>
> *Anastas and Eghbali, 2010*[23]

An intelligent eco-design of chemicals and chemical processes is at the core of Green Chemistry. The knowledge of chemical reaction mechanisms is thereby used for the production of environmentally compatible substances and processes. The overall aim is the creation of inherently safer chemicals in an economically and technologically beneficial manner so that environmental risks are reduced.[17] The environmental risk is defined as the hazard of a chemical combined with its potential exposure. The minimisation of the exposure towards a chemical and a high intrinsic safety (low hazard potential) of the chemical, in case of an accidental release into the environment, will therefore maintain the risk for man and the environment at a low level. The Twelve Principles of Green Chemistry, mentioned above, comprise a set of priorities to create those chemicals with low environmental risk for a sustainable development in chemical production processes. They could already be applied in certain industrial processes and provide an alternative to conventionally less sustainable processes.[24,25] Although the implication of all principles of the Twelve Principles of Green Chemistry is desired, their fulfilment can also be partial. For example, the usage of toxicologically critical substances in closed-operational systems can sometimes be beneficial for a sustainable process considering economical and social aspects in comparison to a less toxic, but highly volatile and flammable, alternative.

The identification and development of inherently safer chemicals that are badly needed for technological applications with low environmental hazard potential is, in general, a tremendous task, as there are already more than 70 million chemicals registered in the database of the

Chemical Abstract Service (CAS)[26] and applied in various industrial processes. The evaluation of chemicals that have the highest impact on the greenness of a process was therefore the most logical starting point for the identification and development of inherently safer substances. The field in which a production of inherently safer chemicals was most needed was the utilisation of solvents.[27] Solvents represent the majority of wastes in synthesis and processes,[28] and conventional organic solvents usually possess properties that are disadvantageous for their operational safety and handling, such as high toxicity, flammability, corrosiveness and volatility. The occurrence of accidents and the release of these substances into the aerial, aqueous and terrestrial environment are most likely.[23] Solventless systems, water based processes, the usage of supercritical fluids or ILs are the best candidates for potentially greener alternatives.[23] Among those, the large substance group of ILs are the chemicals with the highest design potential.

1.3 Design potential of ionic liquids

"[…] we can compare it with a box of Lego® bricks of different colors and sizes, from which we choose an alkyl chain length, a cation and an anion to construct a structure with particular properties. It is clear that with only three different alkyl types, three anions and three cations, we can make 27 ionic liquids, with different properties!"

Seddon et al., 2000[29]

Ionic liquids is the term used to refer to a large group of diverse organic chemicals that are completely composed of ions with an unusually low melting point of less than 100 °C. Although discovered in 1914 and easier to handle than high temperature molten salts (NaCl mp = 801 °C),[30] it was only at the beginning of the 21st Century that a veritable boom in IL research commenced. The first effective industrial application was performed,[31] and they gained popularity as "designer solvents and catalysts" in various chemical processes, such as liquid-liquid extraction, and electrochemical and analytical applications.[32] Due to their ionic composition, which is accompanied by a very low volatility and flammability, they exhibited a much higher operational safety than conventional organic solvents, which is why they were also regarded as "green" alternatives.[33,34] The increasing interest in ILs also led to an immensely increasing number of publications that contain "ionic liquid*" in the title (Fig. 2). Although 20 years ago only very few chemists dealt with ILs,[33,35] the number of publications increased more than exponentially; since the start of the presented studies in 2008, the number of publications more than doubled and in the last 10 years more than 95% of all publications have been released.[36]

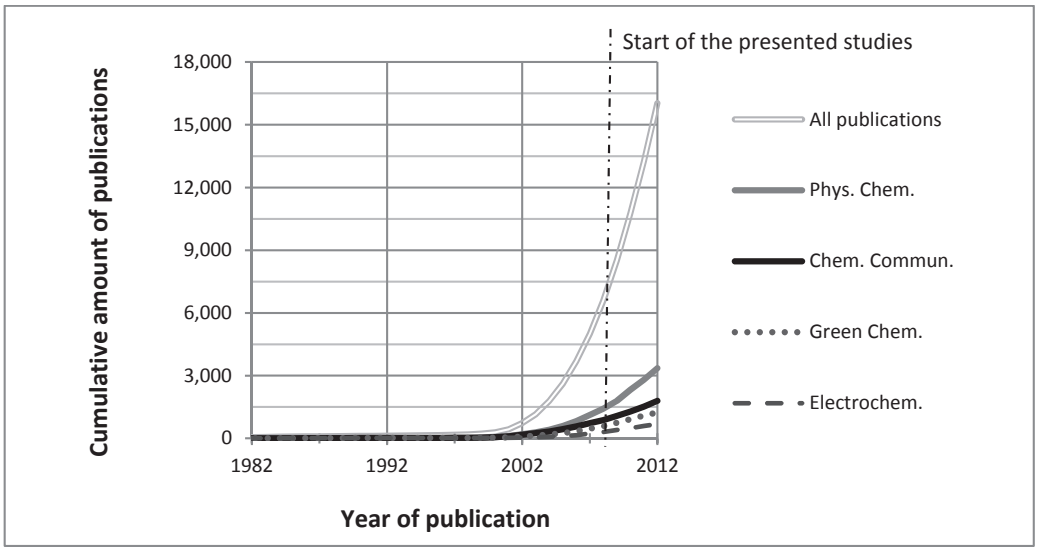


Fig. 2: Cumulative amount of articles, reviews and books with a title containing "ionic liquid*". Searched through Thomson Reuters Web of Knowledge℠ (accessed in January 2013).

Quite recently, the interest in ILs has increased beyond its original frame of electrochemistry,[a] Green Chemistry[b] and chemical novelties,[c] towards physico-chemical properties, such as solubility and reactivity of ILs itself or in ILs as media,[d] which are more and more studied for technical applications. The possibility of designing physico-chemical properties of ILs made this field into a huge playground for synthetic chemists – in fact, the estimated number of potential ILs varies from 10^6 to 10^{12} to up to 10^{18}.[37–39]

The huge variability of ILs results from their specific structural composition, which allows for a high potential of possible variations. ILs are commonly divided into cation and anion, in which especially the cation is additionally varied by using different side chains at the cationic core structure (head group). The most popular IL cationic head groups are organic ring structures, such as imidazolium and pyridinium rings, combined with n-alkyl side chains or those modified with different functional groups, but quaternary ammonium or phosphonium cations with alkyl side chains may also serve as IL cations (Fig. 3 A). Conventional halides or fluorinated/alkyl-chained borates, methanes, amides, phosphates and sulphates are often used as the corresponding anion (Fig. 3 B). The most intensively studied ILs are the ones based on imidazolium cations, namely IM14 and IM12, together with fluorinated anions, such as BF_4^-, PF_6^- and $(C_2F_3SO_2)_2N^-$ (Scifinder® accessed in January 2013). The explanation for acronyms used in the presented studies is given at the beginning (see *Nomenclature of ionic liquids*).

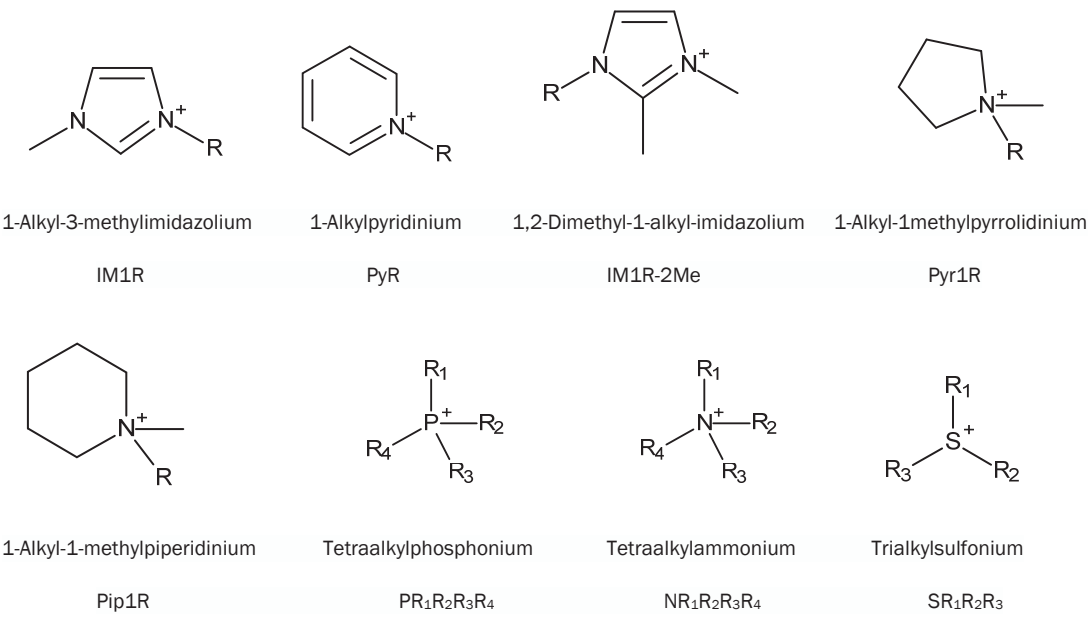
[a] Journals: Electrochemistry, Electrochimica Acta, Electrochemistry Communications, Journal of the Electrochemical Society

[b] Journal for articles about "development of alternative sustainable technologies"[110]

[c] Chemical communications - Journal for articles about "new fundamental knowledge" & "novel applications" in chemical sciences[111]

[d] Journal of Physical Chemistry B, The Journal of Physical Chemistry B, Journal of Physical Chemistry Chemical Physics and Journal of Chemical and Engineering Data

A



B

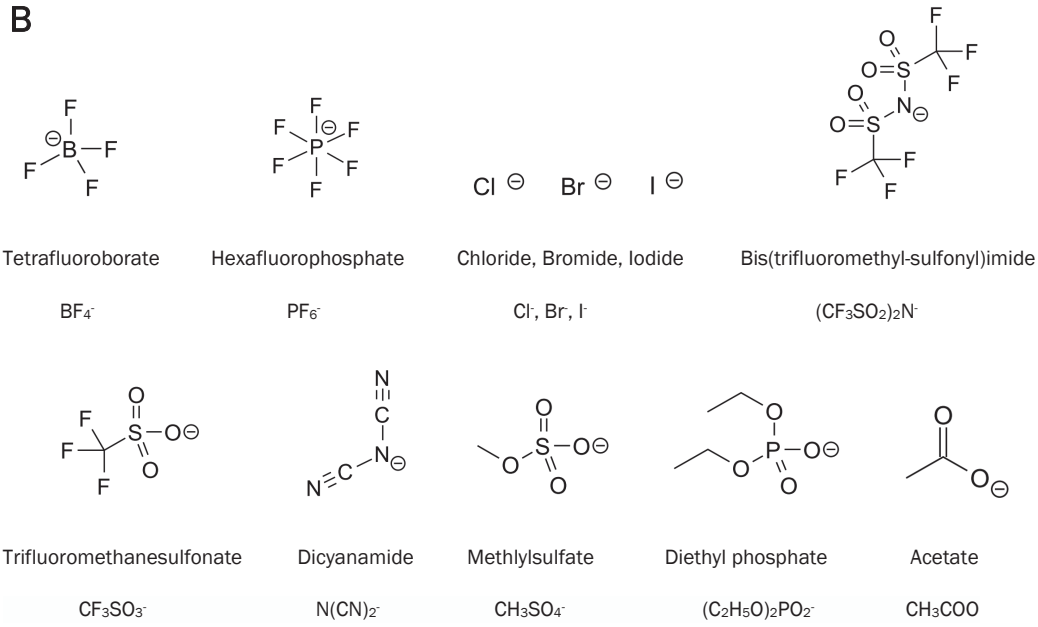
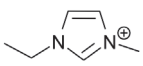
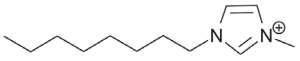
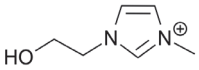
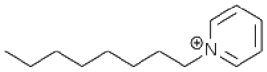


Fig. 3: Commonly used ionic liquid cations (A) and anions (B), their name and ID code (also see *Nomenclature of ionic liquids*) modified from Niedermeyer et al. 2012.[40] Side chains ($R_{1,2,3,4}$) are variable in their length and functional groups.

By varying the different structural elements – cationic head group, side chain and anion - their physico-chemical properties such as the viscosity (Tab. 1) can be tuned.

Tab. 1: Examples of the design potential of ionic liquids. The choice of side chains, cationic core structures and anions influences the viscosity at 25 °C in centipoise (1 cP = 0.001 mPa*s). Data taken from *Yu et al., 2012*.[41] (a) 1-Ethyl-3-methylimidazolium tetrafluoroborate, (b) 1-octyl-3-methylimidazolium tetrafluoroborate, (c) 1-octylpyridinium tetrafluoroborate, (d) 1-ethyl-3-methylimidazolium bis(trifluoromethyl-sulfonyl)imide, (e) 1-octyl-3-methylimidazolium bis(trifluoromethyl-sulfonyl) imide and (f) 1-(2-hydroxyethyl)-3-methyl-3H-imidazolium bis(trifluoromethyl-sulfonyl)imide.

Side chain / Cationic core	Anions: BF_4^-	Anions: $(CF_3SO_2)_2N^-$	For comparison[e]
 IM12	41 cP (a)	37 cP (d)	0.9 cP water 24 cP sulphuric acid 67 cP olive oil 50 cP -750 cP motor oil SAE 10-50 10,000 cP honey
 IM18	302 cP (b)	87 cP (e)	
 IM12OH		91 cP (f)	
 Py8	~350 cP (c)		

The van der Waals interaction potential is increased with increasing side chain length, which leads to a higher viscosity. The addition of a hydroxyl group and the change from imidazolium to pyridinium core also increases the viscosity. Although a high viscosity is disadvantageous for the chemical processing and handling of solvents, it can still be favourable in analytical applications as stationary phase or in the usage as lubricants.[41] If necessary, the viscosity can also be decreased by choosing another anion. A wide range of different ILs is imaginable with such a flexibility in tuning physico-chemical parameters.[32] Even "reversible ILs" can be produced. These

[e] Data on water, sulfuric acid and olive oil (25 °C) was taken from http://www.kayelaby.npl.co.uk/general_physics/2_2/2_2_3.html and from motor oil and honey (20 °C) from http://www.buerkle.de/media/files/Downloads/Viskositaeten_DE.pdf; both accessed in October 2013

ILs, with alkylcarbonate anions, can easily be created and undone by the addition and removal of CO_2 to and from certain base/alcohol mixtures. This can change the polarity of the switchable solvent within a chemical production process, e.g. in the production of polystyrene, and can minimise the energy demand and material consumption of the chemical process.[42] Mixtures of several ILs, with "interesting and useful additions to the properties of ILs",[40] may lead to the ever-increasing applicability of ILs driven by their huge design potential.

1.4 Degradation potential of ionic liquids

"Novel room-temperature ionic liquids with potential sites of enzymatic hydrolysis have been prepared and tested for biodegradable properties."

Gathergood and Scammels, 2002[43]

The previously described variability and designability of ILs make these chemicals promising candidates for "greener" products and processes. The variability, however, also creates a dilemma as soon as the potential environmental hazard needs to be assessed and evaluated for over a million possible ILs.[38] Systematic investigations on structure-activity relationships for the most technological relevant ILs were therefore suggested.[44] The development of ILs could then become a case study for the sustainable design of chemicals.[45] The aim was to develop chemicals that possessed a property profile that is feasible for technical applications, but which at the same time have a reduced hazard potential for human beings and the environment. Among the Twelve Principles of Green Chemistry, there is one idea that comprises many of the impact factors related to the protection of health and the environment:

"Design for Degradation – Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment."[17]

The design for degradation holds the opportunity to reduce the persistency of a chemical and therefore its potential for bioaccumulation and long-range transport within the environment. Inherently safer chemicals are preferably readily degradable in the environment and low in toxicity at the same time. There are many chemicals known, such as DDT, with a low (bio)degradation potential and high environmental persistency ("as much as 50% can remain in the soil 10-15 years after application")[46] that caused severe problems for man and the environment. Therefore the degradation potential has become the key parameter for the determination of "long-term adverse effects on biota".[47]

Such a degradation of chemicals within the aqueous, terrestrial and aerial environment can occur by different processes. In the case of ILs, a release into the aqueous and terrestrial environment is more likely than the risk of air contamination, due to their liquid and non-volatile state. Natural abiotic and biotic processes such as hydrolysis, (photo)oxidation and aerobic and anaerobic microbial degradation, respectively, are thereby the main degradation pathways.[47] Among those,

the biological degradation process was regarded as the most relevant one for the potential transformation of ILs within the environment. Photochemical reactions are surface related, and do not occur in the subsurface, where the removal of persistent organic compounds is dominated by microorganisms.[48] Additionally, the most relevant imidazolium ILs are highly stable, hydrolytically and thermally, for technical applications and recycling processes.[49,50] Therefore a stability of ILs in biochemical degradation processes was anticipated and needed to be investigated.[43]

A chemical is, in general, biodegradable when it can be transformed by living organisms mainly based on enzymatic degradation. The initial transformation of a chemical, known as primary degradation, however, can be initialised not only by biological processes, but also by abiotic processes such as (photo)oxidation and hydrolysis and may make a hardly biodegradable chemical more easily accessible for biological transformation processes. A biodegradable chemical can thereafter be partially or fully mineralised into CO_2, H_2O etc. (Fig. 4). The necessary enzymes can be present within the microbial cell itself or as extracellular polymeric substances in bacterial aggregates (e.g. bacterial flocs and biofilms). Under different environmental conditions, different organisms are able to grow with expression of a variety of enzymes. Long-term exposure of microorganisms towards a chemical can furthermore lead to adaptation processes, such as the induction of specific enzymes, genetic mutation or horizontal gene transfer, each of which enhances the degradative capacity of the entire community or changes the population in terms of the selective growth of certain strains.[51] Whether or not enzymes are present for biological degradation processes of the chemical of concern, then, depends on the type of microorganism, the amount of cells and their state of health. This is limited by growth conditions, such as the availability of essential minerals, temperature, pressure, osmolarity and the presence of oxygen in the environment (oxic and anoxic conditions). Biodegradation data is usually assessed by the determination of primary biodegradation or full mineralisation under aerobic conditions.

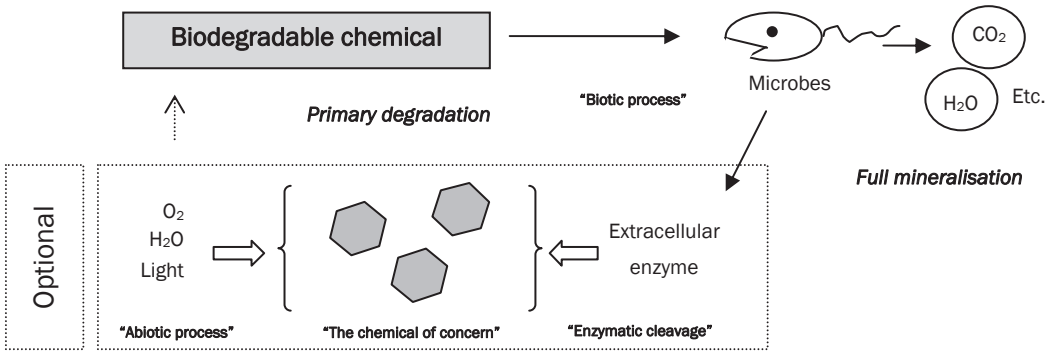
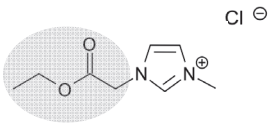
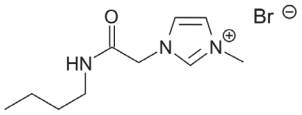
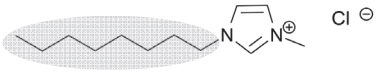
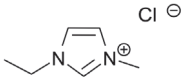
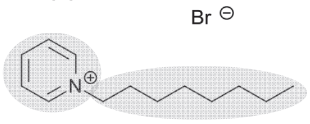
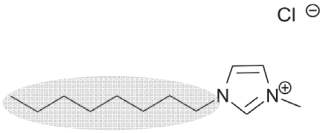
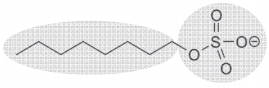
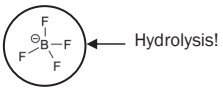
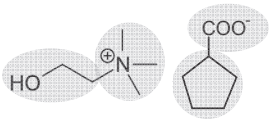


Fig. 4: Schematic illustration of the degradation process of biodegradable chemicals based on the illustration of the degradation process of biodegradable polymers.[52]

The first biodegradation study of ILs was published by Gathergood and Scammels in 2002.[43] Although little was known about the biodegradation potential of ILs, some aspects from the biodegradability of similar compounds such as polymers, plastics and surfactants had been considered. For instance, the presence of long unsubstituted alkyl side chains (≥ 4 carbons) together with end-chained oxygenated functional groups and the insertion of esters or amides had been observed to be advantageous for the enzymatic accessibility of surfactants. The researchers then developed and synthesised new ILs for a systematic approach in which relevant chemical components of ILs were altered.[43] Those structures are exemplified in the next table (Tab 2.) showing the state of the art of the biodegradability of ILs until the beginning of the presented studies in 2008. It illustrates the design potential in respect to an enhanced biodegradability of ILs.

Tab. 2: Observations made for the biodegradation of ionic liquids before the presented studies had begun. The choice of side chains, cationic core structures and anions of ionic liquids influences the microbial degradability. Degradable structures are shaded with grey. Parameter: Ready biodegradability, measured according to OECD guideline 301.

Observation	Biodegradability of ionic liquids	
	Detected	Not detected
#1 The ester preference – since 2004	 IM11COO2 Cl	 IM11CONHBu Br
"The amide functionalised side chains show no improved biodegradability of the alkyl side chains, whereas the incorporation of an ester group enhances the biodegradability."[53]		
#2 The side chain effect – since 2005	 IM18 Cl	 IM12 Cl
"Longer alkyl side chains of the cationic head group (>C6) enhance the biodegradability being limited by an increasing toxicity to microorganisms."[54–57]		
#3 The N-methylimidazolium stability – since 2007 [f]	 Py8 Br	 IM18 Cl
"Cationic head group molecules, such as N-substituted imidazole derivatives and imidazolium are in general supposed to be not readily biodegradable.[55,58] *So, imidazolium-based ILs are only partially mineralized, whereas pyridinium-based ILs with a hexyl or octyl side chain can be fully mineralised."*[54]		
#4 The organic anion degradability - since 2007	 $C_8H_{17}O_4S^-$	 BF_4^-
"Biodegradable anions are usually organic compounds,[59,60] *for inorganic anions, which cannot serve as a carbon source in microbial degradation, other degradation processes are relevant, e.g. hydrolysis."*[45]		
#5 Biodegradable naphtenic acid ionic liquids – since 2008	 N1112OH $(C_5H_9)COO$	
"In naphthenic acid ionic liquids (NAILs) both ions are biodegradable, the choline cation and the surrogate naphthenic acid."[60]		

[f] N-substituted imidazole derivatives since 2002

Based on the observations made regarding the biodegradability of ILs, the applicability of some presumably biodegradable ILs has finally been further investigated in a few chemical processes. IM1C0O4 BF_4^-, for example, was used as media for catalytic reactions.[61] Py1-3COOBu $(CF_3SO_2)_2N^-$ could be applied as solvent for the polymerisation of styrene[62] and also for the preparation of substituted and non-substituted alkynes.[63] However, the focus in the usage of biodegradable ILs was laid on the usage of a biodegradable IL cation and side chain; the low biodegradability of the IL anions and the imidazolium head group was not considered. Therefore the described ILs are probably only partially biodegradable and leave an ultimate stable residual. The only fully mineralisable IL tested was choline lactate, which was used as co-substrate for the enhanced biodegradation of azo-dyes.[64]

Although the biodegradability of ILs and the application of biodegradable ILs had been studied several times, in comparison to the huge number of publications on ILs in general (Fig. 2), the number of those regarding biodegradation studies and applications of biodegradable ILs was rather small in 2008 (Fig. 5) and was still not comparable to the huge growing interest in ILs. Only 14 out of around 6,500 general publications on ILs have been released, which made up only 0.2 % of all publications.

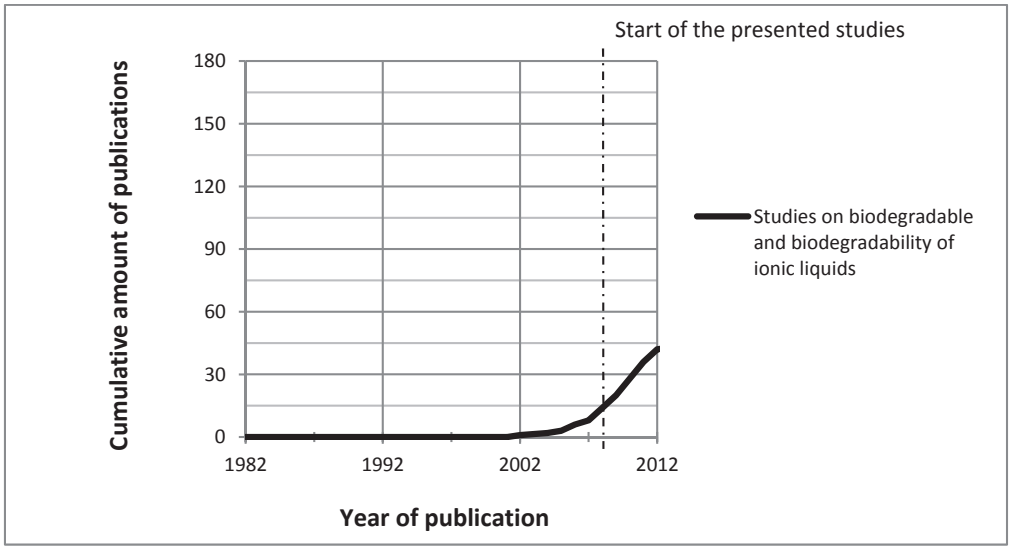


Fig. 5: Cumulative amount of articles, reviews and books with a title containing "biodegr*" and "ionic liquid*" Searched through Thomson Reuters Web of Knowledge℠ (accessed in January 2013).

As a consequence of the importance of biodegradation studies and the low amount of available information, a contribution to the study of the (bio)degradability of ILs needed to be made, considering the observations that had already been published with respect to the molecular composition of the relevant components of ILs (Tab. 2) and the experiments that had already been conducted.

When I began the presented studies in 2008, the experiments had been limited to stringent tests in the context of biodegradation, namely the determination of the ready biodegradability under aerobic conditions (OECD test guideline no. 301).[65] However, metabolic pathways other than aerobic respiration may also play an important role in the biodegradation of ILs. Anaerobic respiration and fermentation are other ways in which to potentially degrade organic chemicals. Anaerobic respiration differs from aerobic regarding the availability of oxygen in the environment. No oxygen is available as terminal electron acceptor in the electron transport chain by which the energy for the cell is derived, but nitrate (NO_3^-), ferric iron (Fe^{3+}), sulphate (SO_4^{2-}), carbonate (CO_3^{2-}) and some organic compounds can serve as alternative terminal electron acceptors under these anoxic conditions. The combination of aerobic and anaerobic respiration is used in wastewater treatment for the degradation and elimination of organic and nitrogen compounds to prevent eutrophication of the receiving water bodies. Biodegradation studies of chemicals that will potentially be present in wastewater treatment are usually conducted with microorganisms from wastewater treatment plants,[66] but pure axenic cultures can also be used for specific bioremediation purposes.[67]

Hydrolytical studies on ILs were even less commonly found than those on the biodegradability. Hydrolysis may be relevant especially for inorganic anions as they cannot serve as carbon source in biological degradation. The technologically relevant IL anions, BF_4^- and PF_6^-, had been found to be hydrolytically instable and may potentially release HF.[68,69] Tris(perfluoroalkyl)-trifluorophosphate ($(C_2F_5)_3PF_3^-$), as a hydrolytically stable IL anion, could be designed as an alternative.[70] However, the investigation of other relevant cyano-based anions and fluoroorganic anions under standardised hydrolytical test conditions (OECD test guideline no. 111) has not yet been tested.[71]

Based on these considerations, the presented studies of the degradation of ILs have been conducted to find chemical structures and conditions under which the selected ILs are degradable, and will not persist in the environment. The combination of (a) changes in the chemical structure of ILs, (b) the environmental conditions, (c) the microbial community and (d) the analytical methods should finally help to evaluate the degradability of ILs for a sound hazard assessment (Fig. 6).

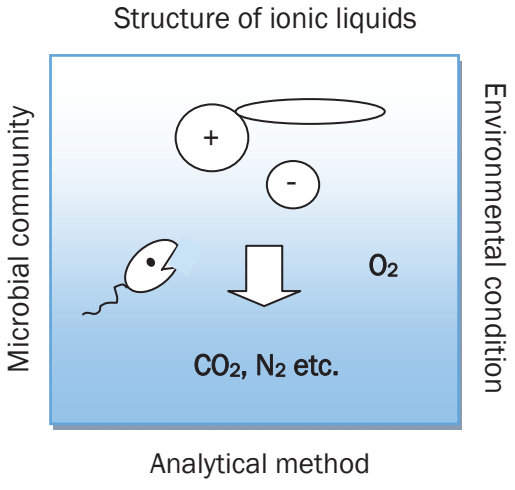


Fig. 6: Schematic illustration of the factors influencing the degradability of ILs that were considered in the presented studies.

1.5 Objectives

The presented studies aim to examine the (bio)degradability of ILs in relation to their chemical structures, their dependency on the environmental condition for bacterial growth and hydrolytic reactions and the microorganisms themselves on the basis of the existing knowledge on the degradability of organic chemicals and ILs. Therefore, experiments have been conducted to investigate the following hypotheses. The test kits containing the selected ILs for each of the studies can be found in paragraph *2.1 Selected ionic liquids*.

Hypotheses

It is anticipated that the degradability of ILs will entail the following aspects:

I. Environmental conditions for biodegradation – presence of oxygen in the environment

ILs are potentially biodegraded when the environmental conditions are changed from oxic to anoxic with nitrate as terminal electron acceptor for anaerobic respiration. The concentration of the ILs used in the anaerobic biodegradation tests shall not inhibit the microbial performance. To verify this assumption, nine IL cations that had previously been examined under aerobic conditions have been tested (test kit no. 1, Fig. 7).

II. IL anions – cyano-based and fluoroorganic anions

Five cyano-based and fluoroorganic anions have not yet been examined on either their aerobic or anaerobic biodegradation potential (test kit no. 2, Fig. 8).

It is generally expected that cyano-based anions are more easily biodegradable than fluoroorganic ones, due to their easier accessible carbon atoms.

Nevertheless, in the case that the cyano-based IL anions are not found to be biodegradable, a change in the bacteria consortia from activated sludge to pure culture of cyanide-degrading bacteria mixture *Cupriavidus spp*. shall enhance their degradability.

Cyano-based IL anions can be degraded hydrolytically, but this may produce hydrogen cyanide.

If a hydrolytic cleavage of the cyano-based IL anions is possible, there are enzymes that can catalyse the hydrolytic reaction.

III. IL cationic head groups - aerobic biodegradation

There were also a number of cationic structural elements that had not yet been investigated systematically under aerobic conditions. The different head groups of cations in particular may lead to an enhanced biodegradation potential of ILs: from imidazolium and pyridinium to morpholinium, pyrrolidinium, phosphonium and piperidinium cationic head groups. All in all, 32 ILs have been tested (Tab. 3).

The overall aim of the presented studies was to determine whether the list of the above aspects apply to the degradation of selected IL cations and anions. It was greatly expected that the study would find factors that influence the “design for degradation” of ILs within the environment to reduce the risk for persistence of chemicals.

2 Methodology

2.1 Selected ionic liquids

To verify or falsify the hypotheses outlined above, we arranged sets of ILs allowing a systematic investigation guided by systematic thinking in terms of structural relationship T-SAR approach. [44]

The selected ILs have been chosen based on two main principles:

1. They should fit into a systematic approach for the discovery of molecular structures to provide information of weak sites for degradation processes.
2. They should be related to technologically relevant ILs to provide data for a sound hazard assessment for chemicals that can potentially be used in application processes.

The following three IL test kits were applied to examine the abovementioned hypotheses:

Test kit no. 1 – Denitrification

To examine the effect of the presence of oxygen in the environment on the biodegradability of ILs, we investigated IL cations that had previously been tested under aerobic conditions. Those were imidazolium, pyridinium and 4-(dimethylamino) pyridinium IL cations (Fig. 7).

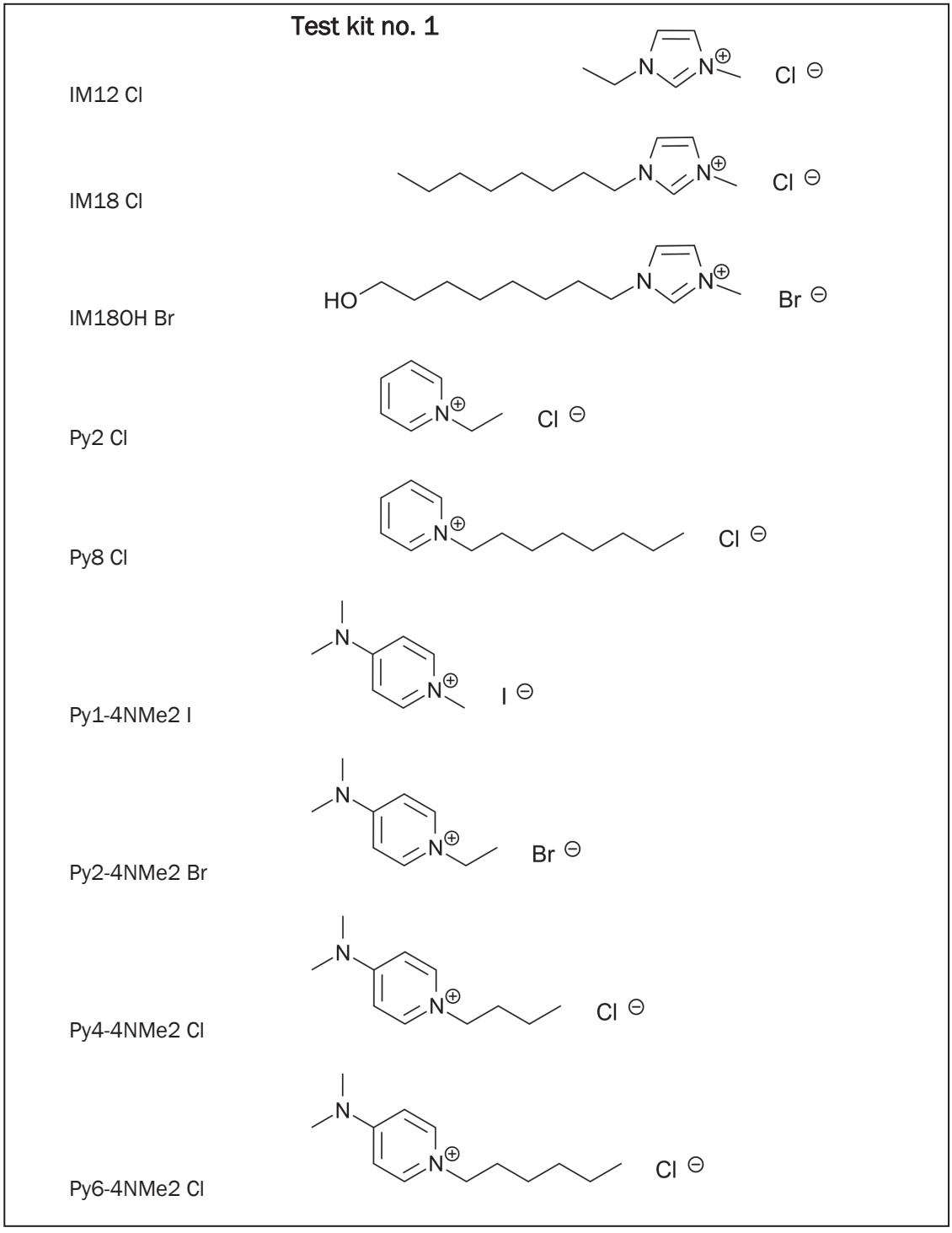


Fig. 7: Ionic liquid cations investigated in this study in respect to their anaerobic biodegradability.

Test kit no. 2 – IL anions

Since only a few organic IL anions had been tested under aerobic conditions, I chose to examine the technologically relevant cyano-based and fluoroorganic anions under both aerobic and anaerobic conditions. For those, the hydrolytical stability was additionally examined. Cyano-based anions were also tested for their biodegradation potential in the presence of cyanide-degrading bacteria (Fig. 8).

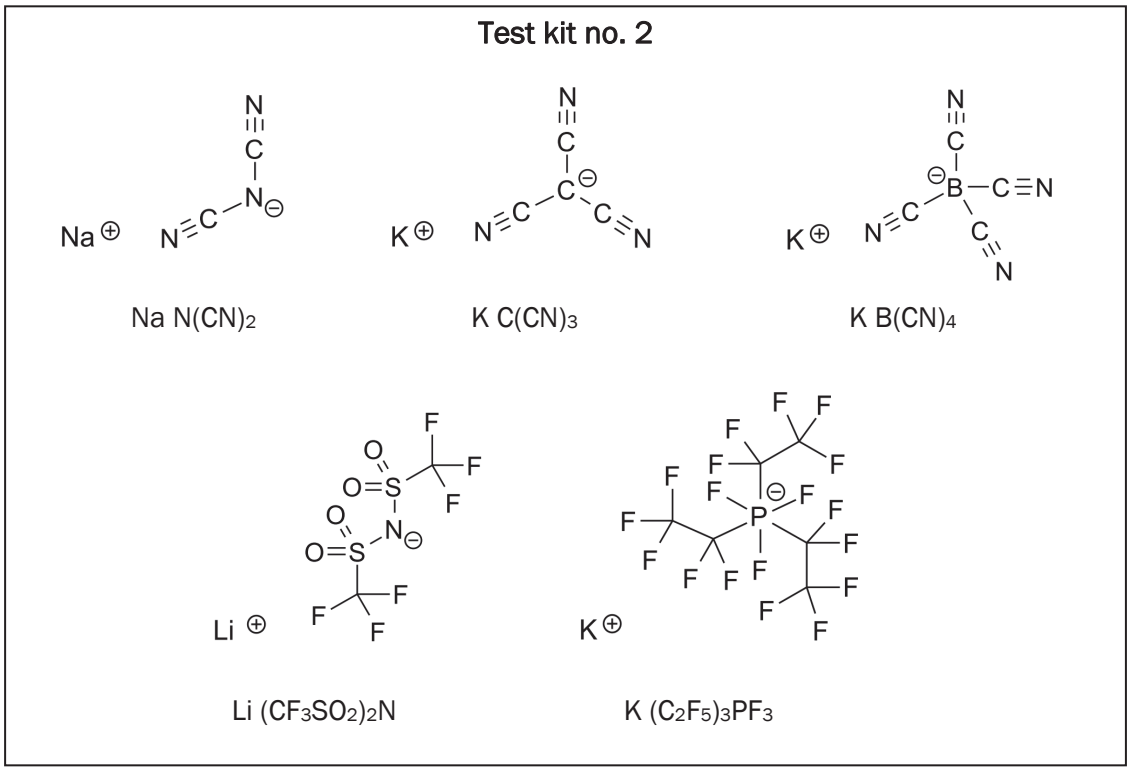
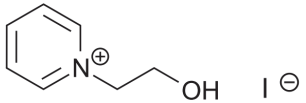
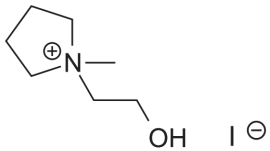
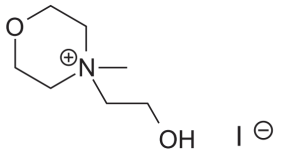
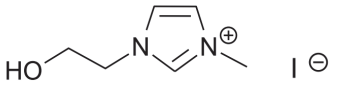
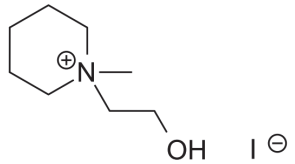
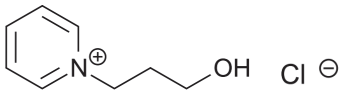
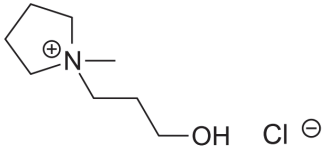
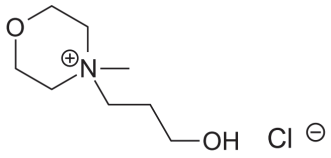
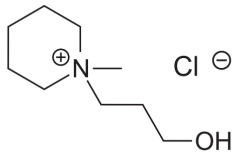
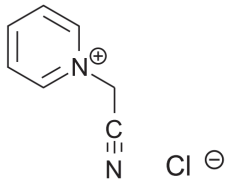
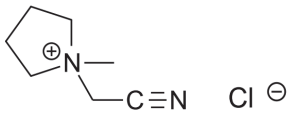
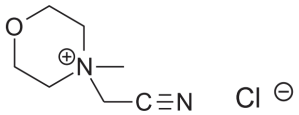
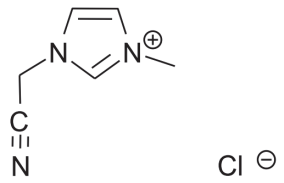
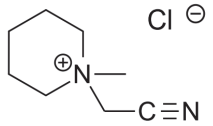
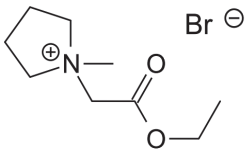


Fig. 8: Ionic liquid anions investigated in this study in respect to their aerobic biodegradation and anaerobic biodegradability and their and hydrolytical stability.

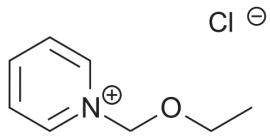
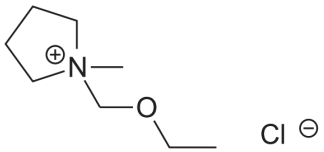
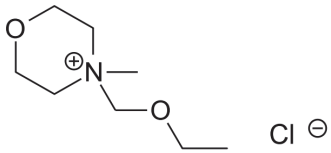
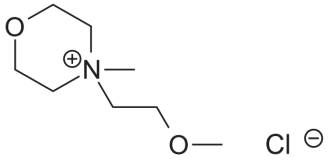
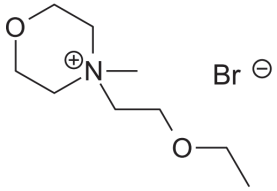
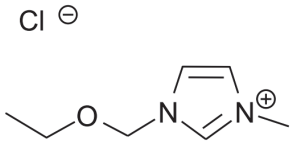
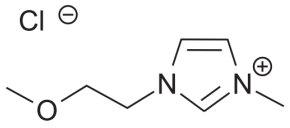
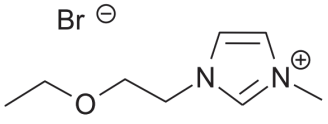
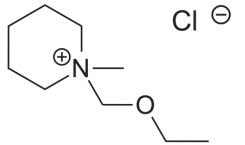
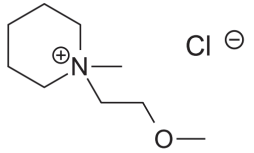
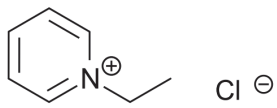
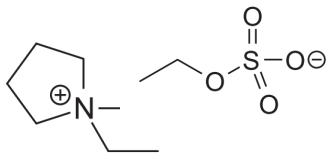
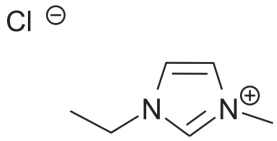
Test kit no. 3 –IL cations

Finally, we investigated IL cations, focusing their different head groups, namely imidazolium, pyridinium, morpholinium, pyrrolidinium, and piperidinium and different side chains mainly alkyl side chains with or without functional groups. The side chains were kept constant among the head groups. In total, 32 cations and anions were examined (Tab. 3).

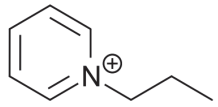
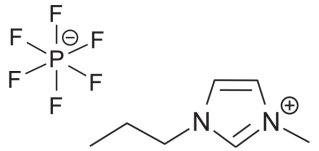
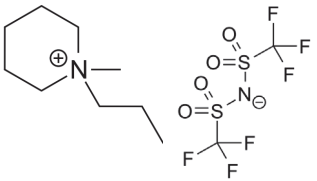
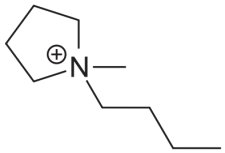
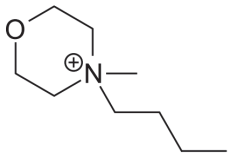
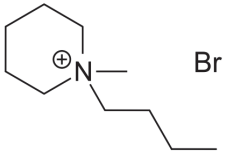
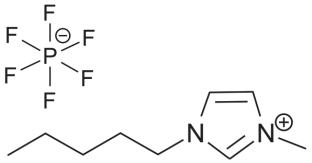
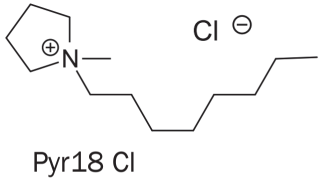
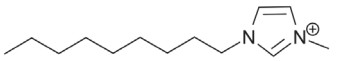
Tab. 3: Ionic liquid cations investigated in this study with respect to their cationic core structure (pyridinium, morpholinium, pyrrolidinium, phosphonium and piperidinium), while keeping the side chain constant. (A) Ethyl hydroxyl, (B) propyl hydroxyl, (C) methyl cyanide and (D) ethoxy carbonyl side chains. Continue page 22.

Test kit no. 3					
A	 Py2OH I	 Pyr12OH I	 Mor12OH I	 IM12OH I	 Pip12OH I
B	 Py3OH Cl	 Pyr13OH Cl	 Mor13OH Cl	*IM13OH Cl* *Already tested*	 Pip13OH Cl
C	 Py1CN Cl	 Pyr11CN Cl	 Mor11CN Cl	 IM11CN Cl	 Pip11CN Cl
D	Py1COO2 Br Not available at our institute	 Pyr11COO2 Br	Mor11COO2 Br Not available at our insitute	*IM11COO2 Br* *Already tested*	Pip11COO2 Br Not available at our institute

Tab. 3 continued. Pyridinium, morpholinium, pyrrolidinium, phosphonium and piperidinium ionic liquid cations with (E) ether and (F) alkyl side chains. Continue page 23.

Test kit no. 3					
E	 Py102 Cl Py201 Cl Not tested yet Py202 Br Not tested yet	 Pyr1102 Cl Pyr1201 Cl Not tested yet Pyr1202 Br Not tested yet	 Mor1102 Cl  Mor1201 Cl  Mor1202 Br	 IM1102 Cl  IM1201 Cl  IM1202 Br	 Pip1102 Cl  Pip1201 Cl Pip1202 Br Not tested yet
F 1st part	 Py2 Cl	 Py12 20S03	Mor12 Cl Not available at our institute	 *IM12 Cl*	Pip12 Cl Not available at our institute

Tab. 3 continued. Pyridinium, morpholinium, pyrrolidinium, phosphonium and piperidinium ionic liquid cations with (F) alkyl side chains.

Test kit no. 3					
F 2nd part	 Py3 Br	Pyr13 Br Not available at our institute	Mor13 Br Not available at our institute	 IM13 PF_6	 Pip13 $(CF_3SO_2)_2N$
	Py4 Br *Already tested*	 Pyr14 Br	 Mor14 Br	*IM14 Cl* *Already tested*	 Pip14 Br
	Py5 Br Not tested yet	Pyr15 Br Not available at our institute	Mor15 Br Not available at our institute	 IM15 PF_6	Pip15 Br Not available at our institute
	Py8 Cl *Already tested*	 Pyr18 Cl	Mor18 Cl Not available at our institute	*IM18 Cl* *Already tested*	Pip18 Cl Not available at our institute
	Py9 Cl Not available at our institute	Pyr19 Cl Not available at our institute	Mor19 Cl Not available at our institute	 IM19 Cl	Pip19 Cl Not available at our institute

2.2 Selected degradation tests

Whilst hydrolysis and aerobic biodegradation could be tested using the OECD guidelines no. 111 and 301 respectively, an anaerobic test system for the examination of the anaerobic biodegradation of ILs under nitrate-reducing conditions is not officially regulated in DIN standards or OECD guidelines. Anaerobic conditions appear as soon as the oxygen is depleted, e.g. in biofilms, in groundwater aquifers or in soils. In the presence of an alternative electron acceptor like nitrate, anaerobic respiration processes, such as denitrification, may occur. Denitrification is also used in wastewater treatment plants as one of the main treatment steps for the elimination of nitrate and organic substances. The general procedure of conducting biodegradation tests under anaerobic conditions was established in the UFT based on the experiences from my former studies on the denitrification of biodegradable polymers, which were gained under the supervision of Angela Boley and Wolf-Rüdiger Müller at the Institute for Sanitary Engineering, Water Quality and Solid Waste Management (ISWA) at the Universität Stuttgart, Germany. In cooperation with the Max-Planck-Insitute for Marine Microbiology, namely Olav Grundmann, the relevant techniques for handling of bacteria were established. The media composition for denitrification processes was based on general media compositions for aerobic biodegradation as it is common practice in anaerobic biodegradation studies.[72] In general, experiments under anaerobic conditions are conducted in closed bottles with butyl-rubber septa in which the sample is covered with a nitrogen atmosphere and can be accessed through the septa by syringes. This test system is based on anaerobic testing techniques used by Hungate in 1950[73] and has been successfully used for the testing of biological degradation of ILs under our testing conditions for denitrification processes. For more experimental details, see Neumann et al. 2010[74].

For the interpretation of biodegradation data, the inhibitory effect on the inoculum needed to be assessed under the anaerobic experimental conditions. The same cultivation system could then be used for the determination of inhibitory effects. In denitrification processes, nitrogen and carbon dioxide gases are released from the reduction of nitrate and the oxidation of carbon. In case of bacteria growth inhibition, the gas production is inhibited and less pressure in the closed vessels is detected. The detection was, therefore, conducted through manometric measurements using a pressure-measuring device. For more experimental details, see Neumann et al. 2012[75]. This was also applicable for the investigation of inhibitory effects of further chemicals from other working groups, such as boron clusters (Prof. Dr. Gabel, Bremen) and nanoparticles[76] (Prof. Dr. Filser, Bremen).

2.3 Selected inoculum

In activated sludge, a diverse microbial community is found. More than hundred different bacteria strains are usually detected in activated sludge samples,[7] but eukaryotic organisms like protozoa and metazoa are also present. The majority of the biomass, however, consists of bacteria. Those bacteria that belong to the *Proteobacteria* phylum are the main contributors to reduce the chemical load of wastewater. Among those gram negative bacteria from different genera can be found, e.g. *Pseudomonas, Acinetobacter, Alcaligenes, Aeromonas,* and various *Enterobacter,* but also *Flavobacterium* and some gram positive bacteria, such as *Arthrobacter*, *Corynebacteria, Micrococcus, Brevibacterium* and *Bacillus.*[77] As these microorganisms originate from the most relevant biological treatment processes in wastewater treatment plants, activated sludge is commonly used for biodegradation tests of organic chemicals.[47,66] The microbial diversity is thereby the key for the removal of a wide range of organic chemicals, as well as inorganic nitrogen and phosphor compounds.

For the biodegradation tests, activated sludge bacteria from a wastewater treatment plant in Delmenhorst were used. The plant treats water from households, industry and agriculture. The treated water is finally discharged into the river Weser as the receiving body. Around 6.5 million m^3 of wastewater per year are treated from around 108,000 inhabitants (Delmenhorst, Ganderkesee, Harpstedt, Stuhr/Wehye).[78] It therefore belongs to the size range 5, which is the largest size range in Germany (>100 inh.). Size 5 and 4 plants are the most common wastewater plants in Germany; together, they treat 90 % of the total number of inhabitants and population equivalents.[79]

As ILs are mainly xenobiotic chemicals which have not yet appeared in the environment in large amounts, the activated sludge consortia has probably not been adapted to those chemicals. Therefore, activated sludge from an industrial wastewater treatment plant of an IL-producing chemical company was used as an alternative inoculum. In case of cyano-based IL anions, an axenic culture of *Cupriavidus spp.,* cyanide-degrading bacteria strain, was tested on its biodegradation potential.

2.4 Selected analytical systems

The analytical identification of the degradation of ILs was another challenge, along with cultural techniques for the inoculum used. In our working group, the quantitative and qualitative measurement of ILs via high-performance liquid chromatographic systems (HPLC) coupled with a Diode-Array detector (UV-Vis) or mass spectrometer (MS) had already been established. For the detection of the fluoroorganic and cyano-based IL anions and IL cations that are not UV active, an additional ion chromatography device (IC) was installed at our institute. Due to the organic and lipophilic nature of some of the selected IL cations and anions, and the biological matrices in the biodegradation test systems, it was challenging to analyse the samples in the IC, which had primarily been made for the detection of inorganic ions. However, the problems could be fixed by changes within the analytical device, the eluent and the sample preparation for most of the selected ILs.

The degradability of a substance can be determined by measuring the change in concentration of the educts, e.g. the chemical itself or the depletion of oxygen under aerobic conditions, and the generation of products, e.g. CO_2, N_2. I chose to measure the concentration of the initial substrate for the determination of primary degradation (using HPLC-UV and IC coupled with a conductivity detector), as any transformation of the parent compound was of interest, even though this may not automatically result in a detectable full mineralisation. The depletion of oxygen was therefore measured for the determination of full mineralisation, if necessary, mainly in the case that a primary degradation step had previously occurred. Additionally, transformation products could be investigated using LC-MS. Because of these difficulties regarding the measurement of valid data, a system of analytical quality management was also established. A method validation procedure based on international and national guidelines and relevant research papers is now available in our working group. Analytical parameters can be determined by measuring a calibration curve with multiple detections and using an excel sheet that comprises statistical calculations for its evaluation. Such a procedure has shown to be essential in getting reliable data and facilitating the determination of reliability, especially when biological matrices and a new analytical device are established. As these considerations were part of the presented studies the excel sheets and the guide can be found appended to this thesis (see Annex I).

3 Publications

Over the years, ten publications have been written on the (bio)degradability of ILs. Eight of them have already been peer-reviewed and released. The other two are already written and have been sent to the editor of a scientific journal, and so are currently under review. The results of the presented degradation experiments are the subject of half of the publications, whereas the other half is related to the subject.

3.1 The presented studies

The publications that belong to the present thesis were mainly published in Green Chemistry or delivered for further reviewing. This highly ranked journal for sustainable approaches to chemistry combines environmental considerations with technical application in industry and research and has gained more and more attention (impact factor: 4.2 in 2007; 6.8 in 2013). At the time of writing (19 September 2013), 804 papers and 43 reviews have been released in this journal dealing with “greener” processes using ILs and their fate and toxicity in the environment. Therefore, we assume that our studies concerning the degradation of ILs for a sound hazard assessment will find their users and add to the previously published knowledge of other research groups in this area.

At this point, three peer-reviewed papers have been published. Two more are under submission.

A.1 **Neumann J**, Grundmann O, Thöming J, et al. (2010) Anaerobic biodegradability of ionic liquid cations under denitrifying conditions. Green Chemistry 12:620. doi: 10.1039/b918453h

A.2 **Neumann J**, Cho C-W, Steudte S, et al. (2012) Biodegradability of fluoroorganic and cyano-based ionic liquid anions under aerobic and anaerobic conditions. Green Chemistry 14:410. doi: 10.1039/c1gc16170a

A.3 Steudte S, **Neumann J**, Bottin-Weber U, et al. (2012) Hydrolysis study of fluoroorganic and cyano-based ionic liquid anions – consequences for operational safety and environmental stability. Green Chemistry 14:2474–2483. doi: 10.1039/c2gc35855g

*A.4 **Neumann J**, Pawlik M, Bryniok D, et al. Biodegradation potential of cyano-based ionic liquid anions in a culture of Cupriavidus spp. and their in vitro enzymatic hydrolysis by nitrile hydratase. Under review in Environmental Science and Pollution Research*

*A.5 **Neumann J**, Steudte S, Cho C-W, et al. Biodegradability of 32 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ionic liquid cations under aerobic conditions Submitted to Green Chemistry*

The results have further been presented as posters or oral presentations on the following national and international conferences on ionic liquids, environmental sciences and Green and Sustainable Chemistry:

Poster #1

Determination of the primary anaerobic biodegradability of Ionic Liquid cations under denitrifying conditions, cometabolism and characterisation of metabolites. J. Neumann , J. Thöming, S. Stolte, University of Bremen/D

BATIL - 2 (BIODEGRADABILITY AND TOXICITY OF IONIC LIQUIDS) - in Frankfurt/Main, Germany , 28 - 29 September 2009.

SETAC Europe 20th Annual Meeting - Science and Technology for Environmental Protection - held in the Palacio de Congresos y Exposiciones - FIBES in Seville, Spain from 23-27 May 2010

Poster #2 & oral presentation #1

Abbau von ionischen Flüssigkeiten. Jennifer Neumann, Chul-Woong Cho, Stephanie Steudte, Jorg Thöming und Stefan Stolte, Universität Bremen/D

1st Forum Junger Umweltwissenschaftler in Burghotel Blomberg , Germany 27 – 29 May 2013

Oral presentation #2

Degradability of ionic liquids – Jennifer Neumann (M.Sc.)

6th International Conference on Green and Sustainable Chemistry (GSC-06) in Nottingham (UK) on 06 Aug 2013

3.2 List of further publications

A range of publications was further released together with other scientists from our institute and the Uniwersytet Gdański (University of Gdańsk, Poland).

For those studies, regeneration processes have been investigated for a reuse of ILs within an application process. It reduces the need for a removal of ILs from wastewater and therefore improves their sustainable use. The first two publications have been released on that topic. The established analytical methods for the determination of organic compounds via LC-MS and IC and also the anaerobic inhibitory test could further be applied within the studies B.3 to B.5. Furthermore, a guide to the calibration of analytical methods in method validation has been established within the working group and for bachelor students of chemistry (B.6). Although these publications and writings have been realised parallel to the PhD studies, they are not part of the presented studies on the degradability of ILs.

B.1 Fernandez JF, **Neumann J**, Thöming J (2011) Regeneration, Recovery and Removal of Ionic Liquids. Current Organic Chemistry 15:1992–2014.

B.2 Siedlecka EM, Czerwicka M, **Neumann J**, et al. (2010) Ionic Liquids : Methods of Degradation and Recovery. Ionic Liquids: Theory, Properties, New Approaches. pp 701–722

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Paper A.1 - Anaerobic biodegradability of ionic liquid cations under denitrifying conditions

Neumann J, Grundmann O, Thöming J, et al. (2010) Anaerobic biodegradability of ionic liquid cations under denitrifying conditions. Green Chemistry 12:620. doi: 10.1039/b918453h

Own contributions:

I established the anaerobic test system after consultation with the microbiologist Olav Grundmann. All experimental work for the publication, as well as the evaluation and interpretation of the results, was performed by myself. The results were then published after discussion with the other co-authors.

Anaerobic biodegradability of ionic liquid cations under denitrifying conditions

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Biodegradability and ecotoxicity of ionic liquids (ILs) are key properties for determining the greenness of IL applications, and have been increasingly investigated during the last few years. Former studies on the biodegradability of ILs were solely focused on the aerobic side. Nevertheless, the anaerobic biodegradation of many compounds plays an important role in the environment. Anaerobic respiration, especially nitrogen reduction, is widespread in the environment and is commonly used for waste water treatment. Therefore, we investigated in this study, whether ILs can be biodegraded under nitrogen reducing conditions. The primary anaerobic biodegradability of nine different imidazolium, pyridinium and dimethylaminopyridinium based IL cations was monitored *via* HPLC-UV over a time period of 11 months. Only for the 1-(8-hydroxyoctyl)-3-methyl-imidazolium cation (IM18OH), and a degradation could be observed and several metabolites were identified using LC-MS. Co-metabolism is sometimes the only way to degrade difficult substances. However, a possible co-metabolism of the substances by using acetate was not observed. All in all, the biodegradability of the tested ILs seems to be even worse under denitrifying conditions compared to aerobic ones. Nevertheless, the present paper aims to fill the gap concerning the biodegradability of ILs in waste water treatment plants. It gives a first insight into the biological degradation of ILs in the absence of oxygen, and provides further data for an appropriate hazard assessment.

Introduction

The popularity of ILs in the light of green chemistry

Ionic liquids (ILs) have gained popularity as designer solvents with high operational safety, particularly due to their low vapour pressure and non-flammability. Operational safety together with the various ways in which anions, cation headgroups and side chains of ILs can be combined, open up a wide range of ILs and applications in industrial processes: be it as organic solvents,[1,2] catalysts,[3] enzyme stabilisers,[4] sensors,[5,6] or potential pharmaceutical ingredients,[7] the application possibilities are manifold. However, increasing application possibilities and usage of ILs also results in a higher amount of final IL waste and its potential release into the environment. The ILs in use should therefore be of high intrinsic safety to reduce environmental hazards according to the principles of green chemistry published by Anastas *et al.* in 1998.[8] A review focusing on the issue of sustainability in the design of ILs has already been published by Ranke *et al.* in 2007.[9] The authors emphasise the welcoming effect of biodegradable ILs for environmental purposes to diminish the risk of bioaccumulation, which is even more important the higher the toxicity and the exposure of an IL is. T-SAR (thinking in terms of structure–activity relationships) based prospective design[10] aims at finding and using chemical structures, which fulfil the principles of green chemistry to avoid environmental hazards whilst maintaining an appropriate technical performance, before large-scale industrial applications for ILs are built up.[11,12]

Aerobic biodegradability of ionic liquids—the main results until now

Following this principle of green chemistry and having stressed the necessity for the determination of biodegradation parameters by Jastorff *et al.* in 2005,[12] the determination of the aerobic biodegradation of ILs has already started. So far, these experiments have been limited to stringent tests for the determination of the ready biodegradability under aerobic conditions. The results indicate that some structural characteristics of ILs enhance or reduce the possibility for an enzymatic cleavage of ILs. One of the main results leads to the conclusion that the biodegradability depends on the length of the alkyl side chain of the cationic head group. The longer the alkyl side chain ($>C_6$) the better the biodegradability is.[13-15] However, this effect does not seem to be endlessly valid due to an increasing toxicity to microorganisms with increasing alkyl side chain length.[16]

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Functional groups, such as amides or esters, were introduced into the alkyl side chain of ILs to make this structural element more susceptible to enzymatic cleavage. The amide functionalised side chains showed no improved biodegradability, whereas the incorporation of an ester group could enhance the biodegradability of the alkyl side chains.[17,18]

Headgroup molecules, such as N-substituted imidazole derivatives, are in general supposed to be not readily biodegradable as it could be shown for, *e.g.*, 1-methyl-, 1-vinyl-, 4-nitro imidazole.[19] For imidazolium based ILs, this observation could be confirmed.[14] Whereas, for pyridinium rings combined with longer alkyl side chains[13] and for 1-undecyloxymethyl-3-hydroxypyridinium saccharinate full mineralisation could be found.[20]

For the determination of the biodegradability of ILs, it is furthermore important to consider not only IL cations but IL anions, too. Biodegradable anions are usually organic compounds such as octyl sulfates,[18] acetate and naphthenic acids like 3-cylcohexylpropionate.[21] For inorganic anions, like BF_4^- or PF_6^-, which cannot serve as a carbon source in microbial degradation, other degradation processes might be relevant, *e.g.* hydrolysis or photolysis.[9]

Exploring further metabolic pathways

These former studies have shown that ILs, which undergo primary aerobic biodegradation, do exist, but there is still a large number of ILs that did not show ready biodegradability in the conducted experiments. Among those were short-chain IL cations, such as the 1-ethyl-3-methylimidazolium cation (IM12), which have—although ecotoxicologically preferred—very low biodegradability at the same time. In such a case, a conflict arises between ecotoxicity and biodegradability.[14] One way out of this conflict might be the biodegradation of ILs through other metabolic pathways than the aerobic one. For example, ILs reaching natural surroundings might not only face aerobic, but also anaerobic environmental conditions, *e.g.* in aquifers, eutrophic lakes, soils or sediments. Some microorganisms, which are well-adapted to anoxic milieus, could be able to degrade ILs under these conditions, *e.g.* by anaerobic respiration or fermentation. A combination of both environmental conditions is commonly used for the purification of water in water treatment plants.

Abiotic degradation

Another way out of the conflict between higher ecotoxicity and lower biodegradability has been examined on a technical level by several authors.[22,23] Advanced oxidation processes (AOPs) shall lead to the oxidation of persistent substances into various break-down products, which are hopefully less toxic than the initial substrate. This technique is mainly used for industrial waste water treatment. It has been shown that ionic liquids can be oxidised electrochemically[14] and by UV photolysis in the presence of hydrogen peroxide[24,25] amongst others. In these recent studies, it has been demonstrated that the oxidation by AOP modifies the imidazolium ring, not the alkyl chain of the molecule. Therefore, a complete degradation could be proven.

The anaerobic biodegradation pathway

The anaerobic microbial degradation process is applied in water treatment and soil remediation, amongst others. It has already been shown to be successful for a range of substances that are recalcitrant for aerobic biodegradation processes.[26] Examples are the anaerobic biodegradation of highly chlorinated hydrocarbons, such as tetrachloroethane (PCE)[27] and the reductive dechlorination of polychlorinated biphenyls (PCBs) in sediments[28-30] or landfill leachate.[31] Although the latter substance group is partly accessible to aerobic biodegradation, highly chlorinated members, which are not readily biodegradable under aerobic conditions, can be made accessible to aerobic biodegradation by anaerobic pre-treatment.[30]

The aerobic biodegradation pathway has further been shown to be unsuccessful for the decomposition of azo dyes, whereas these substances seem to be biodegradable under anaerobic conditions especially when a second organic substrate is added.[32] Such a co-metabolism or co-oxidation process has been defined as an oxidation in which the substrate is oxidised without using the energy derived.[33] It had been reported that several xenobiotics, which are not easily biodegradable, can be degraded by microorganisms *via* co-metabolism, *e.g.* pesticides,[34,35] nonylphenols[36] and aminoaromatic acids.[37]

Anaerobic biodegradation might also be important for substances that are even more biodegradable under aerobic conditions, *e.g.* linear hydrocarbons and aromatic compounds. Once they are exposed to the environment, they face anaerobic conditions in deep aquifers, soils or sediments.[38,39] Additionally, the formation of metabolites during the biodegradation process has to be considered within a proper hazard assessment. Independent of the toxicity of the parent compound, metabolites exhibit their own, sometimes even higher, toxicity as it has been observed, *e.g.*, for the biodegradation products of nonylphenols.[40]

Although the necessity of biodegradable ILs in the context of water treatment facilities has been emphasised several times,[9,16] attempts to further determine the anaerobic metabolic pathways for ILs have not been reported, yet. The present paper aims to fill this gap concerning the biodegradability of ILs in waste water treatment plants and therefore gives a first insight into the biological degradation in the absence of oxygen and the presence of nitrate (denitrification), and provides further data for an appropriate hazard assessment of ILs.

Experimental

Chemicals

Most of the tested ILs were received from Merck KGaA (Darmstadt, Germany), as well as the salts for the mineral salt medium. The synthesis of 1-(8-hydroxyoctyl)-3-methylimidazolium bromide had already been described.[41] Acetonitrile (HPLC grade) was obtained from Fluka (Buchs, Switzerland). Methanol for the HPLC measurements was bought from Acros Organics BVBA (Geel, Belgium).

Selection of ionic liquids

The ionic liquids used in the present study were selected according to established knowledge gained from the primary

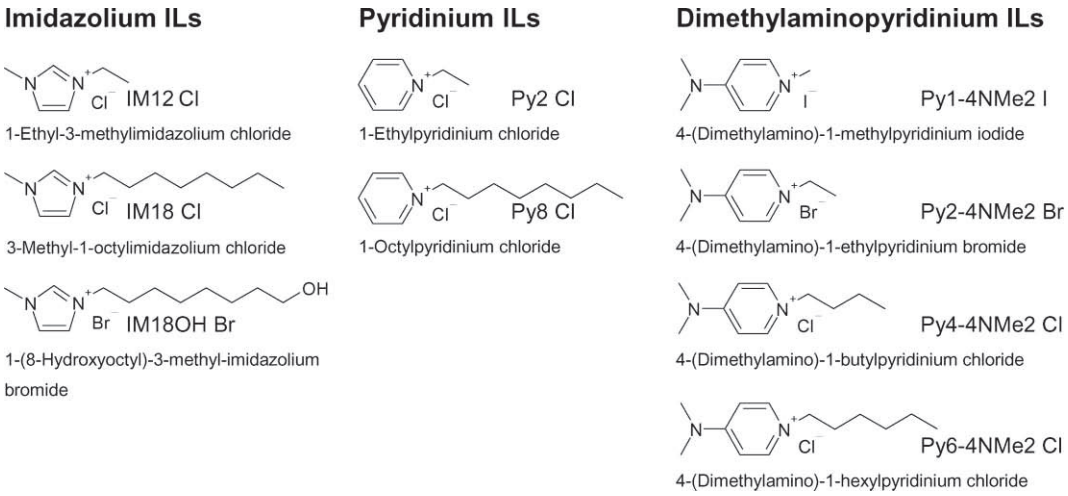

Fig. 1 Ionic liquid structures, acronyms and names used in this study.

biodegradability testing of ILs in the presence of molecular oxygen.[14] According to these results, the effect of the length of the alkyl side chain, the introduction of functional groups and the effect of different head groups have been systematically determined. Therefore, the following ILs were selected (Fig. 1): (i) Imidazolium ILs: 1-ethyl-3-methylimidazolium chloride (IM12 Cl), 3-methyl-1-octylimidazolium chloride (IM18 Cl) and 1-(8-hydroxyoctyl)-3-methyl-imidazolium bromide (IM18OH Br), (ii) Pyridinium ILs: 1-ethylpyridinium (Py2 Cl) and 1-octylpyridinium (Py8 Cl), (iii) Dimethylaminopyridinium ILs: 4-(dimethylamino)-1-methylpyridinium iodide (Py1-4NMe2 I), 4-(dimethylamino)-1-ethylpyridinium bromide (Py2-4NMe2 Br), 4-(dimethylamino)-1-butylpyridinium chloride (Py4-4NMe2) and 4-(dimethylamino)-1-hexylpyridinium chloride (Py6-4NMe2 Cl). The counter-anions were taken from the group of halides due to their low toxicity and high solubility.

HPLC systems

The HPLC-UV system used for the specific analysis of ionic liquid cations was a VWR Hitachi system containing the L-2130 HTA-pump, L-2130 degasser, L-2200 autosampler, L-2300 column oven, L-2450 diode array-detector and the EZChrom Elite software. The LC-MS system utilised for the analytical determination of the degradation products was a Hewlett Packard system Series 1100, with a gradient pump, online degasser, autosampler and a Bruker esquire ESI-MS ion trap detector.

For both systems a hydrophilic interaction liquid chromatography column (HILIC, Multospher 100 Si - 5 µm, 125 × 4.6 mm) with guard column, both purchased from CS–Chromatographie Service GmbH (Langerwehe, Germany), and a cation exchanger CC 70/4 Nucleosil 100-5SA with guard column from Macherey-Nagel (Düren, Germany) were used. The HILIC column provided a good separation of functional groups, *e.g.* for the determination of metabolites. The cation exchanger showed a better performance and was more robust for samples with a matrix composed of high amounts of organic matter from activated sludge. The latter column was therefore preferred for the huge number of samples from the biodegradation test.

The mobile phase consisted of varying solvent proportions for each substance (Table 1). The solvents were acetonitrile (HPLC grade) and aqueous K_2HPO_4 solution. Later the acetonitrile was exchanged with methanol due to a global shortage of acetonitrile, and the analytical methods were adjusted to the new conditions.

The system was operated at a flow rate of 1 mL min^{-1} and 40 °C oven temperature. 10 µL portions of the samples were injected. A detection wavelength of 211 nm was used for quantification of the original compounds based on imidazolium, 254 nm for those based on pyridinium and 288 nm for those based on dimethylaminopyridinium, where they do show maximal absorption.

Primary biodegradation

The primary biodegradation under denitrifying conditions of the test substances was monitored by specific analysis *via* HPLC-UV for 11 months (328 d). By HPLC-UV measurements, the decrease of concentration of the biodegraded parent compound can be followed. Full mineralisation (generation of CO_2, H_2O, N_2) cannot be determined by this method, but would not occur without the determinable first step of primary biodegradation. Mineral salt medium compositions in anoxic media design are commonly adopted from conventional mineral salt media.[42] Therefore, the used mineral salt media composition was adopted from conventional mineral salt media recipes (*e.g.* OECD guideline 301, DIN EN ISO 14851) as it is typically practised for anoxic media design. Only the phosphate buffer concentration was increased to get a higher buffer capacity for the pH increasing denitrifying process. The final mineral salt medium composition was as follows: phosphate-buffer (3.75 g L^{-1} KH_2PO_4 and 8.73 g L^{-1} $Na_2HPO_4{\cdot}2H_2O$) and magnesium and calcium salts (22.5 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 36.4 mg L^{-1} $CaCl_2{\cdot}2H_2O$). As trace element solutions, SL4 and SL6 were used without EDTA. They are listed by the German Collection of Microorganisms and Cell Cultures, *e.g.* in the mineral salt medium N°457. SL4 contains the essential iron ion for microbial growth and SL6 a mixture of essential metals. Ascorbic acid in a non-inhibiting concentration of 0.5 g L^{-1} served as a reducing

Table 1 HPLC methods for selected ILs in the experimental matrix (Flow: 1 mL min^{-1}. $T = 40$ °C)

	CS Multospher 100 Si–5 μm			MN CC70/4 NUCLEOSIL 100-5 SA		
	Acetonitrile	$K_2HPO_{4\,aq}$	$K_2HPO_{4\,aq}$ Concentration	Methanol	$K_2HPO_{4\,aq}$	$K_2HPO_{4\,aq}$ Concentration
Ionic liquid	in (%)	in (%)	in mmole L^{-1}	in (%)	in (%)	in mmole L^{-1}
IM12 Cl	75	25	10	65	35	40
IM18 Cl	80	20	10	60	40	25
IM18OH Br	75	25	10	45	55	25
Py2 Cl	75	25	10	65	35	40
Py8 Cl	80	20	10	60	40	25
Py1-4NMe2 I	75	25	10	65	35	40
Py2-4NMe2 Br	80	20	10	65	35	40
Py4-4NMe2 Cl	80	20	10	65	35	40
Py6-4NMe2 Cl	80	20	10	60	40	25

agent for molecular oxygen. The hot autoclaved medium was degassed by a nitrogen stream. Sodium nitrate ($NaNO_3$) was the nitrate source prepared in 775 mmole L^{-1} stock solutions and kept in a refrigerator at 8 °C. The initial concentration of nitrate in the medium was set to 50 mg L^{-1}. During the course of the experiment, nitrate was replenished whenever its concentration decreased below 5 mg L^{-1} nitrate nitrogen. When nitrate was replenished, the nitrite concentration had been lower than the detection limit. An accumulation of nitrite would finally be toxic to the inoculum, which was not the case in the conducted experiment. The inoculum used was collected from the activated sludge of the communal wastewater treatment plant in Delmenhorst (Germany) in August 2008 (final concentration of 217 ± 10.5 mg L^{-1} total solids in each sampling vessel). ILs were added as the only carbon source in a final concentration of 200 μmole L^{-1}. For the testing of co-metabolism, an equimolar amount of acetate as a second carbon source was added to two further parallel cultures from each IL. The experiment was carried out in 250 mL glass vessels sealed with butyl rubber septa and closed with centre hole caps. The vessels were filled with 200 mL mineral salt medium, 20 mL activated sludge, 2 mL ionic liquid stock solution (20,000 μmole L^{-1}) and a nitrogen atmosphere. The samples were kept at room temperature in the dark. 1.3 mL of each sample were taken at each testing day. The pH value was measured for each sample taken with pH indicator strips from Merck in the range of 6.5–10.0 in 0.3 intervals. The sample was centrifuged for 15 min at 14,500 rpm. The nitrite/nitrate concentration was analysed in the centrifugate by nitrite/nitrate testing strips from Merck (Nitrate-Test/Nitrite-Test Merckoquant®). The final specific analysis of the ionic liquid cation was then conducted by HPLC-UV measurements. An internal standard for each substance was run during each measurement HPLC-UV sequence to identify the IL peak and check the consistency of the analytical method. The internal standard was a defined amount of each analyte. The sampling and the addition of nitrate changed the total volume of the samples and thus the concentration of ILs. The measured concentration was therefore corrected to the calculated volume. The correction could reach a change between 2 to 8% of the detected concentration depending on the amount of samples taken and solution added.

The samples were taken on the initial day of the experiment after inoculation and in shorter intervals at the beginning (2-3 d). After two weeks the sampling was reduced to once a week, and after 60 d the samples were taken once a month. After half a year, the vessels were left for five months until the last samples were taken. All in all, two parallel cultures were run for each testing substance and two for each co-metabolism test.

To be able to evaluate the biodegradability of ILs appropriately, additional control samples had been set up without the testing substances: (1) the blank sample (inoculated sample without testing substance) to control the ground noise and additional peaks from contaminations, (2) a positive control to observe the microbial activity, as the positive control served the easily biodegradable acetate. Microbial activity could be measured by pressure increase and pH increase.

Results and discussion

Toxicity and adsorption

The decrease of IL concentration was used as an indicator for biological degradation. A toxic effect of the used IL itself could be excluded. In the control vessels with acetate and the testing IL in a concentration range of 200 μmole L^{-1}, the microbial activity has been determined by the production of nitrite, depletion of nitrate/nitrite and the increase of the pH-value. No significant toxic effect towards the microbial community has been observed for any of the examined ILs. This means that concentration stability of the tested IL cannot be referred to the toxicity of the IL to the inoculum but to its non-biodegradability under these experimental conditions.

Adsorption of the test substance on activated sludge or the glass vessel surface is another influence on the IL concentration in the sample vessels, which could lead to a false interpretation of the results. If adsorbed, the concentration of the IL would decrease although no microbial activity was involved. Therefore, the adsorption of ILs should have been tested by the addition of $HgCl_2$. The concentration difference between the toxified samples and the samples with microbial activity is seen as the amount of adsorbed IL. However, the bacteria were strongly resistant to the applied concentration of 250 mg L^{-1} $HgCl_2$. A higher concentration of $HgCl_2$ or the addition of NaN_3 might increase a toxic effect and might make a study on the adsorption of IL possible. In this study, it was not further examined experimentally, because in this case, the concentration

of the used ILs did not show any significant decrease of concentration during the first three days, which could be related to an adsorption on activated sludge or the glass vessel surface.

Considering these facts, any decrease in concentration of the test compounds after three days was considered as evidence for biological degradation.

Primary biodegradation and metabolisation

The concentrations of the tested imidazolium, pyridinium and dimethylaminopyridinium cations (Fig. 1) remained stable during the testing period of 328 d, except the concentration of the hydroxylated imidazolium cation (Fig. 2). Therefore, most of the tested ILs were declared as not biodegradable under the experimental conditions. The addition of excess acetate did not help to decrease the measured IL concentration. Thus, the occurrence of a significant co-metabolic process with this substrate is excluded in the conducted experiment (results not shown). A decrease of concentration could only be observed for the 1-(8-hydroxyoctyl)-3-methyl-imidazolium cation (IM18OH). Around 52 to 54% of this substance was already degraded after 9 days. After 34 and 41 days, respectively, no more IM18OH could be detected in the parallel cultures.

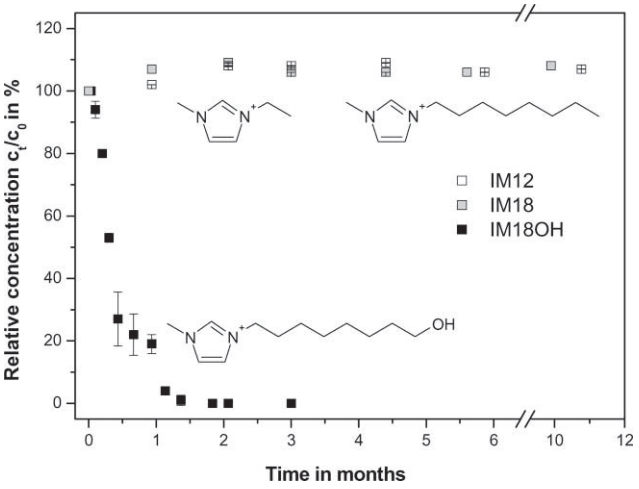

Fig. 2 Mean values with standard abbreviations of the relative concentrations of IM12, IM18 and IM18OH over time ($n = 2$). They are used as examples for the observed results of the investigated imidazolium, pyridinium and dimethylaminopyridinium ILs. The examined ILs were not biodegradable except IM18OH. 1 month = 30 days.

A closer look at the IM18OH samples by MS measurements revealed the structural changes during the observed degradation period. At the beginning of the experiment, the original IM18OH cation (211 m/z^+) could be detected in the corresponding samples (Fig. 3a). In the early phase, a decrease of IM18OH cation has been observed. Substances with a mass-to-charge-ratio of 225 and 197 in the positive mode are now found (Fig. 3b). In the last samples taken, the former observed peaks cannot be detected any more, but a substance with a mass-to-charge-ratio of 169 is found instead (Fig. 3c). The identity of these mass-to-charge ratios was further analysed by MS-MS measurements. For the mass-to-charge-ratio 169, 197 and 225, the 1-methyl-imidazolium fragment (83 m/z^+) could be detected. It is characteristic for imidazolium based cations. Therefore, the detected values presumably belong to the biological transformation products 1-(7-carboxyheptyl)-3-methyl-imidazolium cation (IM17COOH), 1-(5-carboxypentyl)-3-methyl-imidazolium cation (IM15COOH) and 3-(3-carboxypropyl)-1-methyl-imidazolium cation (IM13COOH).

The observed metabolites are the expected products when the alkyl side chain is biodegraded *via* β-oxidation (Fig. 4), which has already been observed for the aerobic metabolism of IM18OH.[14]

Comparing the biodegradation of IM18 cation and IM18OH cation (Fig. 4), one can conclude the predominant degradation mechanism. In aerobic biodegradation processes, the initial oxidation of the octyl side chain of the IM18 cation probably involves molecular oxygen as a reactant. The oxygen is inserted by monooxygenase and the alkyl side chain can be further degraded *via* β-oxidation. Under anaerobic conditions, no molecular oxygen can be inserted by monooxygenase and the biodegradation is not initialised. In contrast, the pre-oxygenated IM18OH can be biodegraded under anaerobic conditions (Fig. 4). However, the present experiments denote no full mineralisation of IM18OH or only a very slow biodegradation under the experimental anaerobic conditions. A residual rest of IM13COOH was still detected even after 10 months of the experiment (Fig. 3c). A similar behaviour had been observed for the imidazolium based ILs under aerobic conditions.[14]

All in all, it can be concluded that the head group of the IL cations does not seem to be the target of the initial enzymatic attack under aerobic as well as anaerobic conditions.

These observations of the formation of metabolites during the biodegradation process are to be considered within a proper hazard assessment. In general, a connection between toxicity and lipophilicity of the chemical substances has been discovered in recent studies.[41] Those ILs, which are less lipophilic, are in general less toxic than those with longer alkyl side chains. The majority of detected metabolites during the present biodegradation process already underwent *in vitro* screening toxicity tests on enzyme inhibition (acetylcholinesterase) and cytotoxicity (IPC-81 leukemia cells) for a first rough toxicity assessment (Table 2).

The results show that the transformation of alkyl side chains of the cationic head group to shorter alkyl side chains containing carboxy or hydroxy groups is advantageous in respect to its reduced toxicity towards cells[12] and aquatic organisms.[43] However, it cannot be excluded until now that other reactive species, such as epoxides, might be generated during transformation. In general, for a profound hazard assessment further tests are necessary. The investigated ILs substituted with alkyl side chains

Table 2 EC_{50}-values of ionic liquids: the substrate IM18OH and its detected metabolites in anaerobic biodegradation. The values demonstrate a reduced toxicity of the initial substrate in comparison to its detected metabolites

	EC_{50}-values in μmol L^{-1}	
Ionic Liquid	Acetylcholinesterase inhibition[44]	IPC-81 leukemia cells cytotoxicity[41]
IM18OH Br	19	229
IM17COOH Br	> 1000	> 3020
IM15COOH Br	No data available	No data available
IM13COOH Cl	> 1000	> 3020

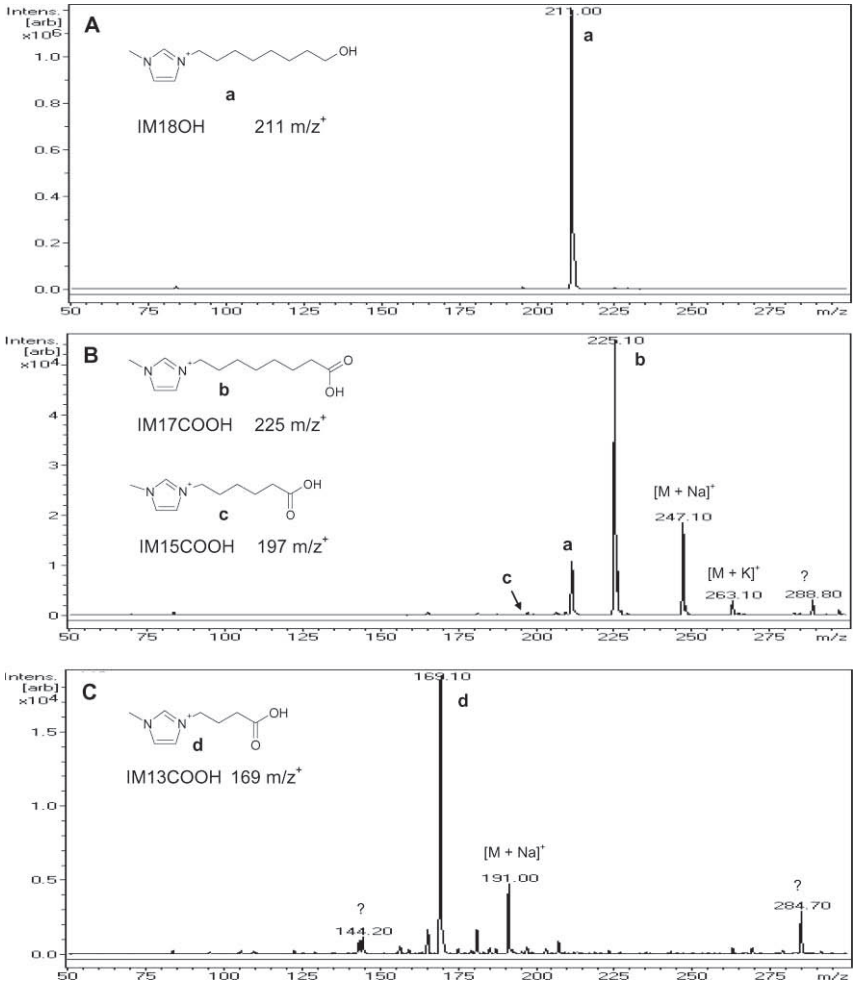

Fig. 3 Mass spectra of the IM18OH samples from the anaerobic biodegradation process at the beginning (A), in the early phase (B) and after 318 days (C). Substrate: IM18OH Br (211 m/z^+). (a) Initial peak with a mass-to-charge ratio 211 m/z^+ belonging to the substrate IM18OH Br, (b) 1-(7-carboxyheptyl)-3-methyl-imidazolium cation (IM17COOH; 225 m/z^+), (c) 1-(5-carboxypentyl)-3-methyl-imidazolium cation (IM15COOH; 197 m/z^+), (d) 3-(3-carboxypropyl)-1-methyl-imidazolium cation (IM13COOH; 169 m/z); $[M + Na]^+$ and $[M + K]^+$ are quasi-molecular ions. The sodium and potassium probably come from the mineral salt medium. Other unidentified peaks '?' appear in a lower concentration than the molecular ion.

were not transformed, and therefore, might not be biodegradable during the denitrification in waste water treatment plants, soils *etc.* Nevertheless, anaerobic biodegradability is still possible, if other bacteria exist that are able to degrade ILs under those conditions. For example, the anaerobic biodegradability of hydrocarbons was not discovered until at the end of the last century bacteria were found that are able to degrade these compounds. It depended on the discovery of a new initialisation step, by which the anaerobians can biodegrade the hydrocarbons with the help of fumarate.[39,45-49] A similar initialisation step for the anaerobic biodegradation of ionic liquids is still to be found.

Conclusion

The biodegradation of ILs under denitrifying conditions has been examined for the first time. It is concluded from the present study that the experimental process does not seem to be a solution to remove the examined alkyl substituted ILs efficiently from the environment. It is assumed that these IL cations cannot be eliminated in the denitrification step of a waste water treatment plant. They might reach the environment if they are not or slowly biodegradable under aerobic conditions or adsorb on activated sludge during water treatment. Therefore, in structural IL design, one cannot rely on anaerobic biodegradation of imidazolium, pyridinium and dimethylpyridinium ILs with an alkyl side chain at the moment. Nevertheless, the hydroxylated imidazolium IL does show activity of primary biodegradation under the experimental denitrifying conditions. Only its headgroup with a short carboxy alkyl side chain remained even after 318 days of the experiment. Although no metabolic pathway that might degrade the imidazolium IL headgroup had been found so far, it might still be advantageous to insert terminal hydroxy groups into long chained ILs either prospectively by structural design or retrospectively through chemical oxidation. Thereby, ILs can be made available to a wider range of microorganisms in oxic and anoxic milieus, but it should also be taken into consideration that such a chemical modification might reduce the industrial applicability. Furthermore, the option to find microorganisms, which are able to degrade ILs, still remains among the huge variety of

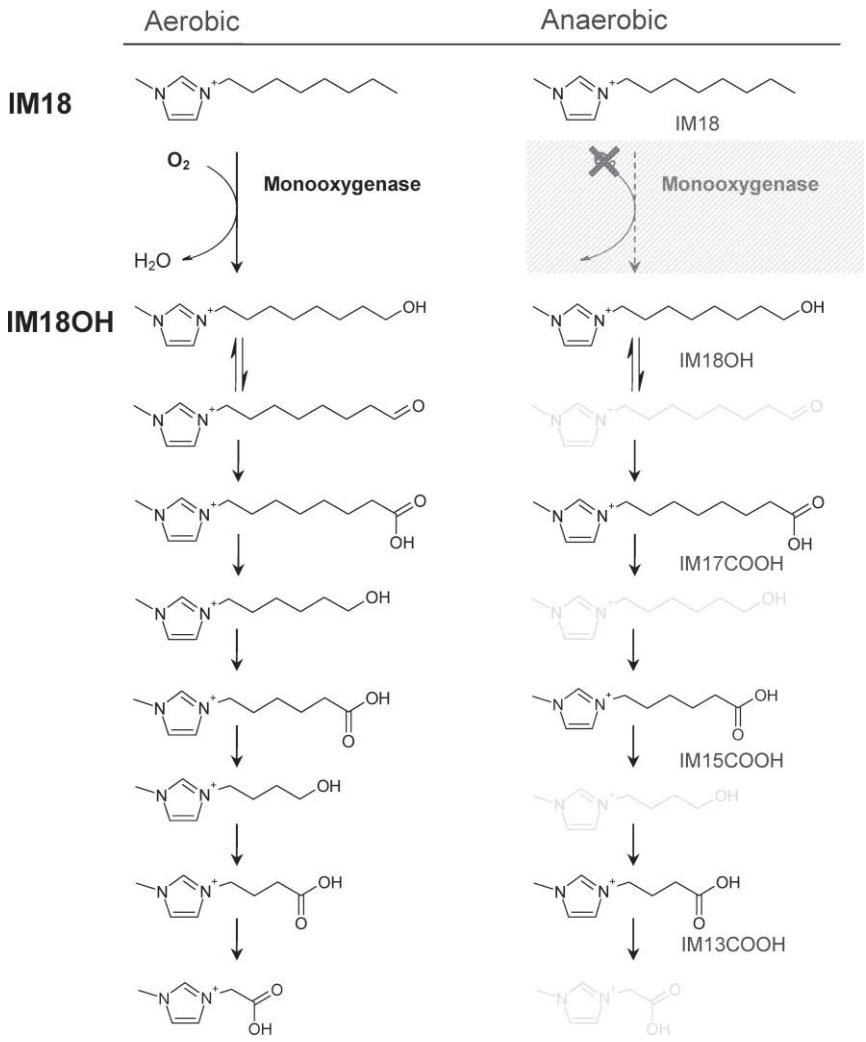

Fig. 4 Simplified scheme of the proposed aerobic[14] and anaerobic metabolic pathway for 3-methyl-1-octylimidazolium chloride (IM18 Cl) and 1-(8-hydroxyoctyl)-3-methyl-imidazolium bromide (IM18OH Br). Without oxygen the anaerobic biodegradation of the alkyl side chain of IM18 is not initialised. The chemical structures in black were found by MS measurements and are further indicated with their acrynoms. The ones in grey are theoretical intermediates.

microbial life. The biodegradation of ILs by obligate anaerobes, for example, still needs to be investigated.

All in all, we expect the described findings to fill the gap in the hazard assessment of ILs and to contribute to a structural IL design that favours sustainable application of ionic liquids.

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Paper A.2 - Biodegradability of fluoroorganic and cyano-based ionic liquid anions under aerobic and anaerobic conditions

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Own contributions:

Apart from the aerobic experiments on inhibition and biodegradation and the programming of MATLAB for the illustration of the anaerobic inhibition results, all anaerobic experiments as well as the analytical measurements by IC, the evaluation and interpretation of all of the results were conducted by myself. For the lab work of the inhibition testing I was partly supported by technical apprentices and students. Furthermore I established the anaerobic inhibition test system and published the results after discussion with the other co-authors.

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PAPER

Biodegradability of fluoroorganic and cyano-based ionic liquid anions under aerobic and anaerobic conditions

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The present study deals with the primary biodegradability of ionic liquids in order to obtain a greater insight into their fate under different environmental conditions. The focus was thereby on the biodegradation potential of ionic liquid anions when undergoing aerobic and anaerobic biological waste water treatment. Five technologically relevant fluoroorganic and cyano-based ionic liquid anions were investigated as alkaline salts (Li $(CF_3SO_2)_2N$, K $(C_2F_5)_3PF_3$ and Na $N(CN)_2$, K $C(CN)_3$, K $B(CN)_4$ respectively). Their biodegradability was determined in activated sludge over a period of around 60 days by specific analysis of the anion using ion chromatography. Additionally, the antimicrobial activity of the test compounds towards the activated sludge organisms was tested in inhibition studies. Because of the technologically desirable chemical, thermal and electrochemical stability of these anions, their biodegradability is questioned. The results seem to support the hypothesis: although the concentrations used did not inhibit the inoculum, none of these anions could be biodegraded under either aerobic or denitrifying conditions. The present paper provides information concerning the biodegradability of ionic liquids in waste water treatment plants and gives a first systematic view of the aerobic and anaerobic biodegradability of fluoroorganic and cyano-based ionic liquid anions and therefore supports further hazard assessment.

Introduction

Ionic liquids (ILs) are generally defined as molten salts that are already liquid below a temperature of 100 °C. The increasing interest being shown by the chemical industry and the scientific community in certain kinds of ILs is due mainly to their excellent organic solvent properties combined with their specific physicochemical properties, such as very low vapour pressure and non-flammability, which offer excellent operational safety. Additionally, their high thermal stability and catalysing properties for many organic reactions are responsible for the popularity of this class of substances.[1–3] Nowadays, their field of application is wide, ranging from solvents in traditional synthesis,[4] catalysts[3] and enzyme stabilisers,[5] through sensors,[6,7] potential pharmaceutical ingredients[8,9] and agents for analytical measurements,[10–12] to applications in gel production,[13] metal deposition,[14] oil extraction[15] and recovery solutions.[16] Even the usage of hypergolic and hydrogen-rich ILs as rocket fuel is being discussed.[17] Such a huge variety of applications are favoured by the large number of combinations in which ILs can be produced. Different cationic head groups, side chains and anions can be arranged to form many different substances. A few hundred are commercially available, several thousand are described in the literature and millions to trillions of ILs are estimated to be potentially accessible.[18]

Because of this vast number of potential ILs and their increasing usage in different applications, the question of their environmental compatibility has arisen: increasing usage leads to a higher probability of their exposure to the environment. Consequently, key parameters in hazard assessment, such as biodegradability and toxicity, for estimating the environmental fate and activity of chemicals are of great importance for preventing negative effects on humans and the environment. In the last two years alone, four reviews on the biodegradation and environmental fate of ILs have been published, in addition

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to those published before 2009.[19,20] Recent reviews include a set of data on the environmental fate and toxicity of ILs, a summary of biodegradability data, methods and applications, and a discussion of the ambiguous image of ILs.[21–23] Whether ILs can be considered green or not is a question that cannot be answered by a straightforward yes or no: ILs as a substance class are simply too diverse in structure and properties. This problem has been further addressed in a recent review, which deals in detail with the manifold chemical structures being researched, listing their biodegradation potential and sites of degradation, and discusses their biodegradability with respect to their chemical structure, ecotoxicity and technical applications.[24]

As the number of publications already indicates, a lot of data on IL biodegradation have already been published, applying ready biodegradability test procedures under aerobic conditions,[4,25–36] mainly OECD guideline No. 301.[37] From these studies it is known that the biodegradability of imidazolium, pyridinium, and 4-(dimethylamino)pyridinium cations is increased when they contain elongated alkyl side chains (C_8 > C_6 > C_4).[29,38] Moreover, improved biodegradability has been achieved by the introduction of functional groups into the side chains, *e.g.* esters.[27] With regard to the cationic core, the pyridinium head group generally exhibits a higher degree of biodegradation than imidazolium head groups. The pyridinium head group itself has been found to be ultimately biodegradable, even when it is linked to shorter side chains (C_4) and under stringent, ready biodegradation test conditions.[39,40] In contrast to the readily biodegradable pyridinium core, imidazolium head groups remain recalcitrant towards biodegradation processes, even though their alkyl side chain can still be biodegraded.[29,38] Since little is known about the biodegradation of ILs under denitrifying conditions, this aspect has been investigated with the focus on the primary biodegradation of IL cations. However, the biodegradability of imidazolium, pyridinium and dimethylpyridinium cations with alkyl side chains in activated sludge from a municipal waste water treatment plant was found to be even worse than under aerobic conditions and could only be performed when the alkyl side chain contained a hydroxy-group that had already been oxidised.[41]

It is furthermore important to stress that a biodegradable and non-persistent IL consists of a biodegradable cation in combination with a(an) (a)biotically degradable anion. Thus, in the design of environmentally benign ILs, not only cations but also anions need to be considered. Until now, most of the ILs investigated have contained inorganic anions (halides, BF_4^-, PF_6^-), which are not relevant to biodegradation tests based on the measurement of the carbon dioxide evolved as a result of substrate oxidation. Therefore, other degradation processes, *e.g.* hydrolysis or photolysis, may be relevant to the inorganic anions mentioned above.[19] Biodegradable anions are usually organic anions, such as alkylsulphates (like methylsulphate or octylsulphate),[27] acetate, and naphthenic acids like 3-cylcohexylpropionate,[28] to name just a few.

For the most common and technologically relevant anions, such as fluoroorganic and cyano-based IL anions, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $N(CN)_2^-$, $C(CN)_3^-$ $B(CN)_4^-$ respectively), systematic investigations into their biodegradability are still lacking. In combination with an appropriate cation, these weakly coordinating anions form ILs with low viscosities, high thermal and electrochemical stability and hydrophobicity (apart from $N(CN)_2^-$), which has been one of the major development aims of researchers in the last decade to obtain even better technological performances. To attain the goal of producing more sustainable ILs, which implies optimal technical applicability on the one hand and a minimum of hazard potential for humans and the environment on the other, we formed a university–industry partnership with Merck KGaA. Together we aimed to fill the knowledge gap in the field of anion biodegradability and focused on the question whether the selected anions are biodegradable when subjected to biological waste water treatment using aerobic and anaerobic environmental conditions. We therefore performed tests under standard aerobic conditions (in accordance with ready biodegradability test procedures) and under denitrifying conditions to expand the diversity of microorganisms and enzymatic reactions. For the aerobic and anaerobic tests, we used an inoculum from waste water treatment plants and measured the course of biodegradation by ion chromatography. It has to be borne in mind that a typical IL cation, whether biodegradable or not, may influence the biodegradability of the anion not only because of the potential antimicrobial activity of the cationic moiety, but also as a result of the reduced water solubility that accompanies the diminished bioavailability of the anion to the microorganisms in the surrounding medium. The selected anions were therefore tested in combination with inorganic counter ions in order to obtain the intrinsic biodegradability of the anion without the influence of a biodegradable organic IL cation. (Fig. 1)

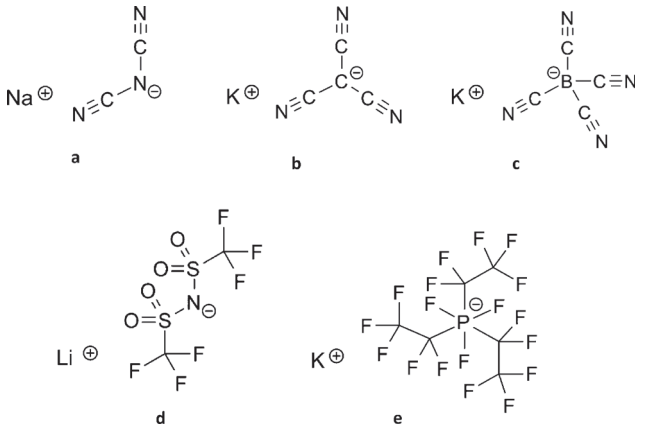

Fig. 1 The ionic liquids investigated in this study: (a) sodium N-cyanocyanamide (Na $N(CN)_2$), (b) potassium tricyanomethanide (K $C(CN)_3$) and (c) potassium tetracyanoborate (K $B(CN)_4$), (d) lithium bis(trifluoromethylsulfonyl)amide (Li $(CF_3SO_2)_2N$) and (e) potassium trifluoridotris(pentafluoroethyl)phosphate (K $(C_2F_5)_3PF_3$).

Apart from the biodegradability of the IL, its toxicity towards the activated sewage sludge environment is important for a sound hazard and risk assessment of ILs. Any adverse effects of ILs on the biological activity of a waste water treatment plant may lead to the inadequate degradation of water contaminants and in the worst case to a breakdown of the biological treatment step of the plant. Moreover, should an IL display inhibitory effects towards the microbial community, the data from biodegradation experiments may lead to an erroneous conclusion. The IL may be declared non-biodegradable, even

though only an excessively high test concentration of the IL was chosen, which inhibited biodegradation. To examine the influence of ionic liquid anions on the inoculum, we performed sewage sludge inhibition tests under aerobic and anaerobic conditions.

Our systematic studies of the biodegradability and antimicrobial activity of IL anions are addressed to the users of ILs in different fields of application to facilitate the selection of environmentally favourable structural elements and hence to contribute to the design of inherently safer ILs.

Materials and methods

Chemicals

All the tested salts of IL anions as well as the salts for the mineral salt medium and the standard eluent for ion chromatographic analysis were received from Merck KGaA (Darmstadt, Germany). Acetonitrile (HPLC grade) for the ion chromatographic measurements was obtained from Fluka (Buchs, Switzerland).

Biodegradation tests

1 Aerobic biodegradability. The aerobic biodegradation was based on OECD guideline No. 301,[37] and run for 58 days. The activated sludge was taken from a municipal waste water treatment plant in Delmenhorst (Germany) in January 2011 and aerated for seven days in order to remove organic substances before inoculation. The medium was prepared with 8.5 mg L^{-1} KH_2PO_4, 21.75 mg L^{-1} K_2HPO_4, 22.13 mg L^{-1} $Na_2HPO_4{\cdot}2H_2O$, 1.7 mg L^{-1} NH_4Cl, 36.4 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 22.5 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ and 0.25 mg L^{-1} $FeCl_3$ (pH 7.2). 90 mL of medium was poured into 100 mL glass vessels and 10 mL activated sludge added. The bacterial colony density referred to 10^6 colony forming units determined *via* Paddle-Tester (Hach Europe, Düsseldorf, Germany). The test substances and a positive control of aniline were prepared in deionised water and injected to a final concentration of 50 μmol L^{-1} and 1.07 mmol L^{-1} (100 mg L^{-1}) respectively, in the inoculated test media and stirred continuously with a magnetic bar in the dark. The evaporated water was made good with an equal volume of deionised water. In order to ensure aerobic conditions, the concentration of dissolved oxygen was measured continuously using an oxygen electrode (WTW Stirr Ox G, inoLab Multi Level, Weilheim, Germany) and kept above 4 mg L^{-1} by regular aeration. For the abiotic test, the inoculated activated sludge was poisoned with 200 μmol L^{-1} sodium azide (NaN_3). Aniline was used as a reference substance for positive biodegradability, since aniline is known to be biodegradable under the chosen test conditions within 14 days.[42]

2 Biological degradation under denitrifying conditions. The primary biodegradation of IL anions under denitrifying (anaerobic nitrate respiration) conditions was also investigated by specific analysis of the anion for 63 days. As a positive control of the occurrence of biological denitrification, acetate was used, which is readily biodegradable under these conditions. Denitrification "nitrate respiration" is defined as "the conversion of nitrate into nitrogen gases under anoxic conditions."[43] The following chemical reaction describes the conversion of nitrate with acetate as the organic carbon source without biomass production as in eqn (1) (taken from Gerardi and modified[44]).

$$8\ NO_3^- + 5\ CH_3COO^- \rightarrow 4\ N_2 \uparrow + 10\ CO_2 \uparrow + H_2O + 13\ OH^- \quad (1)$$

The anaerobic biodegradation test under denitrifying conditions was therefore carried out in 250 mL glass vessels sealed with butyl rubber septa, closed with centre hole caps under a nitrogen atmosphere. The vessels were filled with 200 mL mineral salt medium, 20 mL activated sludge suspension, 0.3 mL IL stock solution (0.2 mol L^{-1}), 6 mL sodium acetate solution (2.05 mol L^{-1}), 4 mL sodium nitrate solution (0.58 mol L^{-1}) and a nitrogen phase. The mineral salt medium composition was adopted from OECD guideline No. 301,[37] and DIN EN ISO 14851, as is typically practised for the growth of facultative anaerobic bacteria.[45] Only the phosphate buffer concentration was increased to achieve a higher buffer capacity, since the number of hydroxyl ions increases when denitrification occurs. The final mineral salt medium composition was as follows: phosphate buffer (3.75 g L^{-1} KH_2PO_4 and 8.73 g L^{-1} $Na_2HPO_4{\cdot}2H_2O$) and magnesium and calcium salts (22.5 mg L^{-1} $MgSO_4{\cdot}7\ H_2O$, 36.4 mg L^{-1} $CaCl_2{\cdot}2H_2O$). As trace element solutions SL4 and SL6 were used without EDTA. They are listed by the German Collection of Microorganisms and Cell Cultures, *e.g.* in the mineral salt medium No. 457. SL4 contains essential iron ion for microbial growth, and SL6 is a mixture of essential metals in low concentrations. Anaerobic conditions were obtained by degasification of the hot autoclaved medium with a nitrogen stream, and these conditions were maintained by the addition of ascorbic acid in the non-inhibiting concentration of 0.5 g L^{-1} serving as reducing agent for molecular oxygen. The inoculum was provided by Merck KGaA (Darmstadt, Germany) in April 2010 from the activated sludge of their industrial wastewater treatment (final concentration of 206 ± 45 mg L^{-1} total solids in each sampling vessel for the biodegradation experiment). The volume of each sample was additionally equalised with deionised water in order to obtain the same volume in each sample. ILs were added in a final concentration of around 300 μmol L^{-1}. Two parallel cultures were run for each test substance. An additional blank sample was set up without the test substances for determining the ground noise and additional peaks from potential contaminants. The samples were then kept at room temperature in the dark.

During the course of the experiment the pressure increase due to microbial activity inside the closed test vessels was measured by a pressure measuring device (V-D3, Schlee GmbH & Co., Germany). Nitrate and nitrite concentrations were regularly measured by nitrite/nitrate testing strips from Merck (Nitrate-Test/Nitrite-Test Merckoquant®). Nitrate was replenished whenever its concentration fell below 150 mg L^{-1}. When nitrate was replenished, the nitrite concentration was lower than the detection limit, thereby preventing an accumulation of nitrite that would eventually be toxic to the inoculum. The pH was measured at the beginning and the end for each sample with pH indicator strips from Merck in the 6.5–10.0 range at 0.3 intervals.

3 Sampling and sampling preparation for instrumental analysis. Samples from the aerobic and anaerobic biodegradation tests were taken on the first day and thereafter at short time intervals for 58 and 63 days respectively. On each testing day,

Table 1 Syringe filters used for sample preparation of the studied anions for ion chromatographic measurements, their recovery rate, eluent composition (acetonitrile in standard IC eluent 3.2 mmol L^{-1} Na_2CO_3/1 mmol L^{-1} $NaHCO_3$), retention time and limit of detection (LOD) and limit of quantification (LOQ)

Anion	Syringe filter used	Recovery rate at 200 µmol L^{-1}	IC method acetonitrile	Retention time	LOD[46] in µmol L^{-1}	LOQ[46] in µmol L^{-1}
$N(CN)_2^-$	Polyamide	99 ± 0%	0%	12–13 min	0.1	0.3
$C(CN)_3^-$	Polyamide	104 ± 6%	16%	21–22 min	0.1	0.3
$B(CN)_4^-$	Regenerated cellulose	98 ± 4%	23%	19–20 min	0.1	0.3
$(CF_3SO_2)_2N^-$	Polyvinylformamide	101 ± 2%	23%	19–20 min	0.1	0.3
$(C_2F_5)_3PF_3^-$	Polyvinylformamide	80 ± 6%	34%	16–17 min	0.1	0.3

6 mL of the sample were centrifuged at 4000 rpm for 10 min (Labofuge 400R, Heraeus instruments GmbH, Germany) in 15 mL polypropylene centrifuge tubes (Sarstedt AG & Co., Germany). Because of the higher sludge concentration in the anaerobic biodegradation tests, these samples were additionally passed through different types of syringe filters according to the best recovery rate of each anion (Table 1). The Rotilabo® syringe filters from Carl Roth GmbH & Co. KG have a diameter of 15 mm and a pore size of 0.2 µm. The samples were stored in a freezer at –20 °C until all the samples had been taken (after 9 weeks). The final specific analysis of the IL anion was then conducted by IC. An external standard for each substance was run during each measurement sequence to identify the IL peak and the consistency of the analytical method. The analytical method has been validated and published.[46]

4 Ion chromatography. The ion chromatograph used was a "Metrohm 881 Compact IC pro" containing a "Metrohm 850 Conductivity Detector" connected to a "Metrohm 863 Compact Autosampler" (Metrohm AG, Switzerland). The "Metrosep A Supp 5" anion exchange column with a "Metrosep RP2 Guard" pre-column was kept at 30 °C in a column oven. A sequential suppressor system consisting of a "Metrohm Suppressor Module" and a "Metrohm CO2 Suppressor" for chemical and carbon dioxide suppression was also part of the system. The device was run at a flow rate of 0.7 mL min^{-1} and the injection volume of each sample was 20 µL. The data were finally evaluated using standard "Magic Net 1.1" software.

5 High-performance liquid chromatography. In order to analyse the concentration of aniline, the reference substance for the aerobic biodegradation tests, high-performance liquid chromatography was performed (Agilent 1100 Series + UV detector). The detector wavelength was set at 254 nm. The elution used consisted of a flow rate of 1 mL min^{-1} and an eluent of 39% acetonitrile and 61% buffer (15 mmol L^{-1} KH_2PO_4 and 30 mmol L^{-1} H_3PO_4). The column was an Obelisc R reversed-phase column (SIELC Technologies, USA).

Inhibition tests

The inhibition of the selected IL anions on the activated sludge microbial community was additionally investigated to find out whether the IL concentrations used had any negative effect on the biodegradability of the IL anion. Therefore, OECD test guideline No. 209,[47] was invoked for aerobic conditions, and an anaerobic inhibition experiment was adapted from OECD test guideline No. 224,[48] under the conditions applied to the denitrifying biodegradation test (as described above).

1 Inhibition under aerobic conditions. In OECD guideline No. 209,[47] inhibition is inferred from the respiration rate of two parallel samples, calculated after three hours of aeration from the measured oxygen consumption using an oxygen electrode (Inolab WTW, Germany) as in eqn (2). The bacteria are grown in synthetic sewage feed based on peptone, meat extract, urea and mineral salts at a pH of 7.5 ± 0.5. Several concentrations of the IL samples are additionally set up to calculate the EC_{50} value.

$$I = 1 - \left(\frac{2R_s}{R_{c1} + R_{c2}} \right) 100 \quad (2)$$

where
I – inhibition in %
R_s – oxygen-consumption rate at the tested concentration of the test substance
R_{c1} – oxygen-consumption rate, control 1
R_{c2} – oxygen-consumption rate, control 2

2 Inhibition under denitrifying conditions. Inhibition under denitrifying conditions of the activated sludge was inferred from the cumulative gas production in closed glass vessels under the same experimental conditions as those used for the anaerobic biodegradation test. Three replicate samples were compared to three replicates of a reference sample without inhibiting agent as in eqn (3) defined by OECD guideline No. 224.[48]

$$I = (1 - P_t / P_c) 100 \quad (3)$$

where
I – inhibition in %
P_t – cumulative pressure at a selected time
P_c – cumulative pressure of the control at the same time

The samples were prepared with sodium acetate as carbon source and sodium nitrate as terminal electron acceptor in a 3 : 1 ratio. Positive controls with 3,5-dichlorophenol as inhibiting agent and negative controls as "no inhibition" reference without the addition of testing agent were also set up. The vessels were stored at 25 °C in temperature-controlled cabinets in the dark. The pressure increases were measured after an equalising time of one hour and then twice a day over the experimental period. The pressure inside the closed bottles rises as a result of biological activity. After a lag phase, the bacteria start exponential growth (log phase). Bacterial growth reverts to a stationary phase as soon as the limits of growth (*e.g.* the substrate amount, pH-value, volume of the glass vessel) are reached. An inhibitory effect is observed if (1) the lag phase takes longer than in the reference samples, or (2) the total amount of gas produced over

the experimental time setting is not achieved. The inhibiting effect itself is highly dependent on the total amount of solids in the sample. The total solids are determined with the aid of an additional vessel set up in parallel with the inhibition samples. It is opened after inoculation and is not further investigated on inhibition, but 5 mL of the sample suspension are passed through a 25 mm diameter 0.45 µm cellulose nitrate filter (Sartorius AG, Göttingen, Germany) by means of a water jet pump and dried overnight at 80 °C. They are then cooled in a desiccator. The weight of the filters with the dry mass was subtracted from their net weight without the dry mass. The average of at least three replicates represents the total concentration of solids. The concentration of the substance is given as the ratio of substance in mg to total solids in mg.

The inhibition experiment was run with IL anion concentrations similar to those in the biodegradation experiment. The endpoint was selected depending on the growth curve of the bacteria: the end of the exponential phase.[49] The measurement data is fitted with the help of MATLAB 7.9.0 according to the formula given for a limited growth curve "logistic function" eqn (4), as is the case in the present study .[50]

$$y = \frac{y^{a}}{1+\left(\frac{y^{a}}{y_{0}}-1\right)e^{-rt}} \quad (4)$$

where
y – cumulative pressure in Pa
y_a – saturation value (asymptote) of the pressure in Pa
y_0 – initial pressure in Pa
r – relative growth rate
t – time in days

The point of inflection (t_i/y_i) is given with a pressure value y_i of half the asymptote value $y_{a/2}$ at the time of inflection t_i. The function of the inflection point for t_i is given as in eqn (5).[50]

$$t_i = \ln (y^{a}/y_0 - 1)/r \quad (5)$$

The point of intersection between the tangent of inflection and the asymptote of the pressure indicates the time t_x at which the linear phase ends and the stationary phase begins. This calculation procedure was applicable to different concentrations of the positive control sample of 3,5-dichlorophenol. Owing to the reproducibility of the calculation at this point and the highest sensitivity for microbial growth inhibition, the inhibition is always measured there.

Results and discussion

The primary biodegradation of the test substances under aerobic and denitrifying conditions was monitored for 58 and 63 days respectively. Specific analysis *via* ion chromatography (IC) using a conductivity detector yielded information on the concentration of the IL anion at a specific time of the experiment. In the following paragraphs the results of aerobic and denitrifying degradation are shown and discussed.

Tests under aerobic conditions

1 Aerobic biodegradation. The aerobic biodegradability of the selected IL anions and the reference substance aniline was investigated over a period of 58 days (Fig. 2).

Table 2 Inhibition data of ionic liquids under aerobic conditions

	Activated sludge inhibition	
Ionic liquid	EC_{50} in µmol L^{-1}	Standard deviation in %
Na $N(CN)_2$	307 000	0.05
K $C(CN)_3$	32 400	
K $B(CN)_4$	37 400	0.02
Li $(CF_3SO_2)_2N$	21 000	0.01
K $(C_2F_5)_3PF_3$	197	0.03

It was observed that the reference substance aniline was biodegraded within ten days (LOD < 5 µmol L^{-1}), indicating the general biological activity of the inoculum. The selected IL anions were not biodegradable under the experimental conditions. The concentrations of IL anions remained stable within the measurement uncertainty levels. Only the concentration of $C(CN)_3^-$ showed a loss of almost 20% (relative concentration on day 57: 81.9% ± 2.6%). However, this could not be related to biological activity, since the abiotic control showed the same decrease of around 20% (relative concentration on day 57: 80.5% ± 2.8%). Why the abiotic control decreased to that extent remains unclear. Abiotic effects, such as sorption and hydrolysis can be considered, but sorption effects that are often responsible for relative concentration losses usually occur within the first few days. Hydrolysis of $C(CN)_3^-$ is also unlikely because of its considerable stability in hydrolytic testing (unpublished data).

Because of the evaporation and topping up of deionised H_2O during the course of the biodegradation experiment and because the sample preparation procedure involves centrifugation, a variation of ± 20% may occur. For $(CF_3SO_2)_2N^-$ the results are in agreement with those of Wells and Coombe,[36] who found 0% biodegradation of $(CF_3SO_2)_2NH$ on measuring the biochemical oxygen demand during 28 days (OECD test method 301F). In any case, none of the IL anions can be classified as readily biodegradable, since the primary biodegradation step measured in the present study is a necessary prerequisite for the complete biodegradation of the compound measured in ready biodegradation studies. Even if 20% of the substance was primarily biodegraded, the biodegradation of the anion could not reach the threshold value necessary for passing the readily biodegradability criterion, *e.g.* 60% of the theoretical biochemical oxygen demand (OECD guideline No. 301F).

2 Aerobic inhibition. The aerobic inhibition tests of the studied IL anions were performed according to OECD guideline No. 209,[47] on the respiration rate of activated sludge from a municipal waste water treatment plant. The EC_{50} values cover different orders of magnitude, ranging from 197 µmol L^{-1} for the most inhibiting substance K $(C_2F_5)_3PF_3$ to 307 000 µmol L^{-1} for the least inhibiting one –Na $N(CN)_2$ (Table 2).

Generally, most of the investigated compounds showed inhibitory effects at concentrations greater than 20 000 µmol L^{-1}. In view of the low IL concentration of 50 µmol L^{-1} in the biodegradation experiments, any negative influence on the inoculum can be excluded. A comparatively strong effect on microbial respiration was found for the most lipophilic anion $(C_2F_5)_3PF_3^-$. This anion is known to be strongly and acutely

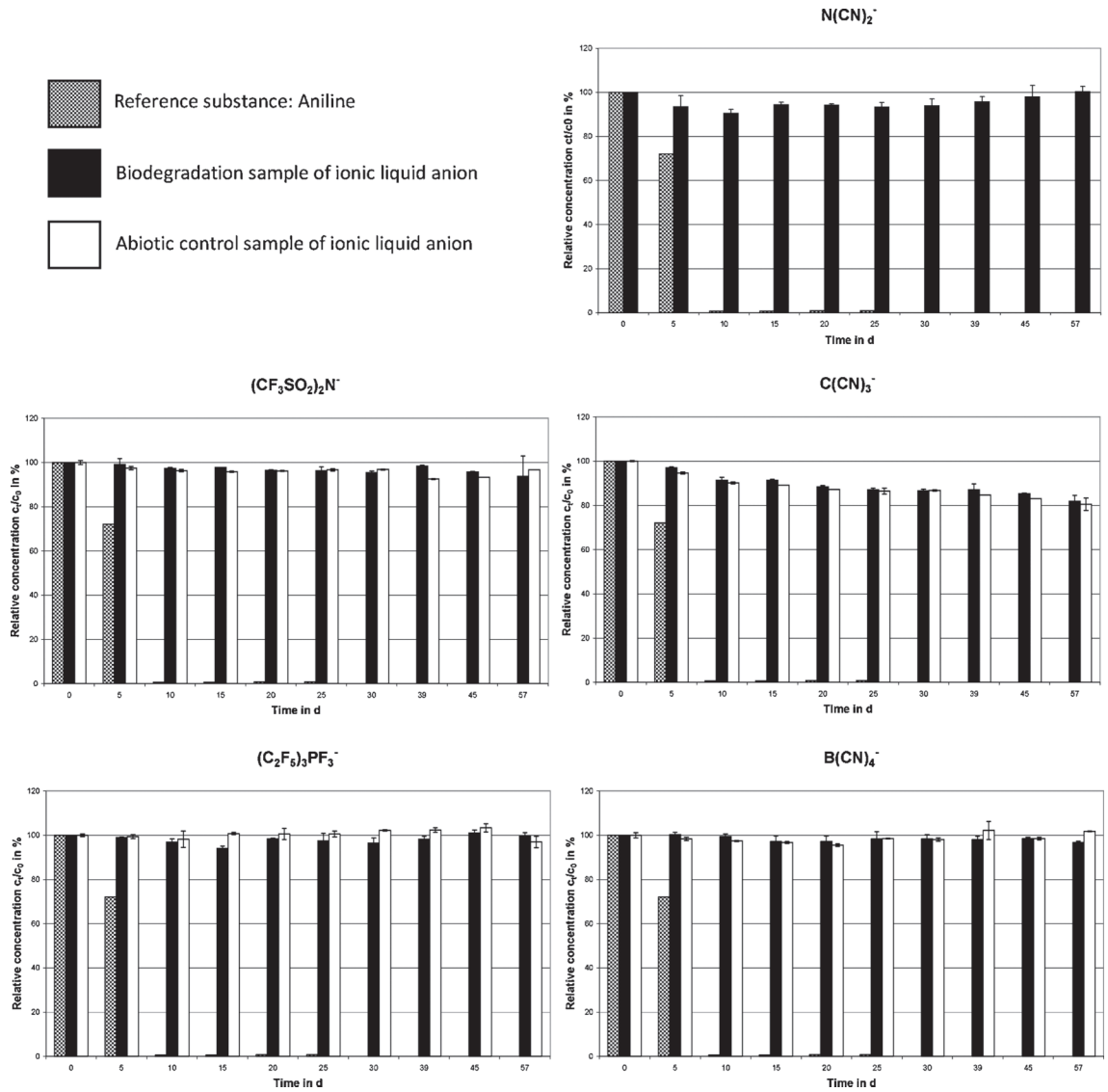

Fig. 2 Relative concentration over time of the investigated cyano-based (right) and fluoroorganic anions (left). The aniline reference (grey column) and the abiotic controls (white column) are illustrated in addition to the IL samples (black columns).

cytotoxic towards IPC-81 cells isolated from rats, and it is suggested that the higher the lipophilicity of the anion, the greater the toxic effect.[51] The fact that IL lipophilicity has a major influence on the inhibition of bacteria has already been demonstrated in the context of IL cations, where those with octyl side chains had a stronger inhibitory effect than those with shorter ethyl side chains.[19] Although K $(C_2F_5)_3PF_3$ was the most strongly inhibiting agent in the inhibition tests, its EC_{50} value is still higher than the concentration used in the biodegradation study. Therefore, non-biodegradability cannot be attributed to inhibition of bacterial growth; it is due rather to the non-biodegradability of the substance under the experimental conditions.

Tests under anaerobic conditions

1 Anaerobic biodegradation. The anaerobic biodegradability of IL anions under denitrifying conditions was monitored for 63 days (Fig. 3).

The microbial activity of the denitrification process could be monitored by measurements of general process characteristics according to eqn (1). Among other things, the cumulative pressure inside the closed sample vessels increases and the nitrate concentration decreases as a result of microbial activity. These characteristics were also observed in the present study (Fig. 3, point diagram), from which it was inferred that denitrification was microbial. However, no significant decreases in concentration over a period of more than two months were found for most of the IL anions. Thus, no biodegradation under anaerobic conditions for the investigated compounds took place. $(C_2F_5)_3PF_3^-$ showed the highest decrease in concentration of 15%. Adsorption on the sludge or glass is often seen as a reason for a non-biological decrease in concentration, but this would only occur within the first few hours or days. The change in relative concentration cannot be related directly to biological activity owing to the cessation of microbial activity after 3 weeks (stationary phase of the cumulative pressure curve

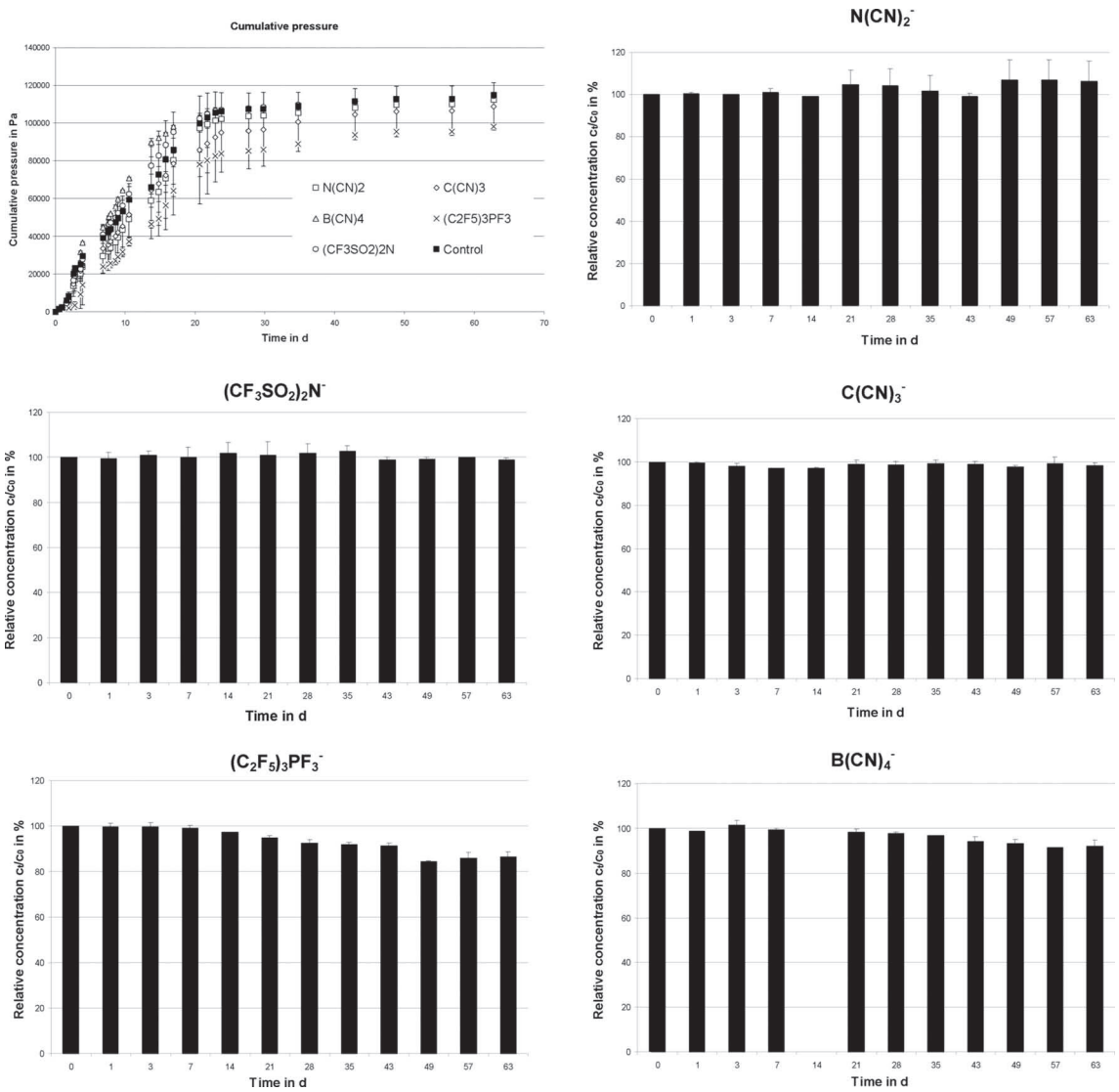

Fig. 3 Point diagram: pressure increase inside the closed bottles over time (positive control). Bar graphs: relative concentration of the studied ionic liquid anions over time.

(Fig. 3, point diagram)); it can, however, be related to analytical limitations of the detection of this IL anion. The signal peak of $(C_2F_5)_3PF_3^-$ appears with a tailing that is strongly influenced by the sewage sludge matrix effects.[52] Furthermore, the samples had to be filtered because of their higher sludge concentration than in the aerobic test vessels. Hence, the recovery rate of $(C_2F_5)_3PF_3^-$ is therefore the lowest (80% ± 6%). Owing to these analytical limitations, the change in relative concentration is assumed not to be related to biological activity. Therefore, the investigated IL anions have no biodegradation potential under the experimental conditions. In the case of $(C_2F_5)_3PF_3^-$ further experiments with an improved analytical measurement technique may be necessary to support or falsify the hypothesis. To ensure that the IL anions were not biodegraded due to an inhibitory effect of the anions, inhibition tests under anaerobic and denitrifying conditions were additionally conducted.

2 Inhibition under anaerobic conditions. The inhibition test was also run under conditions similar to those in the anaerobic biodegradation experiment. No significant inhibitory effect of the investigated cyano-based IL anions and $(CF_3SO_2)_2N^-$ on the bacteria suspension could be observed. (Fig. 4)

The bacteria suspension containing the IL anions did show a cumulative gas production similar to that of the negative control without inhibiting agent at concentrations similar to those in the biodegradation experiment ($N(CN)_2^-$: 0.08 mg mg^{-1}, $C(CN)_3^-$: 0.11 mg mg^{-1}, $B(CN)_4^-$: 0.14 mg mg^{-1}, $(CF_3SO_2)_2N^-$: 0.32 mg mg^{-1}). For the fluorinated compound $(C_2F_5)_3PF_3^-$ the picture is different: at the concentration level of the biodegradation experiment, K $(C_2F_5)_3PF_3$ already shows an inhibition of 82% ($(C_2F_5)_3PF_3^-$: 0.53 mg mg^{-1}). In contrast to the positive control samples containing 3,5-dichlorophenol, which inhibited the samples up to 94.4% with complete cessation of microbial growth, the K $(C_2F_5)_3PF_3$ samples merely retarded growth. However, the cumulative pressure curve finally reaches the total gas production of the negative control samples. The same observation for K $(C_2F_5)_3PF_3$ was made during the anaerobic biodegradation experiment (data not shown). The lag phase of the samples lasted approximately 1.5 days longer in comparison to the control and the other IL anions examined. However, this

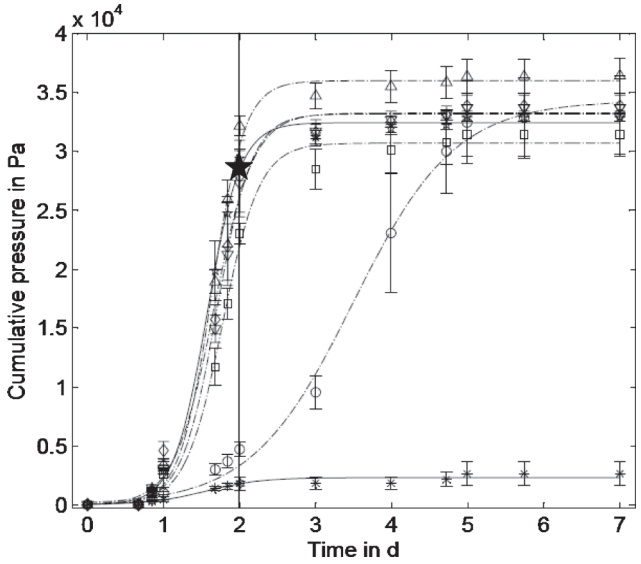

Fig. 4 Cumulative gas production over time at the ionic liquid concentrations similar to those used in the anaerobic biodegradation experiment. Solid lines: ⬮ no inhibition control, *3,5-dichlorophenol inhibition control. Slash dotted lines: △ Na $N(CN)_2$, ▽ K $C(CN)_3$, □ K $B(CN)_4$, ◇ Li $(CF_3SO_2)_2N$, ○ K $(C_2F_5)_3PF_3$.

is unlikely to be the cause for the non-biodegradability of the compound as the inhibition of inoculum was only delayed but not complete.

General biodegradability of cyano-based and fluoroorganic compounds

Although the selected cyano-based IL anions seemed to be non-biodegradable, related substances, such as cyanide and other cyano-based compounds are usually biodegraded along different metabolic pathways. The biodegradation and biotreatment of cyanide and cyano-based compounds has been reviewed several times in recent years.[53–55] Depending on the metabolic pathway and enzymes involved, different end products are possible. In case of the investigated cyano-based IL anions, a similar biodegradation process was expected, but did not occur. As cyanase is proposed for the catalysis of cyanide or cyano-metal degradation,[56] it could be shown that cyano-metal complexes are usually less susceptible to microbial attack.[53] Only highly specialised bacteria are capable of degrading them,[56] and these may not have been present in the municipal and industrial waste water inocula used in this study or did not grow sufficiently under the conditions used. The biodegradability potential of the fluorinated IL anions investigated here is generally considered to be very low. Several studies of chemically related perfluorinated anionic surfactants have been conducted: such compounds have been found to be persistent in the environment or very poorly biodegradable without complete mineralisation.[52] It is aromatic compounds that were defluorinated.[57] Therefore, it is doubtful that $(CF_3SO_2)_2N^-$ and $(C_2F_5)_3PF_3^-$ with perfluorinated alkyl chains undergo defluorination processes.

Summarising the results from aerobic and anaerobic inhibition testing, we may state that the cyano-based compounds and Li $(CF_3SO_2)_2N$ were not significantly inhibited at the concentrations used for the biodegradation experiment, with the exception of K $(C_2F_5)_3PF_3$, which was the most strongly inhibited of all the anions, though not toxic towards the microorganisms. It is the most stable, lipophilic and largest anion of all the anions tested in this study. The reason why these properties turn out to be inhibiting to bacteria cells depends on the cell and its metabolism. Growth occurs as long as many different aspects come together: the supply of sufficient nutrients, the supply of energy for the chemical reactions and enzymes that are able to catalyse the reactions are essential.[43] How K $(C_2F_5)_3PF_3$ inhibits the growth of a bacteria cell could well be a subject for further study. A similar inhibition mechanism as observed for chemically related substances such as perfluorinated surfactants is expected.[58]

Conclusions

All in all, the results of the non-biodegradability of the investigated IL anions under aerobic and anaerobic conditions are similar and support the hypothesis that the chemical stability of an IL anion, desirable in a technical application, also implies a low potential for rapid and complete biodegradability under the chosen test conditions. At the moment it cannot be expected that the studied IL anions will be readily biodegraded in aerobic and denitrifying treatment processes and corresponding environmental situations. In particular, K $(C_2F_5)_3PF_3$, which inhibits growth at the lowest concentration and has a low biodegradation potential, poses the greatest threat in this respect. However, these experiments were conducted under stringent conditions, so further biological testing will be necessary in order to find environmental conditions that will be more supportive of bacterial degradation. Inherent tests that are less stringent,[59] other anaerobic pathways such as fermentation, or abiotic ones such as hydrolysis or photolysis, *e.g.* in surface water, may play an important role in reducing the hazard. For technical applications, one can work in a closed system, thereby diminishing the risk of environmental exposure; separation techniques, *e.g.* nanofiltration,[60] can also be used to eliminate ionic liquid residues from waste water. For poorly biodegradable ILs, AOX[61,62] has been suggested as a special treatment for abiotic processes and can help to make the substances more amenable to biodegradation. Biotic treatment also seems to be possible when other microorganisms are used. A different inoculum using pure cultures of microorganisms, such as *Corynebacterium* sp. and *Sphingomonas paucimobilis*, has been reported to improve the biodegradability of IL cations[63–65] and could be conceivable for IL anions. A different counter-cation than the used alkali metal ions might lead to a different biodegradability and toxicity of the IL. However, it is still to be investigated in how far the combination of cation and anion increases or decreases the biodegradability of the ionic liquid and the inhibition effect on microbial communities.

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Paper A.3 - Hydrolysis study of fluoroorganic and cyano-based ionic liquid anions – consequences for operational safety and environmental stability

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Own contributions:

The mass-spectrometric analysis and the evaluation of the data for the identification of the transformation products and the proposed hydrolytical pathways were performed by me. These results laid the foundation for the understanding of the hydrolytical processes for the interpretation of further investigations on the kinetics and the toxicity of the hydrolysed solutions by the main author. I also supported Stephanie Steudte with discussions before publication.

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PAPER

Hydrolysis study of fluoroorganic and cyano-based ionic liquid anions – consequences for operational safety and environmental stability

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The hydrolytic stability of ionic liquid anions is a key property with regard to their technical applicability and environmental stability. From a technical point of view hydrolytic processes may lead to reduced durability, diminished technical performance and reduced operational safety in that corrosive and/or toxic hydrolysis products are formed. On the other hand, susceptibility to hydrolytic processes is advantageous where environmental stability and persistency are concerned, since hydrolysis is the most important abiotic degradation pathway in the environment. We investigated the hydrolytic stability of the most common ionic liquid anions, dicyanimide $[N(CN)_2]^-$, tricyanmethanide $[C(CN)_3]^-$, tetracyanidoboranate $[B(CN)_4]^-$, bis(trifluoromethylsulphonyl)imide $[(CF_3SO_2)_2N]^-$, trifluorotris(pentafluoroethyl)phosphate $[(C_2F_5)_3PF_3]^-$ and 1,1,2,2-tetrafluoroethanesulphonic acid $[H(C_2F_4)SO_3]^-$, as a function of pH (1, 7, 9 and 13) and temperature. The results show that there was no difference in hydrolytic stability as recorded for 1-ethyl-3-methylimidazolium (IM12) or for the alkali cations. All the anions were stable under neutral and slightly basic conditions (half-lives at 25 °C ⇒1 year). In strongly acidic and basic solutions, however, $B(CN)_4^-$, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $H(C_2F_4)SO_3^-$ were hydrolytically stable, whereas $N(CN)_2^-$ and $C(CN)_3^-$ were not. The kinetics of hydrolysis were recorded and Arrhenius plots were generated for the latter two anions. In addition, their hydrolysis pathways and the resulting products were identified *via* mass spectrometry. The cytotoxicity of hydrolysed IL solutions towards the mammalian cell line IPC-81 and the identified hydrolysis products (pure compounds) was investigated for a first estimate of their toxicological properties.

Introduction

Interest in ionic liquids (ILs), salts with low melting points (<100 °C), has risen in recent years. Depending on the appropriate combination of cations and anions, they can be thermally and electrochemically highly stable, be liquid over a wide range of temperatures, and have negligible vapour pressure. Several applications and potential implementations of ILs have been described, *e.g.* in aluminium plating,[1,2] gas compression,[3,4] dye-sensitized solar cells,[5,6] lubrication formulations[7,8] and separation techniques.[9–11] The cations used are mainly organic *N*-heterocyclic entities like imidazolium, pyridinium, pyrrolidinium and morpholinium, or aliphatic ammonium or phosphonium cations. They are usually asymmetrically substituted with bulky alkyl side chains of different length and/or functionality. The anions cover a wide range of types, from halides, alkyl sulphates, sulphonates, phosphates and phosphonates to perfluorinated compounds like tetrafluoroborate (BF_4^-), hexafluorophosphate (PF_6^-), bis(trifluoromethylsulphonyl)imide $[(CF_3SO_2)_2N^-]$, or trifluorotris(pentafluoroethyl)phosphate $[(C_2F_5)_3PF_3^-]$. The highly fluorinated anions in particular are of exceptional interest owing to their hydrophobicity, low viscosity and broad electrochemical window.[12,13] Recently, the super-acidic anion 1,1,2,2-tetrafluoroethanesulphonic acid ($H(C_2F_4)SO_3^-$) attracted attention because it can be applied in CO_2 absorption[14] or as a catalyst in alkylation, etherification and isomerization reactions.[15] Also, cyano-based anions such as dicyanimide ($N(CN)_2^-$),[16] tricyanmethanide ($C(CN)_3^-$)[17] and tetracyanidoboranate ($B(CN)_4^-$)[18] form ILs with wide electrochemical windows and low viscosities. ILs based on these anions therefore offer more efficient mass transport and higher conductivity, which make them particularly interesting for electrochemical applications. In particular, $B(CN)_4^-$ based ILs have a high potential for applications in dye-sensitized solar cells.[19]

The considerable thermal and electrochemical stabilities of certain ILs are additional reasons for their versatility. From a technical point of view, hydrolytic stabilities are equally important, because hydrolytic processes in aqueous media or caused by air moisture may lead to reduced durability (degradation), diminished technical performance (change of properties,

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formation of corrosive degradation products) and impaired operational safety in that corrosive and/or toxic hydrolysis products are formed. On the other hand, susceptibility to hydrolytic processes is advantageous in terms of environmental stability and persistence, since hydrolysis represents the most important abiotic degradation pathway in the environment. Several IL anions, such as $AlCl_4^-$, PF_6^- and BF_4^-, are known to react with water, leading to the formation of HCl or HF, as the case may be.[20] It was found that the potential of PF_6^- to be hydrolysed is strongly influenced by the type of cation it is attached to. Whereas KPF_6 is not significantly hydrolysed either in acidic (pH < 1), neutral and basic (pH > 12) solutions,[21] $LiPF_6$ was unstable in propylene carbonate–dimethyl carbonate–water solutions; here, HF, $PO_2F_2^-$ and PO_3F^{2-} were identified as hydrolysis products.[22] Studies of primarily imidazolium-based ILs showed that BF_4^- was rapidly hydrolysed in aqueous solutions even at room temperature. In contrast, PF_6^- was more stable under neutral conditions, although $PO_2F_2^-$ was formed as a product of hydrolysis under acidic or high temperature conditions. The side chain of the cation influences the course of hydrolysis for both anions. By changing the cation from 1-butyl-3-methylimidazolium (IM14) to 1-methyl-3-octylimidazolium (IM18) the formation of hydrolysis products was increased, perhaps due to decreasing cation–anion interactions.[23] Swatloski and co-workers were able to isolate the white crystals that were formed during the synthesis and purification of IM14 PF_6. X-ray studies identified IM14 $F\cdots H_2O$ as a hydrolysis product.[24] By replacing three fluorides in PF_6^- with perfluorinated alkyl chains such as C_2F_5, the hydrolytic stability could be increased, since no detectable HF formation was observed after 5 h in boiling water.[12] Using a rapid colorimetric assay, Baker and Baker tracked the hydrolysis of IM14 BF_4, IM14 PF_6, IM14 $(CF_3SO_2)_2N$ and 1-hexyl-3-methylimidazolium (IM16) $(C_2F_5)_3PF_3$ by means of the pH-dependent colour change of a reporter dye. Whereas at 25 °C only BF_4^- showed a pH decrease, this was demonstrated at 50 °C for PF_6^- after 24 h and after one week for $(C_2F_5)_3PF_3^-$ and $(CF_3SO_2)_2N^-$.[25] Even though the hydrolysis of BF_4^- and PF_6^- has been thoroughly investigated, there are few detailed studies investigating the hydrolytic processes undergone by the most common IL anions like $N(CN)_2^-$, $C(CN)_3^-$, $B(CN)_4^-$, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $H(C_2F_4)SO_3^-$ (Fig. 1). Recently, Neumann *et al.* showed that none of the anions (except $H(C_2F_4)SO_3^-$, which has not yet been investigated) is either aerobically or anaerobically biodegradable, which may have unforeseeable consequences for the environment in terms of persistence.[26] Therefore, an investigation of the hydrolytic degradation of the different IL anion species in aquatic systems at the pH values normally found in the environment is crucial for the hazard assessment of ionic liquids.

In order to evaluate the hydrolytic stability of IL anions under environmental conditions as well as in their applications, tests were performed according to OECD guideline 111. The hydrolysis at different pH values (pH 1, 7, 9, and 13) of these anions as inorganic salts (Li^+, Na^+ or K^+) and combined with the 1-ethyl-3-methylimidazolium (IM12) cation is determined by means of ion chromatographic measurements. Half-lives, activation energies and frequency factors can be estimated from kinetic studies at different temperatures. Mechanisms of hydrolysis are

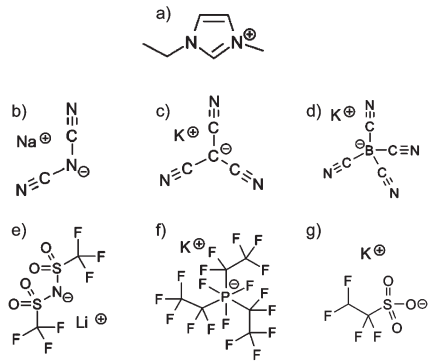

Fig. 1 Structures of (a) the 1-ethyl-3-methylimidazolium (IM12) cation and investigated anions as inorganic salts: (b) sodium dicyanimide ($NaN(CN)_2$), (c) potassium tricyanmethanide ($KC(CN)_3$), (d) potassium tetracyanidoboranate ($KB(CN)_4$), (e) lithium bis(trifluoromethylsulphonyl)imide ($Li(CF_3SO_2)_2N$), (f) potassium trifluorotris(pentafluoroethyl)-phosphate ($K(C_2F_5)_3PF_3$), (g) potassium 1,1,2,2-tetrafluoroethanesulphonic acid ($KH(C_2F_4)SO_3$).

postulated from an MS identification of the hydrolysis products. In the context of environmental and operational safety and for a preliminary hazard assessment, native ILs, hydrolysed solutions and isolated hydrolysis products were tested for cytotoxicity using the promyelocytic leukaemia rat cell line IPC-81.

This comprehensive study is addressed to users of ILs in different fields of applications in order to expand knowledge of the properties of ILs, as well as their behaviour in their applications and in the environment.

Material and methods

Chemicals

Lithium bis(trifluoromethylsulphonyl)imide ($Li(CF_3SO_2)_2N$), sodium dicyanimide ($NaN(CN)_2$), potassium tetracyanidoboranate ($KB(CN)_4$), potassium trifluorotris(pentafluoroethyl)phosphate ($K(C_2F_5)_3PF_3$), potassium tricyanmethanide ($KC(CN)_3$), 1-ethyl-3-methylimidazolium tricyanmethanide (IM12 $C(CN)_3$), 1-ethyl-3-methylimidazolium dicyanimide (IM12 $N(CN)_2$), 1-ethyl-3-methylimidazolium tetracyanidoboranate (IM12 $B(CN)_4$), 1-ethyl-3-methylimidazolium bis(trifluoromethylsulphonyl)imide (IM12 $(CF_3SO_2)_2N$), 1-ethyl-3-methylimidazolium trifluorotris-(pentafluoroethyl)phosphate (IM12 $(C_2F_5)_3PF_3$) were kindly provided by Merck KGaA (Darmstadt, Germany). Potassium 1,1,2,2-tetrafluoroethanesulphonic acid ($KH(C_2F_4)SO_3$) and 1-ethyl-3-methylimidazolium 1,1,2,2-tetrafluoroethanesulphonic acid (IM12 $H(C_2F_4)SO_3$) were obtained from IoLiTec Ionic Liquids Technologies GmbH (Heilbronn, Germany).

Hydrochloric acid, anhydrous sodium carbonate and sodium bicarbonate were purchased from the Sigma-Aldrich Corporation (Deisenhofen, Germany), acetonitrile (HPLC grade) was obtained from VWR International (Darmstadt, Germany), potassium chloride from Merck KGaA (Darmstadt, Germany), sodium hydroxide from Across Organics (Geel, Belgium), potassium dihydrogen phosphate from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and boric acid from Riedel de Haen GmbH (Seelze, Germany).

Hydrolysis

The hydrolysis tests were performed according to OECD guideline 111.[27] The buffer with pH 1.0 was prepared using 47.5 mL 0.2 M HCl, 25 mL 0.2 M KCl and made up to 100 mL with distilled water. 29.63 mL 0.1 M NaOH and 50 mL 0.1 M KH_2PO_4 diluted with distilled water to 100 mL were used to obtain the buffer with pH 7.0. To prepare the buffer with pH 9.0, distilled water was added to a mixture of 21.30 mL 0.1 M NaOH and 50 mL 0.1 M H_3BO_4 in 0.1 M KCl in a 100 mL flask. A mixture of 132 mL 0.2 M NaOH and 50 mL 0.2 M KCl was used to prepare the buffer with pH 13. The pH values of the buffers were checked and adjusted using small amounts of conc. HCl or NaOH. For the preliminary test four replicates of 10 mL solutions containing 100 μM IL and the inorganic salt were prepared in all buffers. The first replicate was measured by ion chromatography (a Metrohm 881 Compact IC system with an online eluent degasser, a 20 μL injection loop, a Metrosep A supp ion exchange column (dimensions – 50 × 4.0 mm ID and 5 μm mean particle size) coupled with a Metrosep A Supp 4/5 Guard and a Metrosep RP Guard, a self-regenerating Suppressor Module (MSM) and a CO_2-suppressor (MCS), a conductometric detector and Metrohm software (MagICNet version 1.1 compact), all purchased from Metrohm, Herisau, Switzerland) to obtain the initial concentration of IL anions using the method reported in ref. 28. The eluent was 3.2 mM Na_2CO_3, 1.0 mM $NaHCO_3$ and varying percentages of acetonitrile, depending on the anion investigated. The flow rate was adjusted to 0.7 mL min^{-1}. The limits of detection and quantification were determined to be <0.10 μM and 0.30 μM respectively.[28] For quantification of the IM12 cation a VWR Hitachi HPLC-UV system was used containing the L2130 HTA-pump, L2130 degasser, L2200 autosampler, L2300 column oven, L2450 diode array-detector and the EZChrom Elite software. As the stationary phase a hydrophilic interaction liquid chromatography column (HILIC, Multospher 100 Si 5 μm, 125 × 4.6 mm) with a guard column purchased from CS–Chromatographie Service GmbH (Langerwehe, Germany) was used with a mobile phase comprised of 65 v/v% methanol (HPLC grade) and 35 v/v% aqueous K_2HPO_4 solution (40 mM). The system was operated at a flow rate of 1 mL min^{-1} and 40 °C oven temperature and a detection wavelength of 211 nm was used for quantification of IM12 with limits of detection and quantification of 8 and 22 μM.

The other three replicates were stored in headspace vials and kept closed under an argon atmosphere for five days at 323 ± 0.5 K in a water bath (GFL mbH, Burgwedel, Germany). Afterwards, the final IL concentration of samples was measured again by ion chromatography or HPLC. If IL hydrolysis was less than 10%, the substance was considered to be hydrolytically stable with a half-life of >1 year at 298 K and no further tests were carried out. If hydrolysis exceeded 10%, advanced kinetic tests were performed: 100 μM of the substance were again dissolved in the buffer, and stored at three different temperatures (283, 298, 310, 323 or 343 K). The concentration was determined using ion chromatography and checked at regular intervals. Assuming pseudo-first order reaction kinetics, the rate constant (k) can be obtained by linear regression according to eqn (1):

$$-\ln\left(\frac{c_t}{c_0}\right) = kt \quad (1)$$

where t is the time, c_t is the concentration of the substance at time t and c_0 is the initial concentration. The half-life of the reaction ($t_{0.5}$) is given by

$$t_{0.5} = \frac{\ln 2}{k} \quad (2)$$

The correlation of temperature (T) and k can be expressed by the Arrhenius equation:

$$k = A\mathrm{e}^{-\frac{E_A}{RT}} \quad (3)$$

where R is the gas constant, A the frequency factor of the reaction and E_A the activation energy of the reaction.

By determining these parameters the rate constant and therefore the half-life of the reaction can be calculated for any temperature.

Identification of degradation pathways

In order to determine free cyanide, a cyanide cuvette test LCK 315 (Hach Lange GmbH, Düsseldorf, Germany) was applied according to the procedure given by the supplier. The CN^- content was determined for samples from preliminary tests of cyano-based anions that underwent hydrolysis. 2 mM solutions of the IL in buffer (pH 1 or 13) were hydrolysed according to results of kinetic studies and measured by mass spectrometry. The pH of alkaline samples was adjusted with conc. HCl prior to MS measurements. The mass spectrometer used was an electrospray ionization MS equipped with an ion trap detector (Bruker-Daltonic GmbH, Bremen, Germany). Mass spectra were acquired in negative and positive ion modes in the m/z 50–300 scan range. The ESI source conditions were set to a capillary voltage of 3500 V, the drying gas flow rate to 5 L min^{-1}, the drying gas temperature to 300 °C and the nebulizer to 70 psi.

Toxicity of hydrolysis products

The cytotoxicity of the hydrolysed solutions and identified hydrolysis products (pure compounds) was determined using the promyelocytic leukaemia rat cell line IPC-81.[29] Cultures of IPC-81 were grown in RPMI medium (with L-glutamine, without $NaHCO_3$, supplemented with 1% penicillin–streptomycin and 1% glutamine, pH 7) with 10% horse serum at 37 °C (5% CO_2).

The cytotoxicity assay was carried out according to Ranke *et al.*[30] Cell viability was measured using a colorimetric assay for 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2*H*-tetrazolium monosodium salt (WST-1) reagent. Each plate contained blanks (no cells), controls (no toxicants), and the test substance in a 1 : 1 dilution series. Stocks of identified hydrolysis products were prepared in a culture medium with max. 0.5% dimethylsulphoxide (DMSO) to improve the solubility of the substances. This DMSO concentration has been proven not to be cytotoxic (data not shown). A 300 mM solution of the inorganic salt in buffer (pH 1) was kept at a constant temperature for a fixed time, corresponding to the results obtained from the kinetic studies, to prepare approximately 50

and 100% hydrolysed solutions. The solution was further neutralized with a small amount of conc. NaOH to pH 7. For the test, IPC-81 cells at a concentration of 15×10^5 cells mL^{-1} (in RPMI with 8% foetal calf serum) were incubated for 44 h in 96-well plates in the presence of the test substance and for an additional 4 h in the presence of WST-1 reagent. Cell viability, assessed as the ability to reduce WST-1, was observed photometrically at 450 nm in a microplate reader (MRX, Dynatech Laboratories, Chantilly, USA). The cytotoxicity of the compounds was expressed as the percentage of cell viability measured as WST-1 reduction compared to controls. Each dose–response curve was recorded for at least 9 parallel dilution series on three different 96-well plates. Positive controls with carbendazim were checked at regular intervals.

Computational calculations

The pK_a predictions were performed using a linear free energy relationship (LFER) based on the free energy differences between the ions and the protonated forms at infinite dilution in water at 25 °C,[31] as implemented in the COSMO*therm* program.[32] The free energies of the species in water, used in this model, are obtained from the COSMO-RS method.[32] COSMO-RS is a quantum chemically based statistical thermodynamics procedure, which enables the calculation of the chemical potential and related properties of molecules in mixtures.

The underlying quantum chemical calculations were performed at the density functional theory (DFT) level, utilizing the conductor-like screening model (COSMO),[33] the BP functional[34–36] with RI (resolution of identity) approximation and a triple-ζ valence polarized basis set (TZVP).[37,38] All structures were fully optimized. The TURBOMOLE program package[39] was used for this task.

Results

Preliminary tests

Preliminary tests for the hydrolytic stability of the inorganic salts and ILs were performed at 323 K for 5 days in different buffer systems (pH 1, 7, 9, 13). The initial and final concentrations of the anion and the 1-ethyl-3-methylimidazolium (IM12) cation were determined by ion chromatographic and HPLC measurements, respectively. No hydrolysis was found for the IM12 cation in all tested buffer systems (data not shown). The recorded hydrolysis rates (in %) for the anions with inorganic and 1-ethyl-3-methylimidazolium (IM12) cations are shown in Fig. 2. In general, there was no significant difference in anion stability between the inorganic salt and the corresponding IL. The type of cation therefore seems to play a minor role in the hydrolysis of the anion. Under environmental conditions (pH 7 and 9), all the anions tested were degraded by <10%. In strongly acidic (pH 1) or basic (pH 13) solutions only $B(CN)_4^-$, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $H(C_2F_4)SO_3^-$ were hydrolytically stable. A high rate of hydrolysis was found for IM12 $N(CN)_2$ at pH 1 (100%) and at pH 13 (81%). The same applied to IM12 $C(CN)_3$, but degradation was greater at pH 13 (100%) than at pH 1 (54%).

Kinetics

In order to calculate the rate constant (k) for IM12 $N(CN)_2$ and IM12 $C(CN)_3$ the course of hydrolysis was tracked (at least 6 points between 10% and 90% degradation) at three different temperatures (283, 298, 310, 323 or 343 K) at pH 1 and pH 13.

The results are plotted according to eqn (1) (Fig. 3). As expected and indicated by the linear correlation, the reaction follows pseudo-first-order kinetics. The slope of the straight line obtained by linear regression represents the rate constant of the hydrolysis.

The results and the half-lives calculated according to eqn (2) are summarized in Table 1. At pH 1 and 283 K the half-life of $N(CN)_2^-$ is about 10.5 h ($3.75 \times 10^4 \pm 983$ s), at 298 K it decreases to <2 h ($6.49 \times 10^3 \pm 35.1$ s) and at 310 K $N(CN)_2^-$ is rapidly hydrolysed to 50% within approximately 20 min ($1.27 \times 10^3 \pm 8.88$ s). Hydrolysis at pH 13 is slower by comparison. The rate constant for the same temperature (310 K) decreases from $5.44 \times 10^{-4} \pm 3.79 \times 10^{-6}$ s^{-1} at pH 1 to $2.16 \times 10^{-6} \pm 5.69 \times 10^{-9}$ s^{-1} at pH 13. Owing to the reduced rate constants, the half-life of $N(CN)_2^-$ in basic media at 310 K, 323 K and 343 K rises to *ca.* 4 days ($3.20 \times 10^5 \pm 841$ s), 1.5 days ($1.21 \times 10^5 \pm 259$ s) and 14 h ($4.92 \times 10^4 \pm 91.8$ s) respectively.

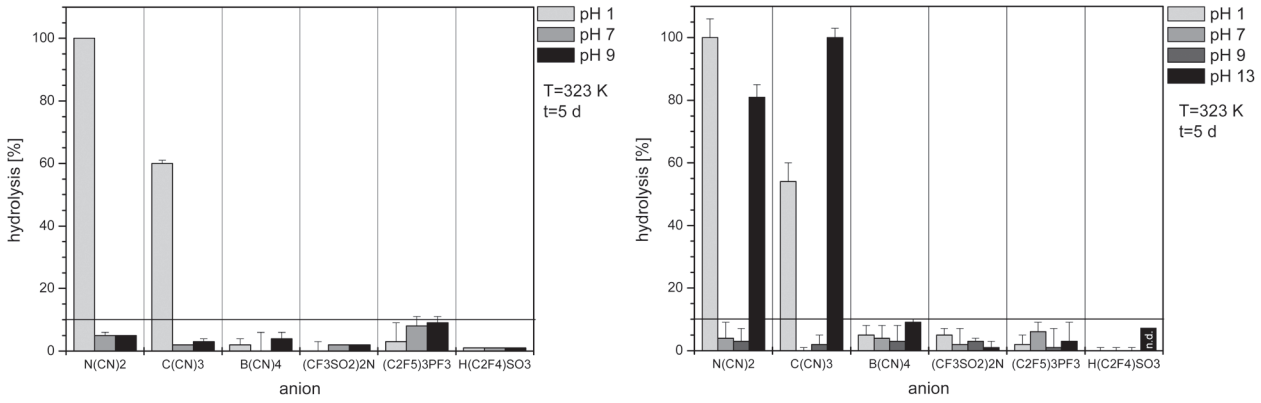

Fig. 2 Results of preliminary hydrolysis tests for different anions ($N(CN)_2^-$, $C(CN)_3^-$, $B(CN)_4^-$, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $H(C_2F_4)SO_3^-$) as alkali salts (left) and IM12 salts (right) at different pH values (1, 7, 9 and 13) (n.d. = not determined).

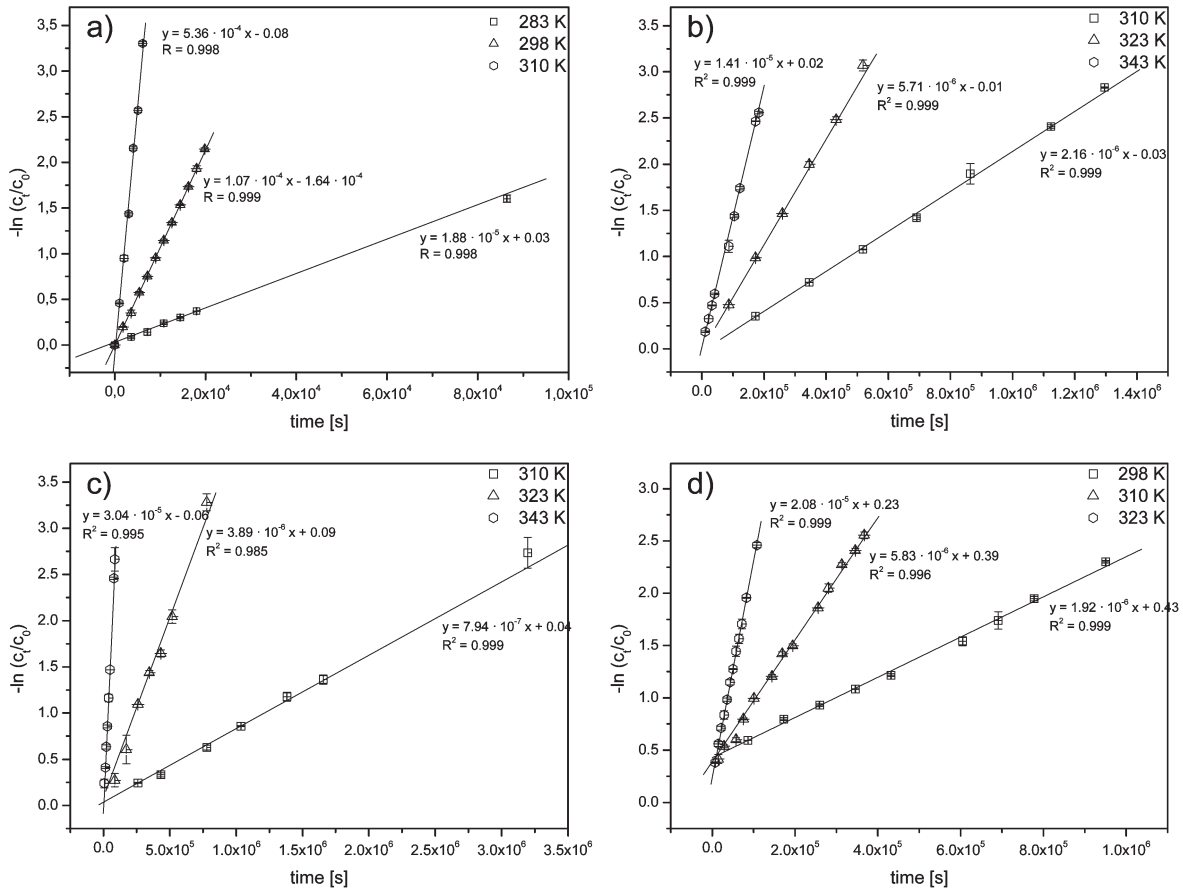

Fig. 3 Hydrolysis kinetics at different temperatures of (a) IM12 $N(CN)_2$ at pH 1; (b) IM12 $N(CN)_2$ at pH 13; (c) IM12 $C(CN)_3$ at pH 1; and (d) IM12 $C(CN)_3$ at pH 13.

Table 1 Reaction rate constants (k) and half-lives ($t_{0.5}$) for the pseudo-1st-order hydrolysis kinetics of the IL anions investigated (cation: IM12) at different pH and temperatures

Anion	pH	T [K]	k [s^{-1}]	$t_{0.5}$ [s]
$N(CN)_2^-$	1	283	$1.85 \times 10^{-5} \pm 4.85 \times 10^{-7}$	$3.75 \times 10^4 \pm 983$
		298	$1.07 \times 10^{-4} \pm 5.77 \times 10^{-7}$	$6.49 \times 10^3 \pm 35.1$
		310	$5.44 \times 10^{-4} \pm 3.79 \times 10^{-6}$	$1.27 \times 10^3 \pm 8.88$
	13	310	$2.16 \times 10^{-6} \pm 5.69 \times 10^{-9}$	$3.20 \times 10^5 \pm 841$
		323	$5.71 \times 10^{-6} \pm 1.22 \times 10^{-8}$	$1.21 \times 10^5 \pm 259$
		343	$1.41 \times 10^{-5} \pm 2.63 \times 10^{-8}$	$4.92 \times 10^4 \pm 91.8$
$C(CN)_3^-$	1	310	$7.94 \times 10^{-7} \pm 8.06 \times 10^{-9}$	$8.73 \times 10^5 \pm 8855$
		323	$3.89 \times 10^{-6} \pm 1.09 \times 10^{-7}$	$1.78 \times 10^5 \pm 4966$
		343	$3.04 \times 10^{-6} \pm 2.03 \times 10^{-7}$	$2.28 \times 10^4 \pm 152$
	13	298	$1.92 \times 10^{-6} \pm 9.46 \times 10^{-9}$	$3.60 \times 10^5 \pm 1772$
		310	$5.83 \times 10^{-6} \pm 3.20 \times 10^{-8}$	$1.19 \times 10^5 \pm 652$
		323	$2.08 \times 10^{-5} \pm 1.08 \times 10^{-7}$	$3.34 \times 10^4 \pm 173$

$C(CN)_3^-$ behaves in the opposite manner. In an acidic environment the hydrolysis rate constant is $7.94 \times 10^{-7} \pm 8.06 \times 10^{-9}$ s^{-1} (310 K), whereas at pH 13 its value is higher ($5.83 \times 10^{-6} \pm 3.20 \times 10^{-8}$ s^{-1}). The half-lives for hydrolysis at pH 1 are *ca.* 10 days ($8.73 \times 10^5 \pm 8855$ s), 2 days ($1.78 \times 10^5 \pm 4966$ s) and 6 h ($2.28 \times 10^4 \pm 152$ s) at 310 K, 323 K and 343 K respectively. As indicated by the greater rate constants, the half-lives in alkaline solutions fall to approximately 4 days ($3.60 \times 10^5 \pm 1772$ s) at 298 K, 33 h ($1.19 \times 10^5 \pm 652$ s) at 310 K and 9 h ($3.34 \times 10^4 \pm 173$ s) at 323 K.

Using the experimentally determined rate constant at different temperatures the activation energies (E_A) and frequency factors (A) of the hydrolysis reactions are accessible (Table 2) *via* the Arrhenius equation (eqn (3)). Even though the hydrolysis of $N(CN)_2^-$ at pH 1 was the fastest (highest rate constant at 310 K), the calculated activation energy was the highest (99.9 ± 0.5 kJ mol^{-1}). The opposite observation was made for the reaction of $N(CN)_2^-$ in alkaline media. Here, the activation energy was the lowest (48.6 ± 0.1 kJ mol^{-1}), but also the frequency factor (359.2 ± 10.9 s^{-1}) of the reaction was small. Generally, the frequency factors found for the reactions under acidic conditions were higher than those recorded at pH 13.

Degradation pathways

To identify the degradation products of the anions a cuvette test for free cyanide anions was performed. None of the samples tested, $N(CN)_2^-$ and $C(CN)_3^-$ in either of the buffer systems (pH 1 and 13), showed any significant CN^- content (<0.1 mg L^{-1}) after 5 days at 323 K.

During the MS measurements, ions with an m/z^- = 84 were additionally detected in the negative ion mode in samples of $N(CN)_2^-$ in both buffer systems (pH 1 and 13). Samples of the $C(CN)_3^-$ anion showed anions with m/z^- = 65, 108 and 126. It is known that nitriles can be hydrolysed by either acid- or

Table 2 Equations resulting from linear regression analysis, obtained by plotting $-\ln(k)$ against T^{-1}, and the coefficient of determination (R^2), as well as the activation energy (E_A) and frequency factor (A) of the Arrhenius equation (eqn (3)), for all the investigated IL anions (IM12 as counter cation) at different pH

Anion	pH	Equation of linear regression	R^2	E_A [kJ mol^{-1}]	A [s^{-1}]
$N(CN)_2^-$	1	$y = 12\,017 \times -31.2$	0.994	99.9 ± 0.5	$3.5 \times 10^{13} \pm 6.9 \times 10^{12}$
	13	$y = 5839 \times -5.88$	0.987	48.6 ± 0.1	359.2 ± 10.9
$C(CN)_3^-$	1	$y = 11\,746 \times -23.8$	0.999	97.7 ± 0.3	$2.2 \times 10^{10} \pm 2.6 \times 10^{9}$
	13	$y = 9159 \times -17.5$	0.999	76.2 ± 0.2	$4.1 \times 10^{7} \pm 3.7 \times 10^{6}$

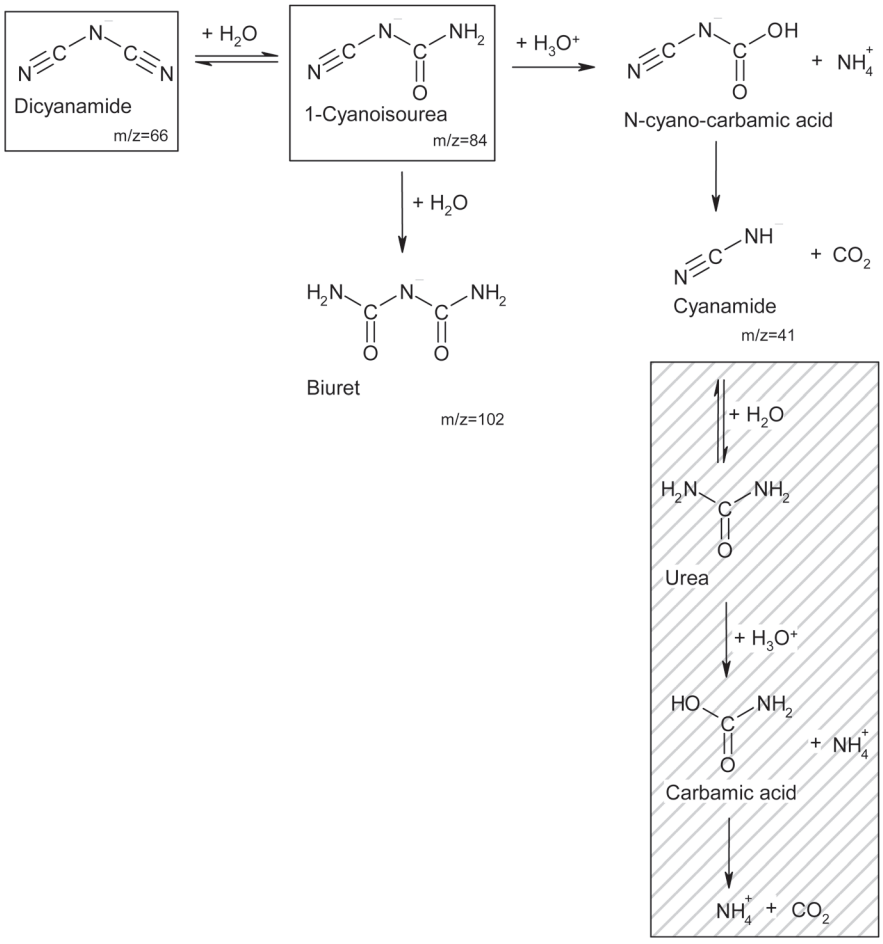

Fig. 4 Postulated hydrolysis degradation pathway for the $N(CN)_2^-$ anion: the framed anions were detected in the negative ion mode of MS-measurements; the assumed degradation pathway is highlighted in the grey hatched box.

base-initiated reactions to form carboxamides, which will further hydrolyse in a second step to carboxylic acids. By applying this knowledge to the mass-to-charge ratios in the MS spectra, possible degradation pathways for $N(CN)_2^-$ and $C(CN)_3^-$ are shown in Fig. 4 and 5 respectively. For both anions only parts of the proposed degradation mechanism could be demonstrated by the MS measurements (framed anions). The part of the pathway that is assumed on the basis of theoretical considerations is shown in a grey hatched box.

Toxicity of hydrolysis products

In order to evaluate the acute toxicological behaviour of the IL and inorganic salts as well as that of selected identified hydrolysis products, *in vitro* cytotoxicity studies were performed using the rat leukaemia cell line IPC-81. The half-maximal effective concentrations (EC_{50}) obtained are summarized in Table 3 (first column). Some of the identified hydrolysis products are common chemicals with high production volumes and further available toxicological data, which are also shown in Table 3.

In general the degradation products and IL anions tested as inorganic salts or combined with IM12 cations exhibited a low acute cytotoxicity. The EC_{50} values of both anions are lower for the IM12 salts than for the inorganic salts. This could be due to the known cytotoxic effect of the IM12 cation.[40] As the results show, $N(CN)_2^-$ has a lower cytotoxic potential than $C(CN)_3^-$. The identified hydrolysis products of each anion exhibited discontinuous cytotoxic behaviour. Whereas no EC_{50} was recorded for

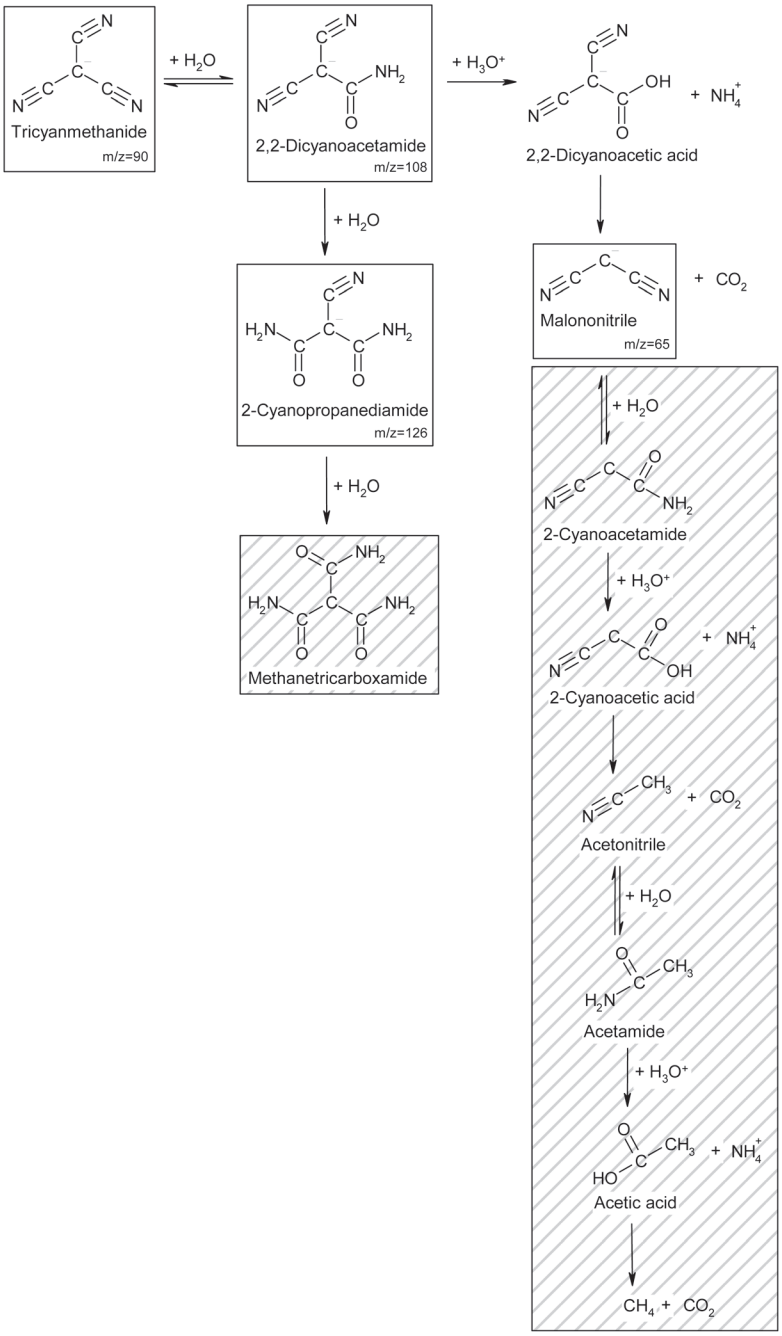

Fig. 5 Postulated hydrolysis degradation pathway for the $C(CN)_3^-$ anion: the framed anions were detected in the negative ion mode of MS-measurements; the assumed degradation pathway is highlighted in the grey hatched box.

the cyanourea sodium salt, carbamylurea (biuret), urea, 2-cyanoacetamide and acetamide up to the highest tested concentration of 10 mM (591–1290 mg L^{-1}), cyanamide (EC50 = 238 mg L^{-1}) and malononitrile (EC50 = 132 mg L^{-1}) displayed elevated acute cytotoxicity. The latter two compounds were also found to be toxic towards rats after oral application. The other compounds were classified as irritants (carbamylurea and urea) or harmful (cyanourea sodium salt, 2-cyanoacetamide, acetonitrile and acetamide) according to their effects after oral application to rodents.

The cytotoxic behaviour of a non-hydrolysed, 50% and 100% hydrolysed solution is illustrated in Fig. 6 for both anions tested as inorganic salts, in order to exclude effects caused by the IL cation. The starting concentration of each solution was set at 100%. The resulting concentrations, obtained by 1 : 1 dilution, were then calculated as a % from this relative concentration. The hydrolysed solutions of both anions turned out to be less toxic than the native anions. The respective EC_{50} values for the $N(CN)_2^-$ anion are 50%, 72% and 68% (relative to the starting concentration of each solution) for the 0%, 50% and 100% hydrolysed solutions. The differences are significantly greater for the $C(CN)_3^-$ anion: whereas an approximately 1 : 8 times diluted solution of the non-hydrolysed solution shows 50% cell viability

Table 3 Results of cytotoxicity experiments with IPC-81 cells and from literature for $N(CN)_2^-$ and $C(CN)_3^-$ anions as inorganic salts and native ILs. Additionally, data for their identified hydrolysis products are shown

Substance	EC_{50} IPC-81 (standard deviation) [mg L^{-1}]	LD_{50} oral (rat) [mg kg^{-1}]	LD_{50} dermal (rat) [mg kg^{-1}]	LC_{50} Daphnia magna [mg L^{-1}]	Hazard symbol
$NaN(CN)_2$ [a]	1250				
IM12 $N(CN)_2$ [a]	1170 (1030–1320)				
Cyanourea sodium salt[b]	>1220				Harmful
Carbamylurea (biuret)[b]	>1110				Irritant
Cyanamide[b]	238 (230–253)	125	84	3.2 (48 h)	Toxic
Urea[b]	>974	8471	8200	3910 (48 h); >10 000 (24 h)	Irritant
$KC(CN)_3$ [a]	718 (555–979)				
IM12 $C(CN)_3$ [a]	258 (234–285)				
Malononitrile[b]	132 (121–145)	14	340		Toxic, dangerous for the environment
2-Cyanoacetamide[b]	>1290	1680[c]			Harmful
Acetonitrile[b]	n.d.	2460	2000[d]	3600 (48 h)	Harmful
Acetamide[b]	>591	7000			Harmful

[a] From chemical database http://www.il-eco.uft.uni-bremen.de/. [b] From MSDS. [c] Not rat, but mouse. [d] Not rat, but rabbit.

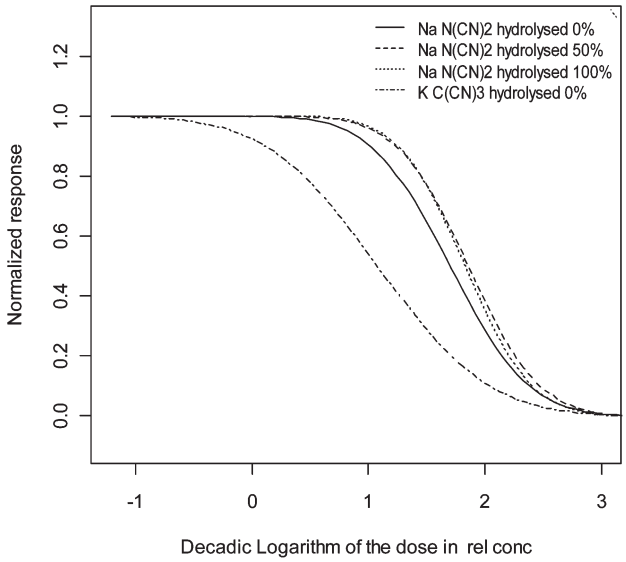

Fig. 6 Comparison of a non-hydrolysed solution of $NaN(CN)_2$ (straight line, EC_{50} = 50 ± 9%), a 50%-hydrolysed solution of $NaN(CN)_2$ (dashed line, EC_{50} = 72 ± 10%), a 100%-hydrolysed solution of $NaN(CN)_2$ (dotted line, EC_{50} = 68 ± 8%) and a non-hydrolysed solution of $KC(CN)_3$ (dashed-dotted line, EC_{50} = 12 ± 2%). The highest concentration for each solution is set at 100%. 50%- and 100%-hydrolysed solutions of $KC(CN)_3$ were inactive (data not shown).

compared to controls (EC_{50} = 12.0%), no half-maximal effect could be observed, even for undiluted solutions of the 50% and 100% hydrolysed samples.

Theoretical calculations

According to the mechanism of degradation the hydrolytic process for $N(CN)_2^-$ and $C(CN)_3^-$ starts with the protonation of the terminal nitrogen. Attempting to explain the finding that the different cyano-based anions exhibit different hydrolytic stabilities, we calculated the pK_b values of the CN-groups. Applying the linear free energy relationship (LFER) based on the free energy differences between the ions and the protonated forms at infinite dilution in water at 25 °C,[31] the pK_b values of the terminal nitrogen of the CN-group were estimated to be 13.9 for $N(CN)_2^-$, 16.7 for $C(CN)_3^-$ and 20.7 for $B(CN)_4^-$. The pK_b values of the central atoms of $N(CN)_2^-$ and $C(CN)_3^-$ were calculated to be less basic than the terminal nitrogen atoms in both cases. The pK_b values are 17.2 for $N(CN)_2^-$ and 18.5 for $C(CN)_3^-$, respectively.

Discussion

Preliminary hydrolysis tests according to OECD guideline 311 at 323 K for 5 days permit a first estimate of the hydrolytic stability of a compound. The results of these tests indicate that the anions $B(CN)_4^-$, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $H(C_2F_4)SO_3^-$ were hydrolytically stable under all the test conditions and no influence of the counter cation (inorganic or IM12) was observed. Thus, the half-lives of these compounds at 298 K (25 °C) can be assumed to be >1 year. Under environmentally relevant conditions (pH 7 and 9) this was also demonstrated for the other two anions tested – $N(CN)_2^-$ and $C(CN)_3^-$. Hence, the hydrolytic degradation of all the anions investigated here as an alternative pathway to their biodegradation is thought to be unlikely. Studies in buffer systems of pH 1 and 13 were performed to cover the two extremes that might occur during technical applications. Although $B(CN)_4^-$, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $H(C_2F_4)SO_3^-$ can be used even under harsh conditions, the rapid hydrolysis of $N(CN)_2^-$ and $C(CN)_3^-$ even at low temperatures reduces their technical applicability.

The kinetic rate constants of the hydrolysis of these two anions were determined; the constants displayed different tendencies. Whereas for $N(CN)_2^-$ k was greater at pH 1 than at pH 13, the opposite was observed for $C(CN)_3^-$ ($k_{(pH\ 1)} < k_{(pH\ 13)}$). This may be explained by the different reaction mechanisms assumed for the hydrolysis in acidic and alkaline solutions. Following the standard textbook reaction mechanisms, hydrolysis is initiated by the protonation of the terminal nitrogen at low pH,

whereas in a basic environment direct attack of the hydroxide ion on the carbon of the nitrile group is the first step. Especially the second variant might be too simple for the anions investigated in this study. The nucleophilic attack of a negatively charged anion by a hydroxide ion is expected to be quite unfavorable and one would expect a more complicated hydrogen bond assisted mechanism instead. Nevertheless, the discussion of the partial charges of the cyano nitrogen atoms can be used for some first considerations: a partial negative charge at the nitrogen favours acidic hydrolysis, and a partial positive charge at the carbon supports alkaline hydrolysis. When considering the resonance structures or by comparing qualitatively the electronegativity and the resulting negative partial charges at the N atoms in the anions $N(CN)_2^-$, $C(CN)_3^-$ and $B(CN)_4^-$, it is decreasing in the given order. Under acidic conditions the reaction rate therefore decreases as well in the same order owing to the lesser protonation capability of $B(CN)_4^-$ than of $C(CN)_3^-$ and $N(CN)_2^-$. This is in accordance with the observations from our experiments and the calculated pK_b values. $N(CN)_2^-$, even though it still has a high pK_b, is the strongest base of the three anions investigated and therefore exhibits a higher protonation potential than the other ions. Additionally, the calculated pK_b values support the proposed reaction mechanism, initiated by the protonation of the terminal nitrogen. As the values are higher for the central atoms, a protonation of the terminal groups is expected to be favoured. For base-induced hydrolysis the partial charge at the nitrile carbon may be regarded as explaining the observed differences in stability. In $B(CN)_4^-$ the partial charge should be slightly negative (electronegativity $B = 2.0$, $C = 2.5$) and therefore reduce the possibility of a nucleophilic attack. In comparison to $N(CN)_2^-$ and $C(CN)_3^-$ this could be an explanation for the observed stability of $B(CN)_4^-$ even at pH 13. However, this simple model cannot be used to explain the higher reaction rate constant for $C(CN)_3^-$ than for $N(CN)_2^-$, since the positive charge should be greater in $N(CN)_2^-$. Another approach that might help to explain the higher rate constant for the hydrolysis of $C(CN)_3^-$ at pH 13 is the larger number of nitrile groups in $C(CN)_3^-$ than in $N(CN)_2^-$ and therefore the greater possibility of an attack. However, more detailed theoretical calculations, taking into account the electronic as well as the steric characteristics of the anions, will be necessary in order to understand this trend. The generally lower frequency factors observed for hydrolysis at pH 13 may be due to the orientation of the colliding entities: a collision between the oxygen of the hydroxide anion and the nitrile carbon leading to a reaction is less likely than a collision between a proton and the nitrile nitrogen.

By means of the CN^- cuvette test and MS measurements we were able to identify the hydrolysis degradation products and postulate a degradation pathway (Fig. 4 and 5). The results of the cuvette test indicate that there is no reaction of water with the central atom and hence no formation of CN^- during hydrolysis. The first step of our proposed hydrolysis degradation mechanism for $N(CN)_2^-$ (Fig. 4) would lead to the formation of 1-cyanoisourea ($m/z^- = 84$). If both cyano groups of the anion were attacked, biuret would be produced. According to the general hydrolysis mechanism of nitrile groups, 1-cyanoisourea can be further hydrolysed to form *N*-cyano-carbamic acid. This compound is unstable, however, and will decompose further to CO_2 and cyanamide. But again, cyanamide is too small ($m/z^- = 41$) to be detected by ESI-MS. Further, assuming that the second nitrile group is hydrolysed in an identical way, urea, carbamic acid and ultimately ammonia will be formed. These degradation products could also be identified during hydrolysis of $HN(CN)_2$.[41] For the $C(CN)_3^-$ anion (Fig. 5) the first hydrolysis step would lead to 2,2-dicyanoacetamide ($m/z^- = 108$), which would be further hydrolysed either at a second nitrile group, resulting in 2-cyanopropandiamide ($m/z^- = 126$), or at the amide group to form 2,2-dicyanoacetic acid. The acid would then decompose to carbon dioxide and malononitrile ($m/z^- = 65$). Further hydrolysis at the second and third nitrile groups with the same mechanism could result in 2-cyanoacetamide, 2-cyanoacetic acid, acetonitrile, acetamide, acetic acid and finally methane. For both anions the presence of the first hydrolysis product (1-cyanoisourea and 2,2-dicyanoacetamide respectively) was observed even after a few days, indicating that subsequent hydrolysis reactions are slow.

It is known that some of the identified hydrolysis products are classified as toxic to man and the environment. In order to compare this potential with the native compounds, a rapid and well-established cellular test system was used to screen the cytotoxic potential of all the substances investigated in this hydrolysis study. The results indicate that most of the identified hydrolysis products have a lower acute cytotoxicity. Studies in solutions of pH 1 were also performed in order to simulate the relevant conditions found in the animal or human stomach. This can be helpful to further predict the toxicological behaviour of the compounds. In fact, hydrolysis of the $N(CN)_2^-$ anion at 37 °C is very fast ($t_{0.5} \approx 20$ min) and will most probably occur in the case of (accidental) oral ingestion. However, the results obtained in the cytotoxicity test for non-hydrolysed and 50% or 100% hydrolysed solutions showed no significant difference in their acute cytotoxicological behaviour, even though some of the identified hydrolysis products are classified as toxic. Since the concentrations of the single hydrolysis compounds in the mixture are not known, this can most likely be explained by the excessively low concentrations. Additional antagonistic mixture effects are possible. But in order to test this hypothesis, further studies for quantifying the hydrolysis products will be necessary.

Conclusion

The hydrolysis of technologically relevant IL anions ($N(CN)_2^-$, $C(CN)_3^-$, $B(CN)_4^-$, $(CF_3SO_2)_2N^-$, $(C_2F_5)_3PF_3^-$ and $H(C_2F_4)SO_3^-$) combined with alkaline or IM12 cations was studied. The results of preliminary tests (323 K for 5 days) showed that none of the investigated anions was hydrolysed under environmentally relevant conditions. Therefore, the hydrolytic degradation pathway does not represent an appropriate alternative to biodegradation for removing these anions from aqueous environmental media. It has to be remembered, however, that these tests were performed under laboratory conditions. Under real environmental conditions, the stability of the investigated anions in aqueous solution could well be affected by such factors as changes in dissolved oxygen, different salinities/electrical conductivity, and the presence of reactive oxygen species.

$N(CN)_2^-$ and $C(CN)_3^-$ were shown to be unstable under strongly acidic or alkaline conditions. This may limit their

technical applicability under such conditions. Kinetic studies, including generated Arrhenius equations, helped to estimate the reaction velocity and half-lives of the compounds at every temperature tested. The hydrolysed solutions of $N(CN)_2^-$ and $C(CN)_3^-$ showed a similar or increased cytotoxicity towards IPC-81 compared to the non-hydrolysed samples, although some identified hydrolysis products are known to be toxic to rodents after oral application. However, further studies, including the quantification of hydrolysis products, will be helpful for acquiring a more detailed hazard assessment. Summing up, the results known to date indicate that the stability of these anions is a matter of great concern in hazard assessments.

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Paper A.4 - Biodegradation potential of cyano-based ionic liquid anions in a culture of Cupriavidus spp. and their in vitro enzymatic hydrolysis by nitrile hydratase

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Own contributions:

The experimental work on the biodegradation by *Cupriavidus spp.* has been performed by Magda Pawlik under my supervision. All experimental work and the evaluation and interpretation of data concerning the enzymatic hydrolysis were performed by me. The results were finally published after discussion with the other co-authors.

Biodegradation potential of cyano-based ionic liquid anions in a culture of Cupriavidus spp. and their in vitro enzymatic hydrolysis by nitrile hydratase

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Abstract

Biodegradation tests with bacteria from activated sludge revealed the probable persistence of cyano-based ionic liquid anions when these leave waste water treatment plants. A possible biological treatment using bacteria capable of biodegrading similar compounds, namely cyanide and cyano complexes, was therefore examined. With these bacteria from the genera *Cupriavidus* the ionic liquid anions $B(CN)_4^-$, $C(CN)_3^-$, $N(CN)_2^-$ combined with alkaline cations were tested in different growth media and using ion chromatography for the examination of their primary biodegradability. However, no enhanced biodegradability of the tested cyano-based ionic liquids was observed. Therefore an in vitro enzymatic hydrolysis test was additionally run showing that all tested IL anions can be hydrolysed to their corresponding amides by nitrile hydratase, but not by nitrilase under the experimental conditions. The biological stability of the cyano-based anions is an advantage in technological application, but the occurrence of enzymes that are able to hydrolyse the parent compound gives a new perspective on future cyano-based IL anion treatment.

Keywords: biodegradation, ionic liquids, cyano groups, axenic culture, biological treatment, hazard assessment, *Cupriavidus spp.,* nitrile hydratase, nitrilase

Introduction

Ionic liquids (ILs) have become an innovative substance group for industry and research purposes. The generic property of being ionic and liquid below a temperature of 100 °C stems mainly from the combination of asymmetric organic cations and anions. The combinability of their different components has led to a vast number of chemicals with different physico-chemical properties. ILs are most commonly used because of their very good solvent and catalytic properties (Welton 1999; Yue et al. 2011). Low vapour pressure and non-flammability are additional common key properties that make for improved operational safety in comparison to conventional solvents. This combination of IL properties has advantages in the fields of chemical synthesis and catalysis (Olivier-Bourbigou et al. 2010; Sheldon 2005), liquid-liquid extraction and enzyme stabilisation (Dreyer and Kragl 2008), electrochemical (Liu and Pan 2011) and analytical applications (Berthod et al. 2008) in that the relevant processes are more effective and safer (Kokorin 2011). However, the environmental risks of IL disposal have not yet been fully examined. Looking at the main source for ILs into the environment, it is probable that

ILs enter the environment via waste water treatment plants when physico-chemical properties and applications of ILs are taken into account (Siedlecka et al. 2010). Adsorption on solid surfaces is also possible as a second source, especially for lipophilic ILs. As non-volatile substances a direct contamination of air is unlikely. Therefore, biodegradability tests using activated sludge from waste water treatment plants have been conducted for a range of ILs with varying results from being low to readily biodegradable, depending on the structural composition of the IL (Stolte et al. 2011). These tests have shown IL anions containing cyano groups to be non-biodegradable (Neumann et al. 2012) and hydrolytically stable under environmental relevant conditions. Only $N(CN)_2^-$ and $C(CN)_3^-$ could be hydrolysed at very strong acidic (pH 1) and basic conditions (pH 13) (Steudte et al. 2012). The technological relevance of the tested IL anions comes from their potential application as alternatives for the commonly used fluorinated anions bis(trifluoromethylsulphonyl)amide $(CF_3SO_2)_2N^-$ (BTA) and trifluorotris(pentafluoroethyl)phosphate $(C_2F_5)_3PF_3^-$ (FAP). The fluorinated anions lower the melting point and the viscosity of the IL, increase its hydrophobicity and widen the electrochemical window for a better technological applicability (Xue et al. 2006). However, these fluorinated ILs are also highly refractive towards abiotic and biotic degradation processes (Ignat'ev et al. 2005; Neumann et al. 2012; Steudte et al. 2012). The cyano-based IL anions have a similar effect to the physico-chemical properties of the IL for technological application, but were shown to be even more effective than the standard fluorinated BTA containing IL in CO_2/N_2 separation by supported IL membranes (SILMs) (Mahurin et al. 2010). $N(CN)_2^-$ was also just recently been studied for a usage in Lithium batteries (Yoon et al. 2013). Furthermore this anion was reported to be a good counterion of IL solvents and catalysts for the processing of alcohols and sugars (Forsyth et al. 2002). Cyano-based ILs have further been reviewed as solvents for liquid-liquid extraction of aromatic hydrocarbons in place of the conventionally used sulfolanes (Meindersma and Haan 2012). Additionally $C(CN)_3^-$ und $B(CN)_4^-$ are of high interest in renewable energy production as electrolyte additives in dye-sensitized solar cells (Kuang et al. 2006; Marszalek et al. 2011).

Since the cyano-based anions, K $B(CN)_4$, K $C(CN)_3$ and Na $N(CN)_2$, have shown not to be biodegradable under aerobic and denitrifiying conditions (Neumann et al. 2012), a mix of two bacteria strains that are capable to biodegrade one of the most stable cyano complexes, Prussian blue $Fe_7(CN)_{18}^{3-}$ (PB), has been tested on its biodegradation potential towards the selected cyano-based IL anions. This bacterial culture mix called KS-7D was investigated for the biodegradation of cyano-based anions in order to reduce their persistence and the risk of their accumulation in the environment (Fig.1).

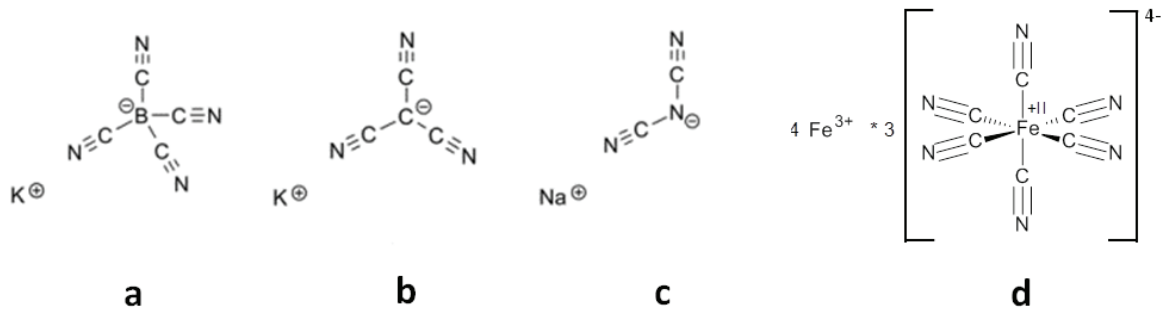

Fig. 1 Molecular structures of the ionic liquids used in this study (a) potassium tetracyanoborate K $B(CN)_4$, (b) potassium tricyanomethanide K $C(CN)_3$, (c) sodium dicyanoamide Na $N(CN)_2$ and the reference substance Prussian blue (PB, $Fe(III)_4[Fe(II)(CN)_6]_3$)

KS-7D is composed of the two bacterial species *Cupriavidus basilensis* and *Cupriavidus eutrophus,* formerly *Ralstonia* spp. and *Wausteria* spp. (Vandamme and Coenye 2004). Both strains are capable of biodegrading cyanide and cyano metal complexes. Free cyanide is hydrolysed by the bacteria to ammonia and formic acid (Bryniok and Trösch 2008), which is one of the metabolic pathways that can be used for the degradation of cyanide and cyano-based compounds (Gupta et al. 2010). This step is catalysed by a cyanide hydrolase without releasing the hazardous intermediate formamide and occurs independently of the presence of molecular oxygen and other co-factors. Ammonia and formic acid also serve as sources of nitrogen and carbon for the bacteria. The optimal growth conditions for the mesophilic KS-7D are a temperature between 30 and 40 °C and a neutral to slightly basic pH of 7.5 to 8.5. These bacteria can also biodegrade ferrocyanide $Fe(CN)_6^{4-}$, ferric cyanide $Fe(CN)_6^{3-}$, and Prussian blue (PB, $Fe(III)_4[Fe(II)(CN)_6]_3$), which is one of the most stable cyano complexes. Cyanide concentrations of up to 1.4 g L^{-1} are still tolerated by KS-7D and no toxic effects are observed below a cyanide concentration of 6.5 mg L^{-1}. This makes biodegradation a potential replacement for physico-chemical processes in the clean-up of cyanide-contaminated process effluents. The successful lab-scale application and the use of KS-7D as a starter culture for full-scale plants for cleaning waste air from flame lamination in the textile industry as well as the use of other, similar bacteria in the biotreatment of cyanide wastes has already been reported (Dash et al. 2009; Sallat and Mählmann 2011). The KS-7D bacteria are microaerophilic betaproteobacteria strains isolated from a former coking plant site. They are niche bacteria that can be found in "soil, root nodules, aquatic environments, and human clinical sources" (Cserháti et al. 2012) with properties that are advantageous for bioremediation purposes. The complete genome of the *C. basilensis* OR16*,* isolated from a Hungarian pristine soil sample, has recently been sequenced due to the large xenobiotic-degrading potential of the *Cupriavidus* genus (Cserháti et al. 2012). Apart from the biodegradation of PB the selected *Cupriavidus* spp. have successfully been investigated for other bioremediation purposes, such as the biodegradation of kraft lignin (Shi et al. 2013), the removal of toxic fermentation inhibitors, e.g. 5-hydroxymethyl furfural (Wierckx et al. 2010), the degradation of bisphenol A (Fischer et al. 2010), the removal of chlorophenols (Zilouei et al. 2006), the degradation of s-triazine (Stamper et al. 2002) and finally for the biodegradation of the first xenobiotic investigated, 2,6-dichlorophenol, to be degradable by the selected bacteria (Steinle et al. 1998). The bacteria in these studies have mainly been isolated from environments near sites that are contaminated with the corresponding chemical.

The use of axenic cultures for the biodegradation of a wider range of ILs has been successful, e.g. using *Sphingomonas paucimobilis* (Abrusci et al. 2011) or *Corynebacteria* for the biodegradation of pyridinium-based ILs (Zhang et al. 2010). In the present study, however, KS-7D did not biodegrade cyano-based IL anions, and changes made to the growth conditions of the bacteria did not improve the result either. Therefore, the question arose whether the bacteria may not biodegrade the anions because they use a combination of siderophores and cyanide hydralase for the biodegradation of PB, but no other nitrile hydrolysing enzyme, such as nitrilase or nitrile hydratase had been reported. Since we know from abiotic hydrolysis studies on the cyano-based IL anions that at least $N(CN)_2^-$ and $C(CN)_3^-$ were hydrolytically instable under harsh pH conditions, we investigated whether the nitrile hydrolysing enzymes and commercially available nitrilase and nitrile hydratase may catalyse the hydrolysis as it has been shown several times for nitrile containing chemicals (Martínková and Kren 2010; O'Reilly and Turner 2003) and could therefore be a hint for future bacteria selection for the biodegradation of cyano-based anions.

Materials and Methods

Precultivation of bacteria in liquid medium

50 mL of a concentrated culture of KS-7D were provided by the Fraunhofer Institute for Interfacial Engineering and Biotechnology Stuttgart (Fraunhofer IGB Stuttgart). 40 mL of it were split into 1 mL aliquots. The remaining 10 mL were used for the first inoculation of the media. The medium composition recipe for the cultivation of KS-7D was followed as provided by Fraunhofer IGB Stuttgart. The analytical grade salts for the media were obtained from Sigma-Aldrich (Germany). The medium components were phosphate buffer at pH 7.5 (180 mg L^{-1} KH_2PO_4, 78 mg L^{-1} $Na_2HPO_4*2H_2O$), 1 g L^{-1} $MgSO_4*7H_2O$, 2 mg L^{-1} Fe(III)-Citrate and trace element solution SL4 (0.3 mg L^{-1} H_3BO_3, 0.1 mg L^{-1} $ZnSO_4*7H_2O$, 0.2 mg L^{-1} $CoCl_2*6H_2O$, 0.03 mg L^{-1} $MnCl_2*4H_2O$, 0.01 mg L^{-1} $CuCl_2*2H_2O$, 0.02 mg L^{-1} $NiCl_2*6H_2O$, 0.03 mg L^{-1} $Na_2MoO_4*2\ H_2O$). 0.72 g L^{-1} fructose $C_6H_{12}O_6$ and 6.5 mg L^{-1} potassium cyanide KCN were specially added for the growth of the cyanide degrading bacteria.

The bacteria were used for the experiments after two days of incubation in order to ensure optimal cell numbers of 10^4-10^5 in 100 mL. For the cultivation of KS-7D 50 mL of such a grown culture were added to 950 mL of medium. The amount of left KCN was checked via Hach Lange cuvette test LCK315 (0.01-0.6 mgL^{-1} CN^-) and was below the quantification limit. The bacteria were kept in an incubator (A120S – LAUDA Dr. R. Wobser GmbH & Co. KG, Germany) at a temperature of 30 °C, where they were gently and continually shaken horizontally at medium speed.

The bacteria from an industrial waste water treatment plant (Merck KGaA, factory Darmstadt, Germany) were not cultivated but received from the plant directly and used within a limited time period of one week. It was taken there, transported in a 10 L container overnight and aerated with oxygen in our lab before use. The industrial activated sludge is not expected to contain the KS-7D bacteria strains.

Cell counter measurement of the bacterial growth

The bacterial growth in the medium composition recipes was examined using cell counter (Z SERIES COULTER COUNTER®, Beckmann Coulter Electronics GmbH, Germany). The particle size range was set to 1.7 – 7.8 µm. The bacterial growth of each cyano-based ILs (at a concentration of 100 $mmol^{-1}$) was investigated in the presence of fructose. The measurements were conducted four times for each sample after 0, 4, 6 and 24 h of cultivation. 1 mL of bacteria suspension was added to 10 mL of isotonic solution (COULTER® ISOTON® II Diluent). The instrument was flashed with isotonic solution three times before each measurement.

The experiments

The question whether IL anions are biodegradable by KS-7D or, if not, can be hydrolysed by isolated enzymes has been addressed with different experimental conditions which are summarized in Tab. 1. A and B more detailed description can be found in the following paragraph.

Tab 1 A. Overview on the biodegradation tests conducted in this study.

Test No.	Type	Analyte	Comment
A	Biodegradation tests on agar plates	Prussian blue	Investigation on the effective operation of the used bacteria strains; Duration: 48 h. Mode of detection: Loss of blue colour.
B	Biodegradation tests in liquid medium	IL anions	Tests using conditions which are increasingly preferred by the used bacteria; Duration: 28 d; Mode of detection: Specific analysis of the anion via ion chromatography
C	Enzymatic degradation test	IL anions	Investigation on the stability of the anions in the presence of nitrile degrading enzymes; Duration: 24 h; Mode of detection: Specific analysis of the anion via ion chromatography and mass spectrometry

Tab 1 B. Overview on the changing test conditions used for the biodegradation test B

Test No.	Medium	T in °C	Bacteria medium ratio in %	C/N ratio	Type of bacteria
B1	Stringent test medium "OECD guideline 301 medium (OECD 1992)"	20	1	5	KS-7D activated sludge (industrial waste water treatment plant)
B2	Optimised test medium for KS-7D "Cultivating medium from Fraunhofer IGB Stuttgart"	30	10	10	KS-7D
B3	Nutrient-rich medium "Same medium composition as for the agar plates, but without agar, so that the medium remained liquid. This medium consisted of peptone, yeast extract and sodium chloride."	20	1	5	KS-7D

A - Biodegradation tests on agar plates

At first the bacteria were incubated on agar plates in order to examine the biodegradation of the positive control PB. Twelve agar plates were set up and PB was added to half of the plates before the agar solidified. Two pairs of PB/non-PB plates were inoculated with (1) pure culture of KS-7D, (2) bacteria from an industrial waste water treatment plant. The remaining pair was not incubated and served as a blind control. The plates were incubated in the dark at 37 °C for 48 h.

B - Biodegradation tests in liquid medium

To examine the biodegradation of the IL anions, the experiment was set up at different growth conditions. The biodegradation experiments were run in 100 mL autoclaved glass vessels. As a blank for the ion chromatographic detection of the IL anions, one vessel was set up that contained medium and bacteria only, without the analyte. For each IL two vessels were used. 100 µmol L^{-1} IL were added to the medium and bacteria. The total C/N ratio, adjusted by the addition of fructose, is related to the concentration of the carbon and nitrogen in the medium and the different IL anions. Thereby the C/N ratio is the carbon to nitrogen ratio of the total of substrates: IL anion and fructose, if necessary.

Different media were used for the experiments (1) OECD guideline 301 medium (OECD 1992) (2) cultivating medium from Fraunhofer IGB Stuttgart and (3) nutrient-rich medium, with different experimental conditions. The first experiment on the biodegradability of IL anions was run at a temperature of 20 °C with 1 % bacteria suspension and a C/N ratio of 5 of the total of substrates (IL anion and fructose). KS-7D and bacteria from an industrial waste water treatment plant were used. In a second run of the first experiment the medium was enriched with more bacteria (10 %), a higher C/N ratio of 10 and a higher temperature of 30 °C. In the second experiment, the enriched conditions in terms of bacteria content (10 %), C/N ratio of 10 and temperature of 30 °C were maintained. This time applied within the original cultivating medium from Fraunhofer IGB Stuttgart. The KCN in this recipe was substituted with a CN-containing IL. For a comparable medium composition of the liquid medium experiment and the agar plates the third experiment was run with the same medium composition as for the agar plates, but without agar, so that the medium remained liquid. This medium consisted of peptone, yeast extract and sodium chloride. A temperature of 20 °C and a C/N ratio of 5 were applied. One experimental run took 28 days. In the first seven days samples were taken daily, and thereafter once a week on days 14, 21 and 28. The samples for the ion chromatographic measurement were centrifuged (RCF 1700, 15 min) and passed through RC-filters (ROTH®). The ion chromatograph (IC) used was an “IC Metrohm 881 Compact pro” (Metrohm, Switzerland) with a “Metrosep A Supp 5” anion exchanger column. The device was run at a flow rate of 0.7 mL min^{-1} and an injection volume of 20 mL. The standard IC eluent for anions (3.2 mmol L^{-1} Na_2CO_3, 1 mmol L^{-1} $NaHCO_3$) was modified with acetonitrile to enhance the detection of the larger and more lipophilic anions $C(CN)_3^-$ and $B(CN)_4^-$. Limit of detection and limit of quantification were below 0.1 and 0.3 µmol L^{-1}, respectively.

C – In vitro enzymatic hydrolysis

To examine the in vitro enzymatic hydrolysis of the selected IL anions, two enzymes were used that are able to catalyze the reaction: (1) nitrilase and (2) nitrile hydratase. Both enzymes are recombinants from *E. coli* and purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany (CAS numbers: (1) 9024-90-2; (2) 82391-37-5). The experiment was conducted according to common nitrilase reactions in organic synthesis (Banerjee et al. 2009; Rey et al. 2004; Robinson and Hook 1964). The reaction medium was therefore a 100 mM phosphate KH_2PO_4/ K_2HPO_4 buffer at pH 7.5 (pH adjustment with KOH). All salts were obtained from Merck KGaA, Darmstadt, Germany. The concentration of the IL anion and the enzyme was 1 mmol L^{-1} and 4 g L^{-1}, respectively. Three control samples were prepared in duplicates in 1.5 mL eppendorf cups: (B1) IL anion in buffer, (B2) nitrilase in buffer, (B3) nitrile hydratase in buffer, (1) IL anion and nitrilase in buffer and (2) IL anion and nitrile hydratase. Additionally three control samples (without enzyme) were arranged. Half of these samples were further preparedfor ion chromatographic analysis. The other half was placed in an Eppendorf AG Thermomixer compact at 35 °C and the lowest speed of 300 rpm overnight (ca. 22 h).

Sample preparation for ion chromatography

1 mL of the sample was transferred into a 15 mL polypropylene centrifuge tubes (Sarstedt AG & Co., Germany). The proteins could then be precipitated by addition of acetone 1:4 (HiPerSolv CHROMANORM for HPLC, VWR International). The centrifuge tubes were then placed into crushed ice for 20 min. The samples were then centrifuged at 3000 rpm for 10 min which makes around 1200 g (Labofuge 400R, Heraeus instruments GmbH, Germany). The supernatant was transferred to round bottom flask and the solution was rotary evaporated in a water bath at 40 °C and vacuum (VV2011, Heidolph Instruments GmbH & Co.KG, Germany). The final residue was diluted in 10 mL deionised water giving the final concentration of 100 µmol L^{-1} for the ion chromatographic analysis. 500 µL of the final sample were diluted 1:1 in methanol (HiPerSolv CHROMANORM for HPLC, VWR International) for mass spectrometric analysis. The mass spectrometer was an esquire ESI-MS with ion trap detector (Bruker Daltonik GmbH, Germany). The samples were directly injected using a syringe pump at a flow rate of 3 µL/min. The nebuliser was run at 5 psi, with a dry gas flow rate of 5 L/min and a drying temperature of 300 °C. The capillary voltage was set to +4000 V. The anions were detected using the negative mode.

Results and discussion

Biodegradation of the positive control on agar plates

The biodegradation of PB was investigated to find out whether the KS-7D bacteria would degrade the analyte as expected and if there was a difference in comparison to industrial sewage sludge. Agar plates were used, since the PB was agglutinating in the liquid medium, forming blue particles, whereas in agar it remained diluted. It could be seen that with both KS-7D and industrial sewage sludge the blue colour of the PB agar reverted to the original colour of the agar. The blind control without inoculum remained blue, showing that without inoculation the analyte remained stable during the incubation time of two days (Fig. 2).

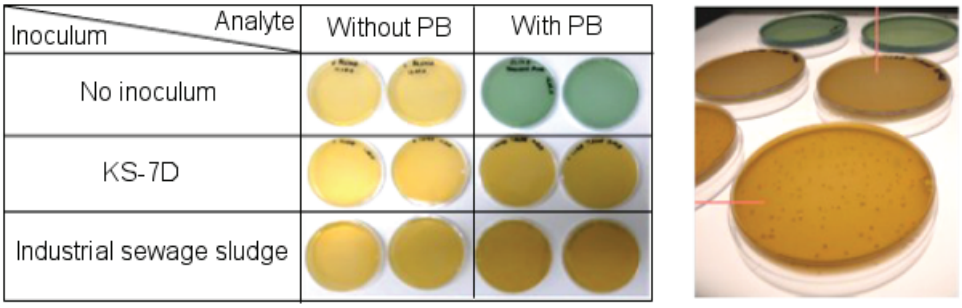

Fig. 2 Left: Results of the biodegradation of Prussian blue (PB) in agar plates by different inocula. Right: Close-up of the bacteria colonies

The results do not match the hypothesis. That KS-7D would degrade PB was expected, but it was not expected that the industrial sewage sludge would do the same, since not many such cases have been reported for metal cyanide complexes (Wehrer et al. 2011). Only a few microorganisms are known to grow on iron cyanide complexes by converting the cyanide into carbon dioxide and ammonia as nitrogen source, among them some *Acinetobacter* sp. (Finnegan et al. 2000), and *Pseudomonas* sp., such as *P. fluorescens* (Dursun et al. 1999) and *P. pseudoalcaligenes* (Luque-almagro et al. 2005). Additionally fungi like the filamentous *Fusarium solani* and *Fusarium oxysporum* have shown to grow on iron and nickel cyanide complexes at normal to acidic pH values (Barclay et al. 1998a). The fungi also hydrolyses the cyanide to ammonia, that serves as nitrogen source, and formate (Barclay et al. 1998b). PB degradation could further be observed in the rhizosphere of cyanogenic plants again with the help of microbial transformation (Kang et al. 2007). Next to the cyanide degradation, the decolouration of PB could also be caused by bacterial reduction of Fe^{3+} ions of the outer complex sphere and not necessarily due to the degradation of the $Fe(CN)_6$- moiety. Such a mechanism has been described for the iron reducing fresh water and marine bacteria *Geobacter metallireducens* and *Shewanella alga* strain BrY, respectively. These bacteria use PB as sole electron acceptor in iron respiration (Jahn et al. 2006).If any of these microorganisms have been involved in the decolourisation of the agar plates inoculated with industrial sewage sludge was not examined. The disappearance of the blue colour in our study, however, proves the biological activity of the used bacteria.

Bacterial growth in the presence of KCN and cyano-based ILs

Recent studies on the toxicity of $N(CN)_2^-$ and $B(CN)_4^-$ towards activated sludge revealed a higher inhibition potential for $B(CN)_4^-$ than for $N(CN)_2^-$ (Markiewicz et al. 2013; Neumann et al. 2012). We tested the bacterial growth of KS-7D in presence of selected cyano-based ILs (100 µmol L^{-1}). In this study the growth of the bacteria via cell counter measurements was followed during 24 h (Fig. 3). $N(CN)_2^-$ showed no inhibition, whereas for $C(CN)_3^-$ and $B(CN)_4^-$ a slight inhibition of bacterial growth was observed compared to KCN. The KS-7D bacteria themselves were able to grow significantly under the experimental conditions in the presence of all selected IL anions. Based on these findings false negative results, with the lack of biodegradation being the effect of the IL's toxicity towards bacteria, are not assumed.

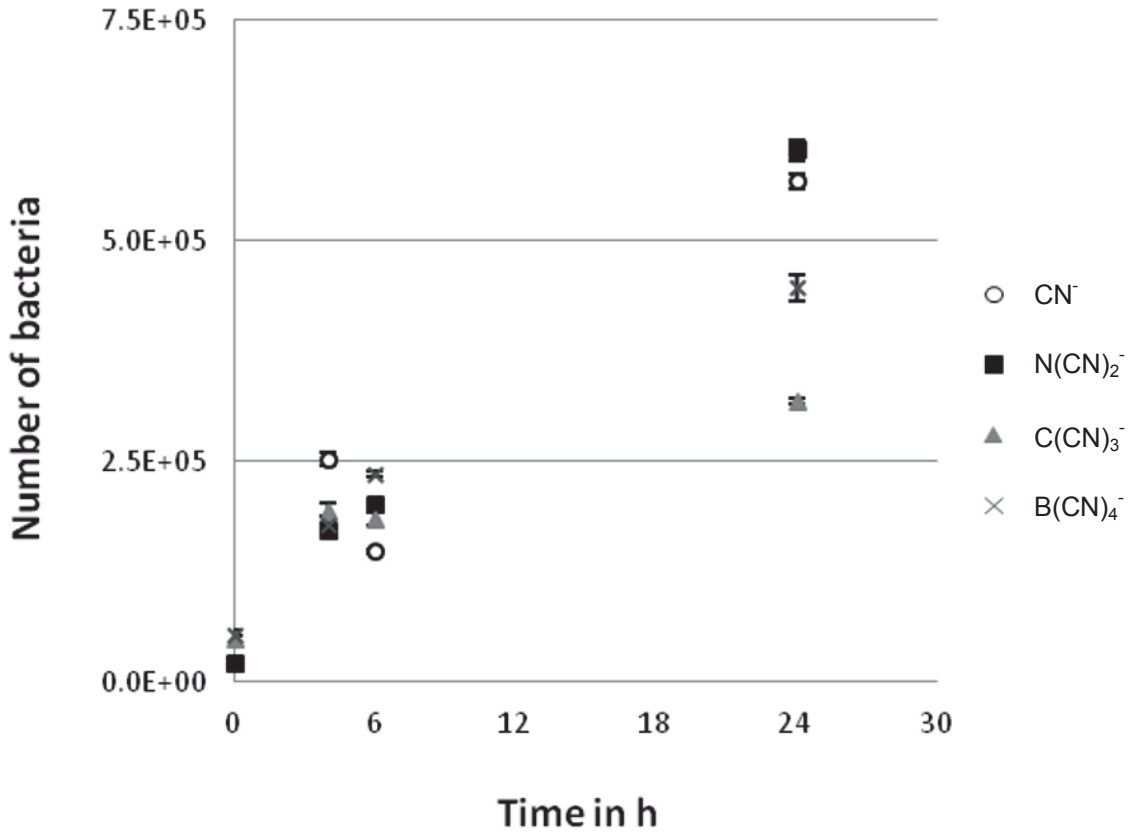

Fig. 3 Number of KS-7D bacteria over time cultivated in medium based on the Fraunhofer IGB recipes containing CN^-, $N(CN)_2^-$, $C(CN)_3^-$ and $B(CN)_4^-$, respectively

Biodegradation of the cyano-based ionic liquid anions

Different growth conditions for the KS-7D bacteria were chosen to enhance their biological activity, and the biodegradability of the cyano-based anions was determined via specific analysis. Under none of the conditions was a significant decrease in relative analyte concentration observed: either at a temperature raised from 20 to 30 °C, together with an elevated bacteria and C/N content (Fig. 4 A, B vs. C, D), or using the same media composition as with the earlier agar plate test (Fig. 4 E). Neither the KS-7D nor industrial sewage sludge bacteria (Fig. 4 A and B) were able to use the anions as carbon or nitrogen sources.

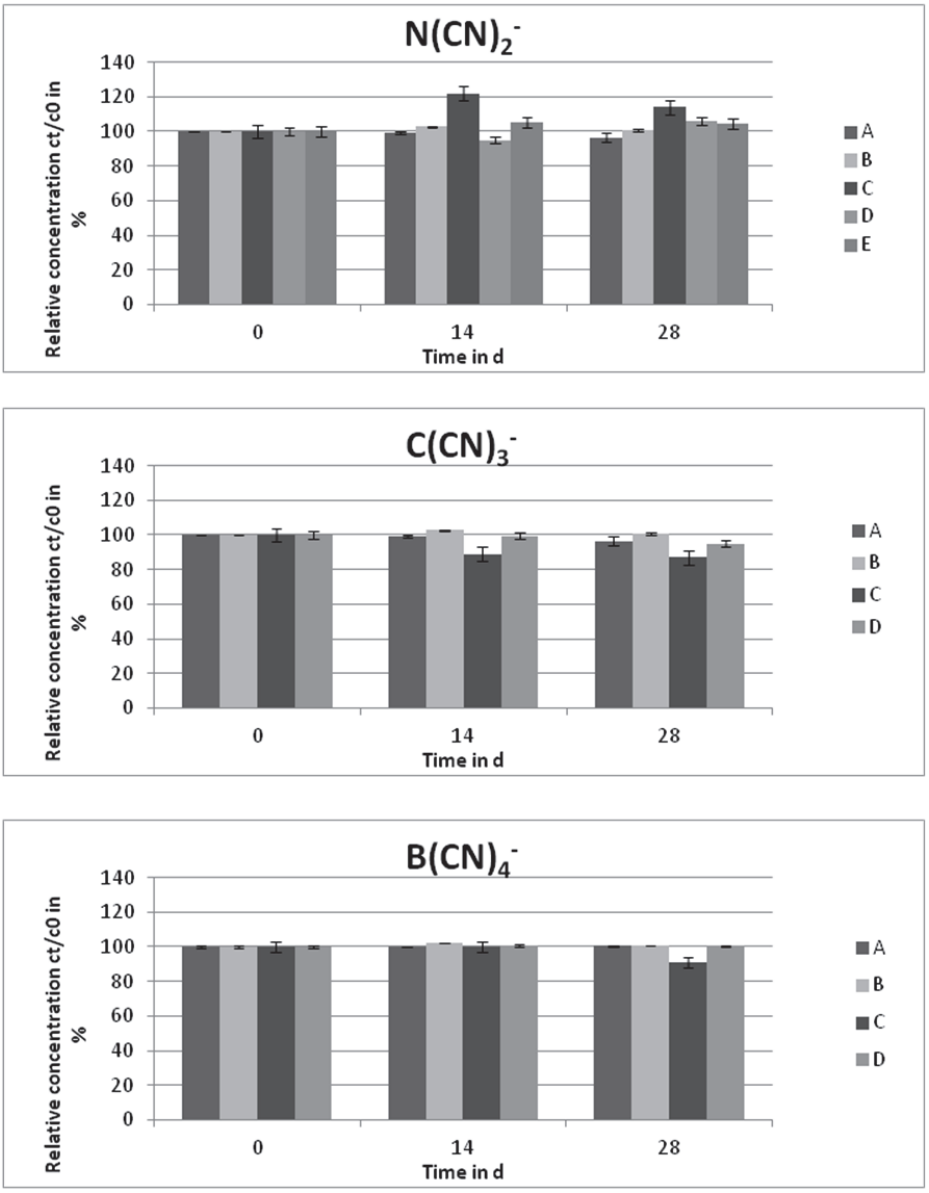

Fig. 4 Relative concentration of N(CN)2-, C(CN)3- and B(CN)4- on different days of the biodegradation test and test conditions:
(A) KS-7D bacteria and (B) industrial sewage sludge bacteria [T=20 °C, OECD test guideline 301 medium, 1% bacteria suspension, C/N ratio 5],
(C) KS-7D bacteria with an enriched medium [T=30 °C, OECD test guideline 301 medium, 10% bacteria suspension, C/N ratio 10],
(D) KS-7D bacteria with an enriched medium [T=30 °C, Fraunhofer IGB Stuttgart medium, 10% bacteria suspension, C/N ratio 10],
(E) KS-7D bacteria with agar medium composition [T=20 °C, liquid agar medium, 1% bacteria suspension, C/N ratio 5]

Without a decrease in relative concentration, the anions are stated to be not primarily biodegradable, where primary biodegradation is the first step in the biodegradation of the whole compound. The difference in relative concentration to up to ± 10-20 % is related mainly to measurement uncertainties that are high in biological degradation tests, especially when matrices with a high organic load are used. The non-biodegradability of cyano-related compounds was also observed in another study in which iron cyanide complexes were investigated for their biodegradability by the addition of cyano-degrading bacteria (Oelsner et al. 2001). The explanation for this is that the bacteria de-adapt as soon as the carbon and nitrogen levels in other components of the medium suffice as nutrients, so that the bacteria no longer need the cyano complexes for their growth. Since the bacteria mixture KS-7D is reported to require an additional carbon source for exponential growth (Bryniok and Trösch 2008) this explanation appears to be unlikely. It must further be considered that the KS-7D bacteria are able to use ferro-cyanide, ferric cyanide and PB not only as carbon and nitrogen source, but as a source of iron, too, and release siderophores (small iron-binding molecules) into the medium to cleave these complex iron cyanides (Schygulla-Banek 1993). It seems that the cyanide hydrolase of KS-7D is now not capable to cleave the ⋹N bond as long as the cyanide is covalently ligated. No molecules, such as the siderophores, seem to exist in KS-7D that can cleave the bond between the central atoms B, C and N, respectively, and the carbon of the cyano groups of the ILs. At the moment no organism has been found that biodegrades cyano-based IL anions.

In vitro enzymatic hydrolysis

We investigated if cyano-based anions are generally susceptible towards in vitro enzymatic hydrolysis using a commercially available nitrilase (NLase) and nitrile hydratase (NHase). Such enzymes are used as catalysts in organic synthesis for the hydrolysis of nitrile groups in pharmaceutical industry and for bioremediation purposes amongst others (Banerjee et al. 2002; Kobayashi and Shimizu 1998; Mascharak 2002; Singh et al. 2006).

In experiments with NLase the concentration of all anions remained stable within 22 h and the experimental uncertainty levels of around 10 %, and no transformation products were found. In contrast, all of the cyano-based anions were hydrolysed to different extent by NHase and corresponding amides that could be detected (Tab. 2). The analytical results are exemplified using the example of $C(CN)_3^-$ (Fig. 5).

Tab. 1 Table of the detected anions (a-h), their formula, the net retention times ($t_{r'}$) and concentrations in ion chromatographic analysis and the corresponding mass-to-charge ratios visible in the mass spectra of the mass spectrometer of the detected IL anion and IL anion transformation products. (t0 - without enzyme treatment (t = 0 min); t1 - immediately after enzyme addition (t = 1 min); t2 - with enzyme treatment (t = 22 h))

Anion		Formula	$t_{r'}$ in min	Relative conc. c_0/c_t in %						Mass-to-charge ratio *m/z* of the IL anion related peaks in mass spectra	
				NLase			NHase			M^-	$[M-43]^-$
				t_0	t_1	t_2	t_0	t_1	t_2	Molecular ion peak	Fragmentation: - HNCO via hydrogen rearrangement
Dicyanamide	a	$N(CN)_2^-$	7.2	100	101	103	100	76	n.d.	66	-
	b	$N(CN)(CONH_2)^-$	0.9	n.d.	n.d.	n.d.	n.d.	15	66	84	n.d.*
Tricyanomethanide	c	$C(CN)_3^-$	6.3	100	104	101	100	3	n.d.	90	-
	d	$C(CN)_2(CONH_2)^-$	1.6	n.d.	n.d.	n.d.	n.d.	89	n.d.	108	65
	e	$C(CN)(CONH_2)_2^-$	0.8	n.d.	n.d.	n.d.	n.d.	3	67	126	83
Tetracyanoborate	f	$B(CN)_4^-$	10.7	100	101	102	100	99	6	115	-
	g or h	$B(CN)_3(CONH_2)^-$	1.7	n.d.	n.d.	n.d.	n.d.	n.d.	5	133	90

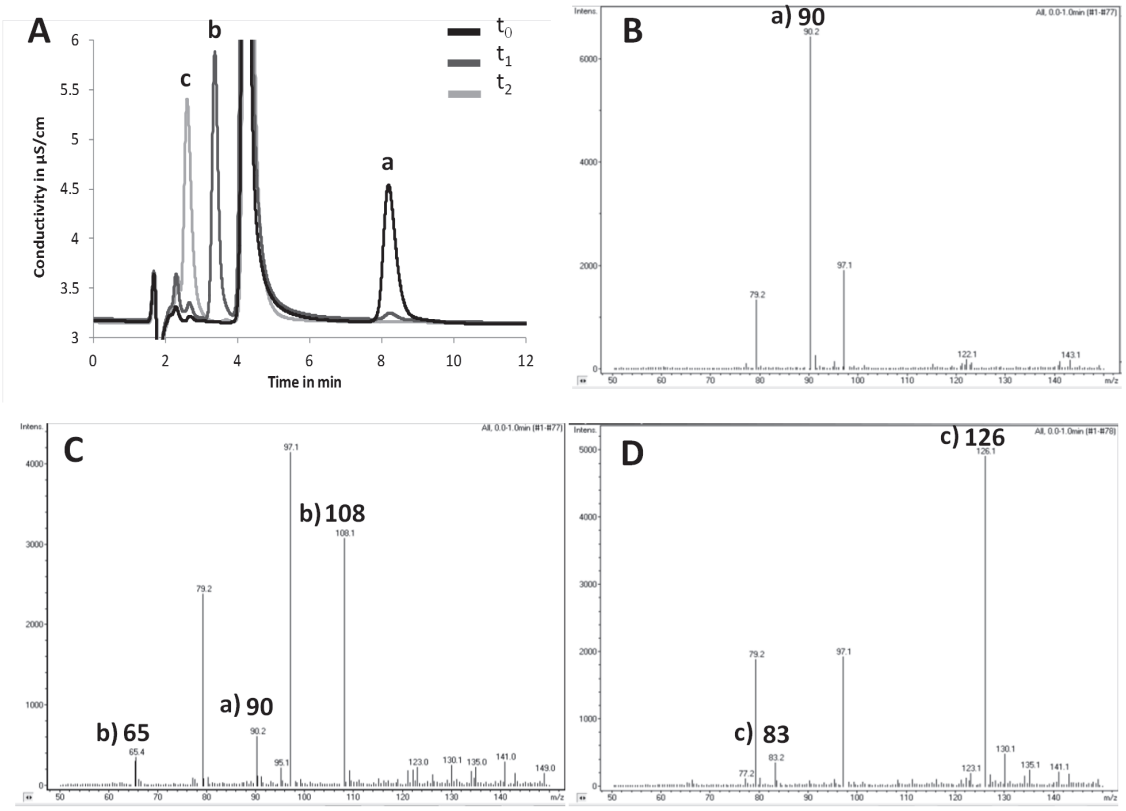

Fig. 5 Results of the in vitro enzymatic hydrolysis of $C(CN)_3^-$ with NHase. (A) Ion chromatogram overlay at three different points of time in the experiment: t0 - without enzyme treatment (t = 0 min); t1 - immediately after enzyme addition (t = 1 min); t2 - with enzyme treatment (t = 22 h), (B) to (D) are the corresponding mass spectra from t_0 to t_2. The compounds detected were: a) $C(CN)_3^-$, b) $C(CN)_2(CONH_2)^-$ and the MS-fragment $C(CN)_2H^-$, c) $C(CN)(CONH_2)_2^-$ and the MS-fragment $C(CN)(CONH_2)H^-$

The sample that was taken immediately after the NHase addition (t_1) already shows a degradation of the parent compound in comparison to the sample without any enzyme addition (t_0). A transformation product that could be identified via MS as the $C(CN)_2(CONH_2)^-$ anion appears just before the phosphate buffer peak at 4.5 min in the IC chromatogram. After 22 h this transformation product disappeared and the next hydrolytical product could be detected, $C(CN)(CONH_2)_2^-$. A similar behaviour has been observed for $N(CN)_2^-$ and $B(CN)_4^-$, where they have been hydrolysed into $N(CN)(CONH_2)^-$ and $B(CN)(CONH_2)^-$, respectively. The proposed hydrolytical transformation pathway for the IL anions is shown in Fig. 6.

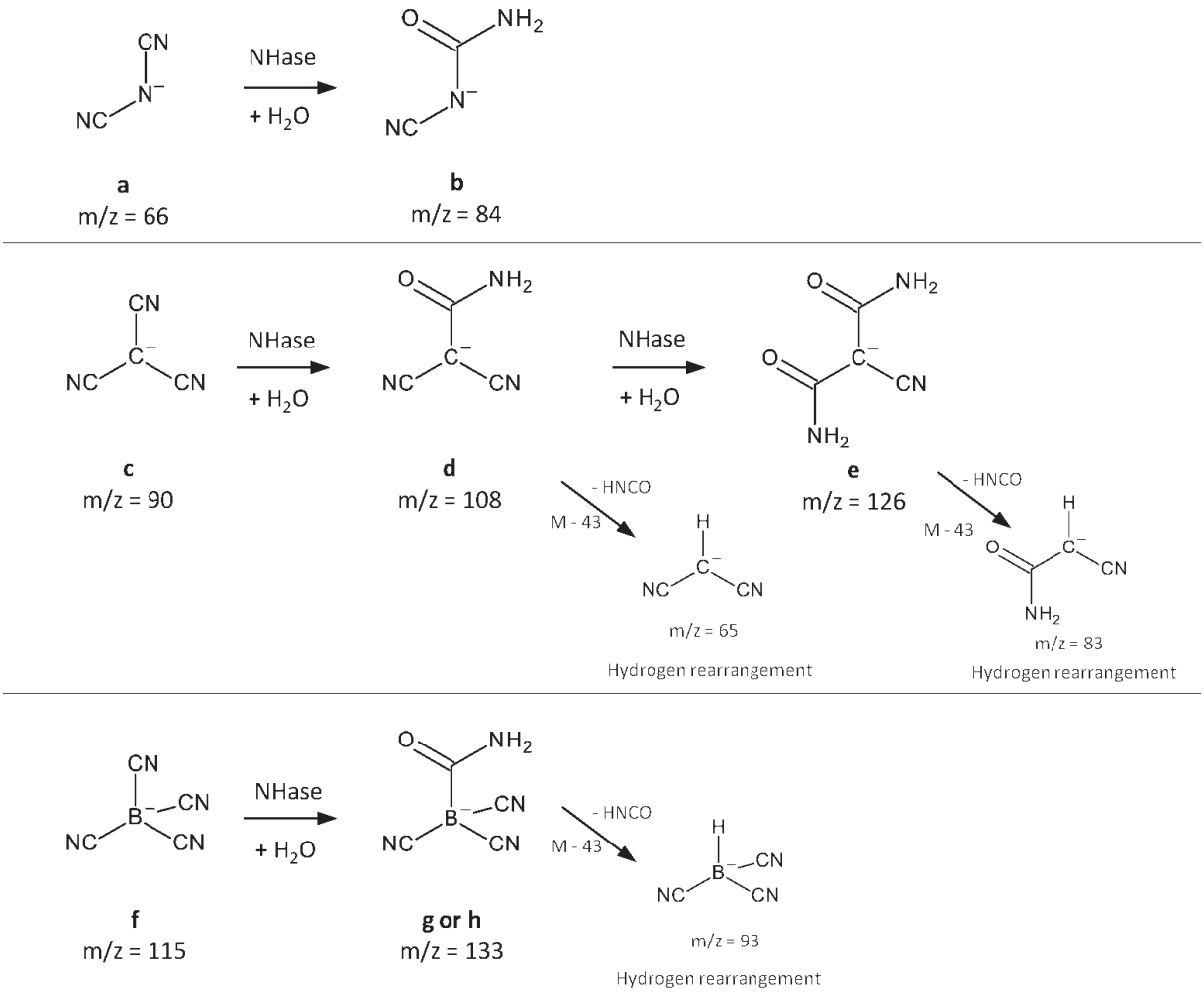

Fig. 6 Proposed in vitro hydrolytical pathway by NHase at pH 7 of the investigated IL anions together with the mass-to-charge ratios (black) and fragmentation pattern detected via mass spectrometry (grey)

The observations revealed that a hydrolysis under pH neutral conditions of the IL anions by NHase lead to the corresponding amides. Even $B(CN)_4^-$ which is stable under harsh conditions could be transformed. The non-hydrolysis by NLase within the experimental run time is assumable due to the completely different mechanisms in NLase and NHase. NLase attacks the C-atom of the nitrile group to form a covalently bonded thiomidate intermediate. This thiomidate is further oxidised and the corresponding carboxylated product is formed. In NHase no such covalent intermediate needs to be formed. There either the cyano group of the substrate is directly attacked by a metal-bound hydroxide ion acting as a nucleophile or the metal-bound hydroxide ion acts indirectly as a base to first activate a water molecule, which then attacks on the cyano group (Banerjee et al. 2002). The nitrile is finally hydrolysed into the amide. Therefore it is assumed that the NHase can more easily access the cyano-based anions. For the degradation of cyano-based IL anions it appears promising to use other NHase containing microorganisms such as *Rhodococcus erythropolis* which are capable to degrade e.g. benzonitrile herbicides (Veselá et al. 2012).

Conclusion

The overall aim of the experiments was to find out whether cyano-based anions can be degraded with the aid of KS-7D bacteria for potential application in waste water treatment or further by nitrile hydrolysing enzymes. At the moment no biodegradation of the cyano-based anions has been observed by the used microorganisms. The capability of nitrile hydratase to hydrolyse the cyano-based anions in vitro now leads to further considerations. Since pure enzymes are relatively instable, they are often more applicable in organic synthesis than wastewater treatment. An alternative may be cross-linked enzymes aggregates (CLEAs) that are made of immobilised and stabilised enzymes and are recycable for multiple uses (Sheldon 2011). The investigated NHase has been especially successfully prepared as CLEAs for "green nitrile hydration in industry" (van Pelt et al. 2008). The use of CLEAs of laccase is also currently under investigation for use as potential treatment procedure for waste water contaminated with endocrine disruptors (Wintgens 2013). Another alternative to the pure enzymes used could be the direct use of bacteria that contain the necessary enzymes for nitrile hydrolysis, such as *Rhodococcus erythropolis* (Vejvoda et al. 2007). In general, bacteria could be advantageous to pure enzymes. They contain a series of different enzymes that can make amides not only from nitriles but also carboxylic acid from the produced amides by amidases, and even a complete mineralisation may be be further realisable. Whether or not any of the mentioned possibilities are applicable for the treatment of wastewater that is contaminated with cyano-based anions will then need to be tested. In terms of a structural design of IL anions, the synthesis of new anions that have an intrinsically higher potential for being biodegradable should also be considered. For example in the case of tetracyanoborate, the borate could already be prepared as a carboxylated anion, e.g. $B(CO_2H)_4^-$ (Bernhardt et al. 2006), and a hydrogenated one, e.g. $BH_2(CN_2)$ (Györi et al. 1983), as well as a dianion $B(CN)_3^{2-}$ (Bernhardt et al. 2011) that could change the accessibility of the molecule for enzymatic attack.

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Paper A.5 - Biodegradability of 32 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ionic liquid cations under aerobic conditions

Neumann J, Steudte S, Cho C-W, et al. Biodegradability of 32 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ionic liquid cations under aerobic conditions.

A revised version of the article was accepted for publication in Green Chemistry in January 2014. The revised article will finally be found on the RSC website.

This thesis includes the version that was submitted to Green Chemistry in October 2013.

Own contributions:

The experimental work on the primary biodegradation of IL cations started with an experiment on a first set of seven IL cations set up by Chul-Woong Cho and measured by me via ion chromatography. I evaluated then the experiment and extended the ideas to the (re)-investigation of some of the measured IL cations and further ILs. I run the corresponding experiments on the primary biodegradability and pre-experiments on the full mineralisation in Karlsruher bottles and in a BOD Track. Further lab work on the full mineralisation of the ILs with WTW Oxitop® devices was conducted by Stephanie Steudte and Carla Sernow. I was further responsible for all of the corresponding analytical measurements, including quantitative chromatographic analysis and identification of transformation products by mass spectrometry, and the evaluation and interpretation of the received data. The results were finally published after discussion with the other co-authors.

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ARTICLE TYPE

Biodegradability of 32 pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium ionic liquid cations under aerobic conditions

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The chemical and thermal stability of ionic liquids (ILs) make them interesting for a large variety of applications in nearly all areas of the chemical industry. However, this stability is often reflected in their recalcitrance towards biodegradation, which comes with the risk of persistence when they are released into the environment. In this study we carried out a systematic investigation of the biodegradability of pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium-based IL cations substituted with different alkyl or functionalised side chains and using halide counterions. We examined their primary degradability by specific analysis and/or their ultimate biodegradability using biochemical oxygen demand tests according to OECD guideline 301F. Biological transformation products were investigated using mass spectrometry. Comparison of the biodegradation potential of these ILs shows that for all five head groups representatives can be found that are readily or inherently biodegradable, thus permitting the structural design of ILs with a reduced environmental hazard.

Keywords: Biodegradation, ionic liquids, hazard assessment, transformation products

Introduction

Ionic liquids

Ionic liquids (ILs) are chemicals with an outstanding design potential. Their components – cationic and anionic molecular core structures with different side chains – can be combined in innumerable ways to create substances with tailor-made physico-chemical properties important for industrial applications. Most of the compounds possess a very low vapour pressure and non-flammability, which are key properties for improved operational safety in comparison with conventional volatile organic solvents.

Depending on the combination of cations and anions, they can be thermally stable, be liquid over a wide temperature range and can have a large electrochemical window, which makes them useful for a variety of technical applications in different fields. Initially used as solvents for synthesis and catalysis in clean technologies, they have been increasingly investigated during the last 15 years.[1–3] Several industrial processes have been established in which the use of ILs is highly advantageous with respect to the productivity of the processes.[4,5] Their range of potential applications extends from the already-mentioned usage as solvents and catalysts to media for electrochemical applications and biosensors.[6–9] ILs have also been considered for utilisation in analytical[10–13] or supercritical fluid applications,[14] gel production[15] or as pharmaceutical ingredients.[16,17] Their application as agents for recovery, extraction and purification purposes[18–20] or as lubricants[21] and hypergolic rocket fluid[22] is also being discussed.

Sustainability considerations

The variety of ILs available for an individual process together with their high operational safety is not only advantageous for the performance and handling of the process, but also for sustainable development.[23] In this context the environmental compatibility of a chemical also plays a major role. The production of substances that simultaneously possess low toxicity and environmental non-persistence is a demanding task for chemists dedicated to sustainable chemistry.[24] Nevertheless, its fulfilment appears feasible in the context of the enormous design potential of ILs.[25–27] The structural aspects that influence the toxicity of ILs have already been identified and have been replaced by less toxic arrangements in one application.[28]

Biodegradable structural elements

Many different studies of ILs for the identification of structural elements which support their overall biological degradation have recently been reviewed.[3,27,29–31]

The biodegradation potential of ILs ranges widely from very good to very poor. Good primary biodegradability has been observed in ILs with longer alkyl side chains (>C6) at the cationic core, whereas the same head group with shorter side chains is only poorly biodegradable: compare, for example, 1-octyl-3-methyl-1H-imidazolium (IM18) with 1-ethyl-3-methyl-

1H-imidazolium (IM12) combined with halide anions (Cl, Br).[32,33] Imidazolium-based ILs in general exhibit reduced biodegradability with respect to their core structure,[32,34] whereas 1-alkyl-3-methylpyridinium-based ILs have an elevated biodegradation potential,[35,32] and many compounds have been classified as readily biodegradable. Apart from the (bio)degradability of the cation, the behaviour of the anion also has to be taken into account. Anions such as PF_6^- and BF_4^- are sensitive to hydrolytic processes,[36] and organic anions such as acetate, and ethyl and octyl sulphate, are readily biodegradable.[37,38] In contrast, anions such as $N(CN)_2^-$, $B(CN)_4^-$ and $(CF_3SO_2)_2N^-$ are known to be neither hydrolytically degradable nor biodegradable under environmental conditions.[39,40] The choice of inoculum using pure cultures, such as *Corynebacterium sp.* and *Sphingomonas paucimobilis*, and the presence or absence of degraders in mixed cultures additionally influences the results towards an enhanced biodegradability of the ionic liquid cation.[41–43]

Thus, a broad biodegradability range of ILs can be created depending (i) on the structural composition with respect to the cationic head group, side chain and counterion, and depending (ii) on the experimental procedure used for the determination of biodegradability data, more specifically the detection either by a specific analytical procedure or sum parameters, the chosen environmental condition and the choice of microorganisms.

The focus of the study is laid on the influence of structural composition and the design potential of ILs.

Missing data

The identification of the biodegradable substructures of ILs has been a mammoth task. Although many commercially available pyridinium and imidazolium-based ILs have been tested, some relevant data for a systematic investigation of other head groups are still missing. To date only N-alkyl-N-methylmorpholinium and 4-benzyl-4-methylmorpholinium have been examined, head groups that are not readily biodegradable.[44] A few phosphonium ILs with alkyl and functionalised side chains were also refractory to biological degradation under ready biodegradability test conditions.[45,46]

As yet, no systematic investigation of the biodegradability of other head groups has been undertaken. We therefore examined a set of 32 ILs with differently substituted head groups – pyrrolidinium, morpholinium and piperidinium ILs – together with some pyridinium and imidazolium ILs (Tab. 1 - Tab. 6). We screened the primary biodegradability of IL cations, tracking the course of degradation using liquid chromatography, determined (where possible) transformation products with mass spectrometry, and investigated the extent of mineralisation of selected ILs by applying the sum parameter biochemical oxygen demand (BOD). Several of the IL cations under investigation were fully degraded and some of them match the criteria for being classified as readily biodegradable. In experiments with a prolonged standard test duration (> 28 d) some more compounds underwent biodegradation, showing the importance of microbial adaptation. Even during the degradation of one IL, microbial adaptation occurred as diauxic growth when head groups and side chains were successively biodegraded.

Our systematic studies of the biodegradability of ILs are addressed to the users of ILs in different fields of application to facilitate the selection of environmentally favourable structural elements and hence to contribute to the design of inherently safer ILs.

Materials and methods

Chemicals

All the tested ILs as well as the salts for the mineral salt medium and the standard eluent for ion chromatographic analysis were obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (HPLC grade) for the ion chromatographic measurements was obtained from VWR International GmbH (Darmstadt, Germany).

Biodegradation tests

1 Manometric respirometry

The manometric respirometry test was performed according to OECD guideline 301F.[47] The biological oxygen demand (BOD) of the substance was determined for 28 d (but prolonged as soon as a significant increase in oxygen consumption had been detected) using a BOD measurement system (OxiTop©, thermostatically controlled from WTW GmbH, Weilheim, Germany). The extent of mineralisation could be inferred from this test: if 60% biodegradation was exceeded within a certain time frame, the compound was classified as "readily biodegradable".

The activated sludge was taken from the municipal wastewater treatment plant in Delmenhorst (Germany), filtered through grade 1288 filter discs (Sartorius AG, Göttingen) and aerated for ten days to remove organic substances from sewage treatment before inoculation. The medium was prepared with 8.5 mg L^{-1} KH_2PO_4, 28.5 mg L^{-1} $K_2HPO_4 \cdot 3H_2O$, 33.4 mg L^{-1}, $Na_2HPO_4 \cdot 2H_2O$, 0.5 mg L^{-1} NH_4Cl, 36.4 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 22.5 mg L^{-1} $MgSO_4 \cdot 7H_2O$ and 0.25 mg L^{-1} $FeCl_3$ (pH 7.4). An additional 1.16 mg L^{-1} allylthiourea have been added in order to inhibit nitrification. The bacteria suspension was set up using 20 % supernatant from the aerated activated sludge and 80 % mineral salt medium solution. The concentrations of the test substances were chosen according to their expected oxygen demand, which were in a non-inhibitory concentration, usually below 850 µmol L^{-1}.[48] Moreover, blank samples (inoculated media without test substance) and controls (inoculated media with benzoic acid) were also prepared. In this test a bacteria number of 10^4 cells L^{-1} was applied (determined by Paddle-Tester; Hach Europe, Düsseldorf). Sodium hydroxide was used to absorb the evolved carbon dioxide inside the sample vessels. The vessels were closed with gas-tight stoppers and stored in the dark at 20 ± 0.5 °C. The oxygen consumption was determined manometrically. Biodegradation of the test substance was calculated by the oxygen uptake (OD) for the test substance (corrected by the oxygen demand of the blank samples) with respect to the theoretical oxygen demand (ThOD) of the substance and the amount of substance present

in the sample. For the calculation of ThOD the cation was considered without including its inorganic halide anion. The chemical formula can be given as $C_cH_hN_nO_oP_p$ (eq.1):

$$ThOD = \frac{16\left[2c+\frac{1}{2}(h-3n)+\frac{5}{2}p-o\right]\frac{mg}{mg}}{molecular\ mass\ of\ test\ substance\ \frac{mmol}{mg}} \quad \text{eq.1}$$

The reference substance benzoic acid was always degraded within 10 d, consuming around 80 % of the ThOD and showing that the test was working reliably.

2 Primary biodegradation

The stock solutions of the test substances were prepared in deionised water. Each test substance was used at a final concentration of 50 µmol L^{-1} in the sample vessel. The medium and sample vessels were prepared as described above. Additionally, one sample vessel was prepared for each experimental set up with aniline as positive control at a final concentration of 1.07 mmol L^{-1} (100 mg L^{-1}). Aniline was used as a reference substance for positive biodegradability, since aniline is known to be biodegradable under the chosen test conditions within 14 d.[1] In this study aniline was fully biodegraded within ten days (limit of detection < 5 µmol L^{-1}, data not shown), indicating the general biological activity of the inoculum. The vessels were stirred continuously with a magnetic bar in the dark. The evaporated water was made good with an equal volume of deionised water.

The concentration of the test substance was determined by ion chromatography. The primary biodegradation was then calculated from the concentration of the substance on a specific day in comparison to that on day zero. The maximum deviation was calculated from the standard deviations of the ion chromatographic measurement according to common error propagation.

Examination of the analyte concentration and metabolites

Sampling and sample preparation for instrumental analysis

The samples for the specific analysis were taken on the first day and thereafter at short time intervals for 28 d. On each testing day, 6 mL of the sample were centrifuged at approx. 3000 g (Labofuge 400R, Heraeus instruments GmbH, Germany) in 15 mL polypropylene centrifuge tubes (Sarstedt AG & Co., Germany). The samples were stored in a freezer at -20 °C until all of the samples had been taken. The final specific analysis of the IL cation was then conducted by ion chromatography.

3 Ion chromatography

The ion chromatograph used was a "Metrohm 881 Compact IC pro" equipped with a "Metrohm 850 Conductivity Detector" connected to a "Metrohm 863 Compact Autosampler" (Metrohm AG, Switzerland). The "Metrosep C 4 50" cation exchange column with a "Metrosep C 4 50 Guard" pre-column was kept at 30 °C in a column oven. The analytical method was developed on the basis of previous studies.[49] 5 mmol L^{-1} nitric acid (HNO_3) with 25 % acetonitrile (ACN) were used as eluent. The device was run at a flow rate of 0.9 mL min^{-1} and the injection volume of each sample was 20 µL. The analytical method was developed to analyse most of the IL cations with one eluent solution. The data were evaluated using standard "Magic Net 1.1" software. An external standard for each substance was run during each measurement sequence to identify the IL peak and to check the consistency of the analytical method. Analytical quality parameters have already been published for other IL cations – these are generally in the range of < 0.33 µM and < 1 µM for the limit of detection and limit of quantification, respectively.[49]

4 Liquid chromatography - mass spectrometry

In order to analyse potential degradation products, liquid chromatographic separation combined with mass spectrometric analysis was subsequently undertaken if necessary. The samples were diluted in methanol and deionised water (50:50) and analysed using an HP 1100 HPLC coupled to a Bruker Esquire ESI-MS ion trap detector. The chromatograph was run with 55 % of 5 mmol L^{-1} sodium formate pH 3.4 and 45 % ACN at a flow rate of 0.5 mL min^{-1}. Column: Multohigh 150 x 4 mm, 100 Si-5 µ Hilic (CS-Chromatographie Service GmbH, Place).

Mass spectra for cations were acquired in positive ion mode in the scan range of m/z^+ 50–200. The ESI source conditions were set with a drying gas flow rate of 11 L min^{-1}, drying gas temperature at 350 °C and nebulizer at 70 psi. Generally, all samples after the biodegradation experiments have been investigated with LC-MS to identify (if applicable) transformation products.

5 Interpretation of the data

The following effects that can lead to a false interpretation of the data were excluded:
(1) Excluding false positive results: the declaration of an IL as biodegradable although it is not may be due to the adsorption of the analyte on the bacteria and glass vessels, as well as to systematic deviations of the analytical test system. However, adsorption effects would only be observed within the first few days[50] and often in samples with a high organic load. As we used a negligible amount of sewage sludge flocs in the filtered test solutions, no adsorption effects were observed in our experiments. Furthermore, an abiotic sample was additionally run as an adsorption control. Systematic deviations in the analytical measurements were reduced by adjusting the results so as to relate them to the external standards run with each analytical sequence. Substances detected as being primarily biodegradable were further examined for their full biodegradability to confirm the results of the first run.

(2) Excluding false negative results: apart from the stringent test conditions, inhibition effects can also lead to a false classification of the data as "non-primarily biodegradable". However, these can be excluded at a concentration of 50 µM in primary biodegradation tests and below 850 µmol L^{-1} in BOD tests at which inhibitory effects are unlikely to be observed.[48]

Results

The primary biodegradation of the test substances under aerobic conditions was monitored for 28 d. Specific analysis by ion chromatography (IC) using a conductivity detector yielded information on the concentration of the IL cation at a specific time of the experiment. If an IL cation was not degraded we assumed that it was not "readily biodegradable" in accordance with OECD 301. If primary biodegradation was observed, we used WTW OxiTop® devices measuring the biochemical oxygen demand (BOD) to determine the full mineralisation of the IL. In this test a compound was classified as readily biodegradable if it exhibited > 60 % degradation in relation to the theoretical oxygen demand (ThOD) within 28 d and an exponential growth phase of 10 d. In this study this result was attributable solely to the cation, because most of the IL anions investigated contained inorganic moieties ("halides") (Tab. 1 - Tab. 6) that were irrelevant to biodegradation tests based on the measurement of oxidisable carbon in the molecule. The same held true for PF_6^- and the non-biodegradable anion $(CF_3SO_2)_2N^-$.

The results of the primary biodegradation and full mineralisation experiments are given in Tables 1-6. For some ILs the standard test duration of 28 d was prolonged (in total up to 60 d) to investigate their long-term behaviour.

Biodegradability of head groups

1 Pyrrolidinium compounds

In primary and mineralisation experiments the biodegradation rate of pyrrolidinium compounds varied between 0 and 100 % and was dependent on the substituted side chain (Tab. 1).

No biodegradation was found for the ether (Pyr11O2 Cl) and ethyl (Pyr12 2OSO3) derivatives, whereas Pyr13OH Cl and Pyr18 Cl could be classified as readily biodegradable, exhibiting > 60 % degradation within 28 d. Pyr11COO2 Br was completely primary degraded, but 20 % of the ThOD was attained after just 5 d. No further degradation took place until the end of the standard experiment (28 d). The test procedure was prolonged, however, and after 37 d the whole compound was fully mineralised (78 %) (Fig. 1).

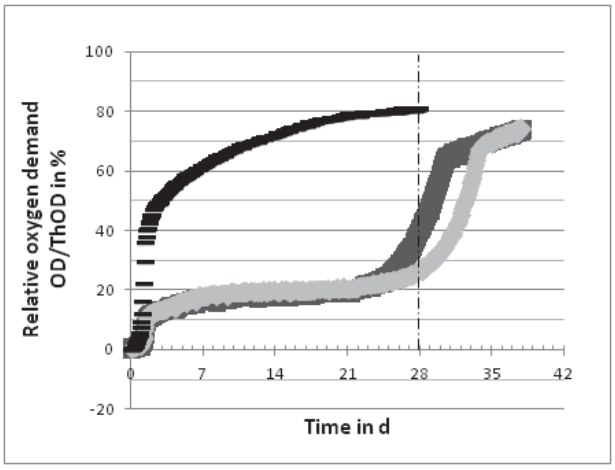
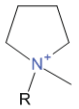
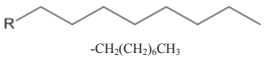
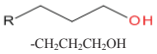
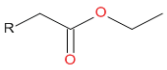
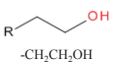
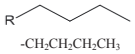
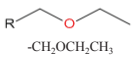
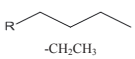


Fig. 1: Biodegradation curves (duplicates in grey: ■ and ◆) derived from BOD measurements for Pyr11COO2 Br. The black line (▬) represents the reference compound benzoic acid.

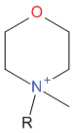
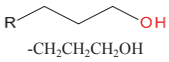
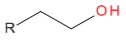
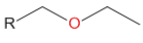
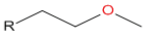
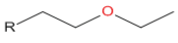
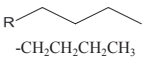
The MS measurements of samples taken from the BOD experiment showed that after 5 and 28 d no parent compound could be detected any more, but that a degradation product with $m/z^+ = 144$ and the corresponding sodium and potassium adducts ($m/z^+ = 144 + 23 - H^+ = 166$ and $m/z^+ = 144 + 39 -$

Tab. 1: Structural formula and results of pyrrolidinium-based ionic liquid cations obtained from primary and BOD experiments.

	R	Anion	Substance identifier	Classification OECD* (...biodegradable) and biotic hydrolysis	Primary Biodegradation in % (28 d)	Biological Oxygen Demand in % (28 d)	Reference
	 -$CH_2(CH_2)_6CH_3$	Cl	Pyr18 Cl	Readily	100	68-70	This study
	 -$CH_2CH_2CH_2OH$	Cl	Pyr13OH Cl	Readily	0-100*	66-69	This study
	 -CH_2CN	Cl	Pyr11CN Cl	Biotic hydrolysis	100	2	This study
	 -$CH_2C(=O)OCH_2CH_3$	Br	Pyr11COO2 Br	Inherently	100	20 (28 d); 78 (37 d)	This study
	 -CH_2CH_2OH	I	Pyr12OH I	Inherently	5-6 (28 d); 100 (50 d)	5-20 (28 d); 0-56 (60 d)	This study
	 -$CH_2CH_2CH_2CH_3$	Br	Pyr14 Br	Inherently	-	5-23 (28 d); 57-82 (42 d)	This study
	 -$CH_2OCH_2CH_3$	Cl	Pyr11O2 Cl	Not readily	-	0	This study
	 -CH_2CH_3	$CH_3CH_2OSO_3$	Pyr12 2OSO3	Not readily	0	-	This study

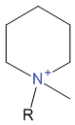
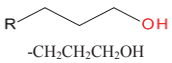
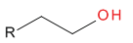
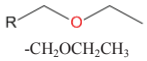
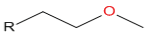
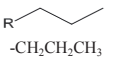
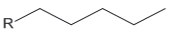
*In the majority of test bottles the compound was primarily degraded to 100%

Tab. 2: Structural formula and results of morpholinium-based ionic liquid cations obtained from primary and BOD experiments.

	R	Anion	Substance identifier	Classification OECD* (...biodegradable) and biotic hydrolysis	Primary Biodegradation in % (28 d)	Biological Oxygen Demand in % (28 d)	Reference
	 -$CH_2CH_2CH_2OH$	Cl	Mor13OH Cl	Inherently	13-14	20-42 (28 d); 65 (42 d)	This study
	 -CH_2CN	Cl	Mor11CN Cl	Biotic hydrolysis	97	0	This study
	 -CH_2CH_2OH	I	Mor12OH I	Not readily	37-41*	0	This study
	 -$CH_2OCH_2CH_3$	Cl	Mor11O2 Cl	Not readily	0	-	This study
	 -$CH_2CH_2OCH_3$	Cl	Mor12O1 Cl	Not readily	1	-	This study
	 -$CH_2CH_2OCH_2CH_3$	Br	Mor12O2 Br	Not readily	0	-	This study
	 -$CH_2CH_2CH_2CH_3$	Br	Mor14 Br	Not readily	0	-	This study

*Results of the primary degradation test could not be confirmed by repeated measurements in full mineralisation experiments and mass spectrometric measurements

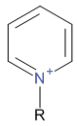
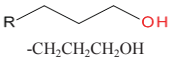
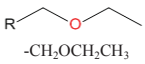
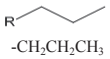
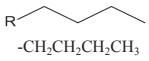
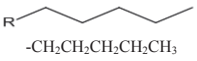
Tab. 3: Structural formula and results of piperidinium-based ionic liquid cations obtained from primary and BOD experiments.

	R	Anion	Substance identifier	Classification OECD (...biodegradable) and biotic hydrolysis	Primary Biodegradation in % (28 d)	Biological Oxygen Demand in % (28 d)	Reference
	 -$CH_2CH_2CH_2OH$	Cl	Pip13OH Cl	Inherent and ultimate	-	77-81*	This study
	 -CH_2CN	Cl	Pip11CN Cl	Biotic hydrolysis	100	5	This study
	 -CH_2CH_2OH	Cl	Pip12OH Cl	Not readily	-	27-31 (28 d) 84-85 (60 d)	This study
	 -$CH_2OCH_2CH_3$	Cl	Pip11O2 Cl	Not readily	0	-	This study
	 -$CH_2CH_2OCH_3$	Cl	Pip12O1 Cl	Not readily	2	-	This study
	 -$CH_2CH_2CH_3$	$(CF_3SO_2)_2N$	Pip13 (CF3SO2)2N	Not readily	-	3	This study
	 -$CH_2CH_2CH_2CH_3$	Br	Pip14 Br	Not readily	-	0	This study

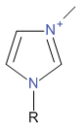
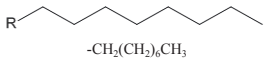
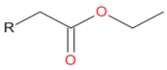
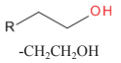
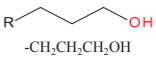
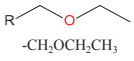
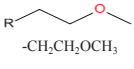
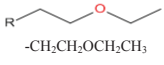
*Not within a 10-day window

$H^+ = 182$) were formed (data not shown). This suggests that the ester bond was enzymatically hydrolysed (in abiotic samples the compound remained stable; data not shown) and Pyr11COOH ($m/z^+ = 144$) was released and fully mineralised after an additional adaptation period of 20 d (Fig. 1). Degradation rates below 25 % and strong deviations between replicates were observed for Pyr12OH I and Pyr14 Br; however, prolonging the test to 60 d enabled both compounds to be fully mineralised. According to OECD Pyr11COO2 Br, Pyr12OH I and Pyr14 Br could be classified as "inherently biodegradable with pre-adaptation". Complete primary degradation and no BOD were observed for Pyr11CN Cl; similar findings have been made for other head groups with a cyanomethyl side chain (Tab. 1 - Tab. 6) and will be presented in a separate paragraph.

Tab. 4: Structural formula and results of pyridinium-based ionic liquid cations obtained from primary and BOD experiments. The data from this study are complemented with data from the literature.

	R	Anion	Substance identifier	Classification OECD (...biodegradable) and biotic hydrolysis	Primary Biodegradation in % (28 d)	Biological Oxygen Demand in % (28 d)	Reference
	 -CH_2CH_2OH	I	Py2OH I	Readily	100	58-65	This study
	 -$CH_2CH_2CH_2OH$	Cl	Py3OH Cl	Inherently	-	51	This study
	 -CH_2CN	Cl	Py1CN Cl	Biotic hydrolysis	100	0	This study
	 -$CH_2OCH_2CH_3$	Cl	Py1O2 Cl	Not readily	-	0	This study
	 -CH_2CH_3	Cl	Py2 Cl	Not readily	0	-	Stolte et al. 2008
	 -$CH_2CH_2CH_3$	Br	Py3 Br	Not readily	-	1	This study
	 -$CH_2CH_2CH_2CH_3$	Br	Py4 Br	Not readily	-	1-3	Harjani et al., 2009
	 -$CH_2CH_2CH_2CH_2CH_3$	Br	Py5 Br	Not readily	-	0	This study

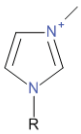
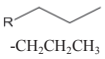
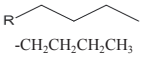
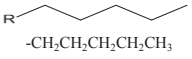
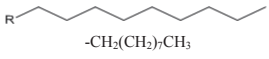
Tab. 5: Structural formula and results of imidazolium-based ionic liquid cations (functionalised side chains and an octyl side chain) obtained from primary and BOD experiments. The data from this study are complemented with data from the literature.

	R	Anion	Substance identifier	Classification OECD (...biodegradable) and biotic hydrolysis	Primary Biodegradation in % (28 d)	Biological Oxygen Demand in % (28 d)	Reference
	 -$CH_2(CH_2)_6CH_3$	Cl	IM18 Cl	Inherently	100	-	Stolte et al. 2008
	 -$CH_2C(=O)OCH_2CH_3$	Br	IM11COO2 Br	Inherently	-	21	Gathergood et al., 2004
	 -CH_2CN	Cl	IM11CN Cl	Biotic hydrolysis	0 100	- 0	Stolte et al. 2008 This study
	 -CH_2CH_2OH	I	IM12OH I	Not readily	0	-	Stolte et al. 2008
	 -$CH_2CH_2CH_2OH$	Cl	IM13OH Cl	Not readily	0	-	Stolte et al. 2008
	 -$CH_2OCH_2CH_3$	Cl	IM11O2 Cl	Not readily	0	-	Stolte et al. 2008
	 -$CH_2CH_2OCH_3$	Cl	IM12O1 Cl	Not readily	0	-	Stolte et al. 2008
	 -$CH_2CH_2OCH_2CH_3$	Br	IM12O2 Br	Not readily	0	-	Stolte et al. 2008

2 Morpholinium compounds

None of the investigated morpholinium compounds showed significant primary biodegradation apart from the Mor13OH cation (Tab. 2). At the end of the primary biodegradation with Mor13OH a slight decrease in concentration (12 - 13%) was observed, so an additional BOD experiment of prolonged duration was performed. In these experiments oxygen consumption began after 25 d and reached levels from 20 to 42 % biodegradation (deviation between replicates). After 42 d total biodegradation of 55 to 65 % degradation was observed, leading to the classification “inherently biodegradable with pre-adaptation”.

Tab. 6: Structural formula and results of imidazolium-based ionic liquid cations (alkyl side chains) obtained from primary and BOD experiments. The data from this study are complemented with data from the literature.

	R	Anion	Substance identifier	Classification OECD (...biodegradable) and biotic hydrolysis	Primary Biodegradation in % (28 d)	Biological Oxygen Demand in % (28 d)	Reference
	 -CH_2CH_3	Cl	IM12 Cl	Not readily	0	-	Stolte et al. 2008
	 -$CH_2CH_2CH_3$	PF_6	IM13 PF6	Not readily	-	2	This study
	 -$CH_2CH_2CH_2CH_3$	Cl	IM14 Cl	Not readily	0	-	Stolte et al., 2008 Wells & Coombe, 2006 Romero et al., 2008 Garcia et al., 2005 Merck MSDS, 2010
	 -$CH_2CH_2CH_2CH_2CH_3$	PF_6	IM15 PF6	Not readily	-	0	This study
	 -$CH_2(CH_2)_7CH_3$	Cl	IM19 Cl	Not readily	-	0	This study

3 Piperidinium compounds

Among the piperidinium compounds tested only Pip13OH Cl and Pip12OH Cl were fully mineralised (Tab. 3).

Pip13OH Cl was degraded up to 77 % after 28 d. The 60 % pass level was exceeded, but not within a ten day window. The compound was thus classified as "inherently, ultimately biodegradable" rather than "readily biodegradable". The biodegradation of Pip12OH Cl did not exceed the 60 % pass level after 2 8 d, but after a prolongation of the test duration to 60 d more than 80 % degradation were observed, leading to the classification "inherently biodegradable with pre-adaptation".

4 Pyridinium compounds

The N-alkylpyridinium cations (Py2, Py3, Py4 and Py5) and the ether-containing compound (Py1O2 Cl) did not exhibit significant levels of biodegradation, whereas the hydroxylated compounds Py2OH and Py3OH showed good biodegradation rates of 58-65 % and 51 %, permitting classifications of "readily biodegradable" and "inherently biodegradable", respectively (Tab. 4).

5 Imidazolium compounds

The corresponding imidazolium-based ILs with similar side chains as used for the experiments described above have already been examined by different working groups (Tab. 5). In this study the biodegradability imidazolium cations with odd-numbered N-alkyl substituents (propyl, pentyl, nonyl) was investigated, but no biodegradation was observed for any of these compounds (Tab. 6).

6 Degradation of the cyanomethyl side chain

Regardless of the head group, compounds with a cyanomethyl side chain (Pyr11CN Cl, Mor11CN Cl, Pip11CN Cl, Py1CN Cl, and IM11CN Cl) exhibited complete primary degradation within 28 d, but no oxygen consumption in BOD measurements. LC-MS analysis of the 28 d samples revealed the occurrence of several mass-to-charge ratios suggesting the hydrolysis of the CN group. The corresponding carboxamide ($m/z^+ = 159$) and carboxylic acid detected as sodium ($m/z^+ = 182$) and potassium adducts ($m/z^+ = 198$) were observed in the mass spectrum (Fig. 2).
The methylated cation R+-CH2-C(=O)O-CH3 (Fig. 2) is based on an esterification reaction of the free acid and with the methanol solvent under analytical conditions (pH 3.5). The same detection pattern was observed for all analogous IL cations: Py1CN, Pyr11CN, Mor11CN, IM11CN and Pip11CN. None of the compounds in the abiotic samples was degraded, indicating that microorganisms are involved in the cleavage of the cyano group. In the case of Pyr11CN the corresponding acid (Pyr11COOH) formed was the same transformation product as that obtained in the experiments with Pyr11COO2. Pyr11COOH was found to be fully mineralised in a prolonged biodegradation test.

Discussion

Structure-biodegradability relationships

Most of the degradable compounds are among the pyrrolidinium cations. When substituted with hydroxyl-containing side chains and when enough time is allowed, full mineralisation (side chain and core) takes place. The biodegradability of 1-alkyl-1-methyl pyrrolidinium compounds increased with lengthening side chain, changing from "not readily biodegradable" (ethyl) to "inherently biodegradable with pre-adaptation" (butyl) and "readily biodegradable" (octyl). A similar trend has been reported for 1-alkyl-3-methylimidazolium and 1-alkyl-3methyl-pyridinium-based cations.[32,33] Unfortunately, the influence of the side chain could not be investigated for the other non-aromatic head groups, since the compounds with an octyl side chain were unavailable.

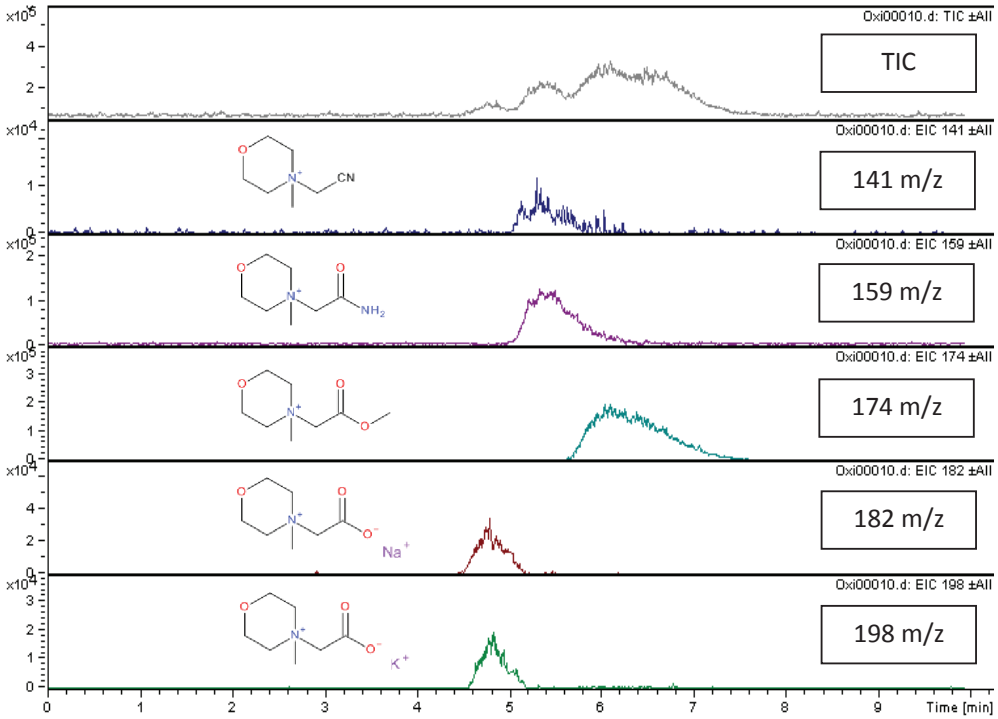


Fig. 2: The total ion current TIC over time from LC-MS measurements of the 28 d sample from the BOD biodegradation experiment on Mor11CN Cl. The extracted ions presumably belong to the molecular structures shown.

Good biodegradation rates of IL compounds with substituted hydroxyl side chains were not only obtained for pyrrolidinium cations, but also for morpholinium, piperidinium and pyridinium ones. Several of these compounds were fully mineralised, especially when a hydroxypropyl or even a hydroxyethyl residue was attached. However, the hydroxyethyl side chain formed only in the case of pyridinium a readily biodegradable IL cation and in contrast, in the case of morpholinium a non-biodegradable compound. The improved biodegradability of compounds containing alcohol groups – a potential target of enzymatic degradation – has been reported for several other substance classes[26] for instance, imidazolium[29] and ammonium-based ILs.[51] Apart from the above-mentioned hydroxylated compounds, none of the other investigated morpholinium, piperidinium and pyridinium compounds were mineralised. In comparison with the other head groups the imidazolium group exhibited the lowest biodegradation potential. None of the functionalised compounds was degraded and only IM18 Cl was primarily degraded, but the core proved to be recalcitrant towards biodegradation and only the side chain was degraded via β-oxidation, yielding 1-(3-carboxyethyl)-3-methyl-imidazolium.[33]

In this study several odd-numbered alkyl side chains were investigated to check whether β-oxidation then leads to products such as 1-(3-carboxypropyl)-3-methyl-imidazolium that might be further degraded (full mineralisation of the ring). This hypothesis was not confirmed and none of the propyl, pentyl and nonyl compounds were biodegraded.

With respect to the side chains a microorganism enabled primary degradation of the cyanomethyl side chain, and the identified transformation products suggest enzymatic hydrolysis via nitrile degrading enzymes such as nitrilases or nitrile hydratases together with amidase. These enzymes are commonly applied as catalysts in organic synthesis for the hydrolysis of nitrile groups in the pharmaceutical industry and for bioremediation purposes, amongst others.[52–55] Recently, IL anions such as $N(CN)_2^-$, $C(CN)_3^-$ and $B(CN)_4^-$ were hydrolysed to their corresponding amides with isolated nitrile hydratase.[56]

Variability and adaptation time

The data presented in this study show some inconsistencies in comparison with the literature data, and in some cases there were evident discrepancies between our independently performed experiments (e.g. Pyr12OH I and Pyr13OH Cl). This does not imply that our data have no validity or reliability, but that such measurements have a restricted reproducibility based on biological variability. In particular, the nature of the inoculum in terms of spatial and temporal variations is a very variable factor in the assessment of biodegradability.[57] Hence, during every experiment and within each test vessel a unique microorganism community is present, which may result in completely different degradation results. For example, we found no primary degradation of the IM11CN cation in our previous study,[33] but this did occur in the present study. Moreover, primary biodegradation of the IM19 cation was expected, since IM18[33] and IM1-10[58] are degradable. Here it remains unclear whether the different results are due to the molecular structure (odd-numbered side chain) or to the microbial community involved. The same holds true for observations made for pyridinium compounds. In studies performed independently by Docherty et al.[59] and Pham et al.,[60] vastly different biodegradation rates and different degradation pathways were found for one IL cation (N-butyl-3-methyl-pyridinium). In the first study there was no biodegradation within 43 d,[32] but after re-examination[59] by the same group, mineralisation was nearly complete (88 % within 41 d); this

was corroborated in primary biodegradation studies by Pham et al.[60] In the present study N-butylpyridinium, for example, was investigated and no biodegradation was observed, but whether a different substitution pattern of the pyridinium core or the microorganisms were responsible for degradation/recalcitrance is a question that remains unanswered. The results obtained for Pyr11COO2 suggest that the microbial composition changes during the test (Fig. 1). In the first step the ester is degraded enzymatically, after which there is a long lag phase of around 20 d, followed in the second step by additional exponential growth and another lag phase. It can be assumed that other microorganisms or enzymes are involved in the degradation of the side chain and the subsequent decomposition of the core. Such a symbiosis of different microorganisms from a test community has been described for the biodegradation of aliphatic and aromatic hydrocarbons.[61] As a consequence of different substrate availabilities, the subsequent activation of enzymes resulting in the diauxic growth of microorganisms has been successfully modeled for different sugars and organic anions.[62]

For several compounds (Pyr11COO2 Br, Pyr12OH I, Pyr14 Br, Pip12OH Cl, Mor13OH Cl) the prolonged test duration leads to an increased biodegradation rate. Long-term exposure of microorganisms to ILs is presumably associated with adaptation processes, such as the induction of specific enzymes, genetic mutation or horizontal gene transfer, which enhance the degradative capacity of the entire community or change the population in terms of the selective growth of certain strains.[63] Such long-term adaptation processes and an increased biodegradation potential (including the complete degradation of the imidazolium ring) were recently observed for IM18, too.[64] Moreover, cultures of axenic bacterial strains, such as *Corynebacterium* sp. and *Sphingomonas paucimobilis*, or isolated enzymes have successfully enhanced the biodegradability of certain IL cations[41,42] or IL anions[56], and a wide range of IL structures can be generally targeted for microbial degradation.

Conclusion

Comparison of the biodegradation potential shows that for all the five head groups investigated compounds can be found that are readily or inherently biodegradable. It is highly likely that chemicals classified as "readily biodegradable" are biodegradable in the environment with just a low risk of persistence, or even none at all. This cannot generally be assumed for inherently biodegradable chemicals, although they do have a reduced risk of being persistent. From the structural design point of view, both the type of head group as well as the substituted side chain influence the degradability of the IL cation. When the same side chain is substituted, pyrrolidinium and pyridinium cores generally display better degradation rates in comparison to piperidinium and morpholinium cations. Imidazolium appears to be the most refractory head group. The general ability of microorganisms to adapt to IL cations again reduces the risk of persistence. The degree of mineralisation, however, also seems to depend strongly on the inoculum and the test/environmental conditions. The underlying science behind IL biodegradation, namely the microbiological processes and the microorganisms involved, is not yet well understood, so detailed investigations are needed. The environmental risk of poorly degradable ILs can be eliminated when these are used in closed systems or can be reduced when residues are removed from waste water by separation techniques such as nanofiltration[65] or advanced oxidation processes.[66,33]

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4 Results, recapitulatory discussion and perspectives

Over the years, the evaluation of ILs in respect to their environmental impact became more and more differentiated within the scientific world. From the generalised declaration of ILs being "green solvents", it has been evident that not all ILs are green and not all are toxic.[37,80] A contribution to the question, "what makes ILs degradable from an environmental and structural chemical point of view?" was supposed to be made by the presented studies. It is assumed that the knowledge of the degradation conditions and of chemical structures, which are easily accessible to biological attack and hydrolytical cleavage, could enable chemists to design ILs that do not persist and accumulate in the environment.

The hypotheses for these presented studies were tested by examination of around 50 ILs in more than six years of accumulated (bio)degradation studies and several thousand analytical measurements. In the following paragraph, the results are summarised according to the formulated hypotheses and discussed with respect to future research purposes.

4.1 Exploring the degradability of ionic liquids from an environmental and structural point of view

4.1.1 Environmental condition – presence of oxygen in the environment

"ILs are potentially biodegraded when the environmental conditions are changed from oxic to anoxic with nitrate as terminal electron acceptor for anaerobic respiration and the concentration of the ILs used in the anaerobic biodegradation studies does not inhibit the microbial performance."

Nine IL cations, which had already been examined under aerobic conditions, have been investigated anaerobically to test the hypothesis of the influence of molecular oxygen within the environment on the biodegradation process (test kit no. 1, Fig. 7). Five IL anions that had not yet been investigated regarding the biodegradation potential (test kit no. 2, Fig. 8) have additionally been tested under aerobic and anaerobic conditions. The presented hypothesis could not be verified for the complete set of selected IL cations and anions. In this paragraph I will discuss the results of the biodegradation test of the IL cations. The results of the degradation tests for the IL anions are further discussed, separately in paragraph 4.1.2.

Looking at the IL cations, almost none of them were primarily biodegraded under the experimental anaerobic conditions, except the imidazolium IL that contained a hydroxylated octyl side chain (IM18OH Br). The detected metabolites that were generated during the biological degradation process of IM18OH Br gave rise to the assumption that the side chain of the cationic

head group was biodegraded by β-oxidation as it had previously been suggested for the same cation under aerobic conditions.[55] In the same way, the unsubstituted octyl side chain of the imidazolium cation (IM18) can be oxidised under aerobic conditions in which the reaction is initialised by monooxygenase (ω-oxidation) that uses molecular oxygen. However, under denitrifying conditions, no molecular oxygen is present and the unsubstituted octyl side chain of IM18 Cl could not be biodegraded. It is suggested that without oxygen the monooxygenase cannot catalyse the hydroxylation of the side chain for further β-oxidation and an alternative initiating mechanism, such as fumarate addition,[81] was not detected in the conducted studies under the experimental conditions.

These have been the first and, up until now, the only studies on the biodegradation of ILs under denitrifying conditions. The expectation that the change of the environmental condition with respect to the oxygen availability leads to an enhanced biodegradation could not be fulfilled. However, the presented studies could provide the first illumination regarding the fate of ILs in denitrification processes in which the lack of oxygen can reduce the biodegradation potential of the tested IL cations with n-alkyl side chains. Whether or not this is also applicable beyond the experimental conditions in wastewater treatment or within the environment, and if there are other microorganisms that can degrade the tested IL cations, even under obligate anaerobic conditions and independently of the presence of oxygen, needs further investigation.

4.1.2 IL anions – cyano-based and fluoroorganic anions

4.1.2.1 Biodegradability under aerobic and anaerobic conditions

"Cyano-based anions are better biodegradable than fluoroorganic ones due to their easier accessible carbon atom."

In addition to the IL cations previously mentioned, IL anions that are potentially technologically relevant have been investigated on their biodegradability under aerobic and anaerobic conditions. All of the five fluoroorganic and cyano-based IL anions (test kit no. 2, Fig. 8) were shown to remain biologically stable under the experimental conditions. The expectation that cyano-based anions could serve as a biodegradable alternative to the highly stable fluoroorganic ones could not be met. A complete inhibition of the growth of bacteria by the examined IL anions can be excluded. Since molecules that contain cyano-groups are known to be degraded by microorganisms,[82] it is anticipated that these microorganisms were not present in the used inoculum. For the fluoroorganic anions, such a microbial reason for the non-biodegradability is also imaginable, but far less likely, because of "the high strength of the C–F bond and the supposed innocuousness of fluorinated chemicals" and because furthermore most defluorinating microbial processes take place in fluorinated aromatic compounds.[83] Therefore, microorganisms known for their ability to

degrade cyano-compounds were selected and investigated regarding their biodegrading potential towards the cyano-based anions. This led to the second part of the hypothesis.

4.1.2.2 Biodegradation potential of cyano-based anions using cyanide-degrading bacteria

"The cyano-based IL anions are biodegradable by a change in the bacteria consortia from activated sludge to a pure culture of cyanide-degrading bacteria mixture Cupriavidus spp."

An axenic culture of the cyanide-degrading bacteria *Cupriavidus spp.* was used as potential microbial degrader for cyano-based IL anions. *Cupriavidus spp.* had previously been observed to degrade one of the most stable cyano-complexes, Prussian blue.[84] Such a microbiological approach is less close to the conditions in a wastewater treatment plant than using a mixed bacterial culture from activated sludge, but it is useful for the identification of potential bacteria for bioremediation processes in the case of an accidental release of the chemicals of concern into the environment.[85]

Against the expectations, the biodegradation of the three cyano-based IL anions $N(CN)_2^-$, $C(CN)_3^-$ and $B(CN)_4^-$ was not observed. Whether or not the chemical structure of the cyano-based IL anions in general or the degradation process within the *Cupriavidus spp.* bacteria strains was not applicable for microbial attack of the tested anions is unknown. However, looking at the degradation of Prussian blue, it is probable that the degradation process within the *Cupriavidus spp.* bacteria strains does not fit to the cyano-based anions. In the biodegradation of Prussian blue, siderophores initiate the enzymatic degradation by ligating the iron of the metal cyano complex. The released hydrogen cyanide (HCN) is further degraded by cyanidase.[84] However, such a metal-drawing mechanism is not applicable in the covalently bonded IL anions with N, C and B as central atoms. A similar mechanism for these non-metallic central atoms does not seem to exist in *Cupriavidus spp.* and cyanidase did not catalyse the degradation of the covalently bonded nitrile groups. Biotic degradation processes are therefore either not applicable or not yet found for the cyano-based anions. Abiotic processes, such as hydrolysis or oxidation processes, can now be investigated further. Among those, the hydrolytical reaction could be of concern if HCN was released and increased the toxicity of the IL anions in the environment and technical application.

4.1.2.3 Hydrolytical stability

"The cyano-based anions may be degraded hydrolytically in the environment with a potential release of hydrogen cyanide."

Since the first assumptions regarding the potential biodegradability of the cyano-based anions could not be verified either with activated sludge bacteria or using an axenic culture of

Cupriavidus spp., the hydrolytical stability of the anions and the potential release of HCN were further investigated. The same process has been conducted for the previously examined non-biodegradable and presumably hydrolytical stable fluoroorganic anions to exclude a release of HF from these compounds.

The abiotic degradation study on the cyano-based and fluoroorganic anions confirmed the stable state of all tested anions under environmentally relevant aqueous conditions (pH 7-9). However, for $N(CN)_2^-$ and $C(CN)_3^-$ the hypothesis regarding the hydrolytical degradation potential could partly be confirmed. Under strong acidic and alkaline conditions, they did undergo hydrolytical reaction processes. The formation of HCN as an environmental concern was not observed, but the formation of other relevant substances instead.

The hydrolysis products were identified and came from a two-step reaction: first, the transformation of the nitrile group into the corresponding amide, and second, the further formation of a carboxylic acid from the amide intermediate. A complete hydrolysis of $N(CN)_2^-$ and $C(CN)_3^-$ could finally lead to their complete mineralisation into ammonium and carbon dioxide, and methane and carbon dioxide, respectively. In the presented studies, however, the first transformation products were the most prominent ones, since they could still be detected after the first few days. Those detected amides of the first hydrolytical step were 1-cyanoisourea ($N(CN)(CONH_2^-)$) and 2,2-dicyanoacetamide ($C(CN)_2(CONH_2^-)$) in the hydrolysed solution of $N(CN)_2^-$ and $C(CN)_3^-$, respectively. As pure substances they were similarly or less toxic than their parent compounds towards a rat leukaemia cell line (IPC-81). Further hydrolysis of the amides then lead to the release of ammonium and carbon dioxide and the intermediates cyanamide $NH_2(CN)$ and malononitrile $CH_2(CN)_2$, respectively. However, the latter are declared as toxic, and malononitrile in particular is additionally dangerous for the environment.

Although no HCN could be detected, some of the transformation products are already classified as toxic to man and the environment and should be used with great care. For technical applications, strong acidic and basic conditions shall therefore be avoided in the case of $N(CN)_2^-$ and $C(CN)_3^-$, not only due to the operational safety criteria, but also to avoid corrosive effects within the technical process. $B(CN)_4^-$ remained stable. The high hydrolytical stability of all of the tested anions under neutral environmental relevant conditions is a major point of concern for the hazard assessment of these compounds since these compounds might be persistent in the environment.

From the latter study, we can see for the first time that there were pH conditions that change the structural composition of $N(CN)_2^-$ and $C(CN)_3^-$. With respect to the biodegradation potential, this may initialise further microbial action that was not yet tested. Furthermore, these extreme pH values are no standard environmental conditions. Under more common neutral pH values, nitrile-hydrolysing enzymes may catalyse the process.

4.1.2.4 *In vitro enzymatic hydrolysis*

"The hydrolysis of cyano-based IL anions can be catalysed enzymatically."

Since the abiotic hydrolysis of $N(CN)_2^-$ and $C(CN)_3^-$ was possible under harsh conditions, the question arose whether this abiotic degradation process could be enhanced in vitro by bacterial enzymes which catalyse hydrolytical reactions at neutral pH values. This would be especially important for future treatment procedures of cyano-based anion contaminated wastes. Therefore, the hydrolysis of the cyano-based anions was tested using nitrilase (NLase) and nitrile hydratase (NHase), respectively, as catalysts. In contrast to previous studies in which all of the cyano-based anions have shown to be stable under pH 7-9, in the presented studies, the hypothesis on the in vitro enzymatically-catalysed hydrolysis could be verified. All of the three tested IL anions could be hydrolysed into their corresponding amides by NHase within less than 22 h. The other tested enzyme NLase did not catalyse the reaction under the experimental conditions. Whether or not the different enzymatic mechanism or the test duration was not sufficient in the test with NLase is unknown by now.

The discovery of an enzyme for the transformation of cyano-based anions represents a good starting point for further research. Although the results showed that the hydrolytical transformation of the cyano-based anions by NHase was generally possible, it is not known to what extent they can be transformed if the experiment was extended to a longer run time. For example, the catalysis of other molecules that contain several nitrile groups, such as 2,6-pyridinedicarbonitrile, showed that the fastest transformation step catalysed by NHase towards the amide was the first.[86] Transformations of further cyano-groups were much slower and took several days (up to 118 h). Therefore, investigations into the kinetics of the hydrolytical process are still needed. In case the observations from the study on 2,6-pyridinedicarbonitrile were transferable to the cyano-based anions, a transformation even by NLase would then be possible when the experimental run time was extended. However, pure enzymes are relatively instable and are often more applicable in organic synthesis than wastewater treatment. An alternative may be cross-linked enzymes aggregates (CLEAs) that are made of immobilised and stabilised enzymes and are recycable for multiple uses.[87] The investigated NHase has been especially successfully prepared as CLEAs for "green nitrile hydration in industry".[88] The use of CLEAs of laccase is also currently under investigation for use as potential treatment procedure for endocrine disruptors - contaminated wastewater.[89] Another alternative to the pure enzymes used could be the direct use of bacteria that contain the necessary enzymes for nitrile hydrolysis, such as *Rhodococcus erythropolis*.[86] In general, bacteria could be advantageous to pure enzymes. They contain a series of different enzymes that can make amides not only from nitriles but also carboxylic acid from the produced amides by amidases, and even a complete mineralisation may be further realisable. Whether or not any of the mentioned possibilities are applicable for the treatment of wastewater that is contaminated with cyano-based anions will then need to be tested. Although the structural

composition of the cyano-based IL anions seems to be inaccessible for microbial attack by the microorganisms that were present in the used activated sludge samples, a possible enhanced biodegradation process may be possible with nitrile-hydrolysing bacteria.

However, readily biodegradable ILs are still to be preferred over poor biodegradable compounds when inherently safer chemicals shall be created. Therefore, a modification of the chemical structure of the anion itself may be considered. For example, the insertion of more easily attacked side groups as in $B(CO_2H)_4^-$ and $BH_2(CN_2)^-$ might help to enhance their biodegradation potential and can be subject for further investigations. The fluoroorganic anions are supposed to be of greater concern with respect to their potential persistency. They were not only biologically, but also hydrolytically, stable. If their persistency should be confirmed by further investigations, an appropriate waste management system and treatment process will be needed; in case their usage is necessary, since technical, economical and social aspects for their application predominant over the ecological disadvantage of being potentially persistent and more toxic towards aquatic organisms than cyano-based anions. Finally, although the general biological stability of the cyano-based anions is an advantage in technological application, the occurrence of enzymes that are able to hydrolyse the parent compound gives a new perspective on future IL contaminated water treatment.

4.1.3 Chemical structure – cationic head groups

"Different head groups of IL cations may lead to an enhanced biodegradation potential of ILs: from imidazolium and pyridinium to morpholinium, pyrrolidinium, phosphonium and piperidinium cationic head groups."

In the biodegradation studies presented above, IL cations and anions had been tested under different oxygen conditions with little to no effect on their biodegradation potential. In the study that is presented in the next paragraph, I finally observed a range of IL cations to be (readily) biodegradable under aerobic conditions.

In total, 32 different ILs that had not yet been tested systematically on the influence of their different IL head group components, namely pyrrolidinium, morpholinium, piperidinium, imidazolium and pyridinium were examined (test kit no. 3, Tab. 3). The previous focus on the side chain of the cationic head group was shifted towards the effect of these head groups on the overall biodegradability of the cation. As a main result, the change in IL cationic head group did change the biodegradability of the investigated IL cations as expected, but only in combination with a side chain that was suitable for microbial attack. For example, it had previously been observed that the imidazolium head group combined with a hydroxy propyl side chain was not primarily biodegradable (IM13OH Cl).[55] When the head group of the IL cation was changed to pyrrolidinium, pyridinium, piperidinium or morpholinium (Pyr13OH Cl, Py3OH Cl, Pip13OH Cl and Mor13OH Cl), it could not only be primarily biodegraded, but also fully mineralised. Among those,

Pyr13OH Cl was even biodegraded within the criteria for being classified as readily biodegradable. However, an improved biodegradation potential could not be observed for any cationic head group and side chain combination. When these head groups were combined with the butyl side chain, only the pyrrolidinium cation (Pyr14 Br) could be biodegraded. Although the best biodegradation potential and moreover a complete biodegradability was exhibited by the pyrrolidinium cations, the IL cation with a short n-alkyl side chain together with a pyrrolidinium head group (Pyr12 Cl) was still not biodegradable, which is also true for the corresponding pyridinium and imidazolium head groups (Py2 Cl, IM12 Cl).[55] The same low biodegradation potential was observed for any of the ether (R = 102, 201 or 202) containing cations with different head group combinations. With respect to the side chains a microorganism enabled primary degradation of the cyanomethyl side chain, and the identified transformation products suggest enzymatic hydrolysis via nitrile degrading enzymes such as nitrilases or nitrile hydratases together with amidase. In the case of Pyr11CN the corresponding acid (Pyr11COOH) formed was the same transformation product as that obtained in the experiments with Pyr11CO02. Pyr11COOH was further found to be fully mineralised in a prolonged biodegradation test. Whether the same was true for Pyr11CN needs to be checked under less stringent conditions. Although the pyrrolidinium, piperidinium and morpholinium head groups did not enhance the biodegradation potential of ILs in general, members were found among all of the tested IL head groups, including pyridinium and imidazolium ones, that were (primarily) biodegradable under the experimental conditions.

The most probably reason for such a huge difference in the biodegradation potential of different head group and side chain combinations is presumably connected to the chemical properties of the molecular structures themselves, but also to the microbial community that degrades the chemical compounds. The biodegradation of the IL cations mostly took place in two steps of side chain and head group degradation, which were often accompanied with longer lag phases (>20 d). Therefore, it is assumed that different bacteria or enzymes are involved. Long lag phases are usually observed when the microbial community needs some adaptation time, e.g. for the expression of specific enzymes.[90] From our studies on the IL anions, we have seen that the use of different enzymes can lead to an enhanced biodegradation potential. However, at the moment the unknown actual bacteria composition (the “black box”) of the activated sludge community used in the biological test system only gives limited reproducible data due to bacterial variability under the stringent conditions and limited information on the involved microorganisms. Hence, in the case of the IL cations, a pre-adaptation of the activated sludge and the examination of the microbial community may help to lighten up the biological processes involved in the test systems. The knowledge of different biodegradation potentials in different ecosystems, such as rivers, lakes, sediments/soils etc., is further crucial in respect to future exposure areas.[91] The combination of an appropriate eco-design on the chemical compounds and the composition of the degrading community shall then help to design inherently safer substances.

From this study, it is further evident that until now, the most popular IL cationic head group N-methylimidazolium has been the one with the poorest biodegradation potential of all of the examined IL cations. The observed resistance of the head group to biological attack fits well alongside former studies on its non-ionic precursor methylimidazole and its derivatives. In these, "all N-substituted imidazole derivatives were poorly biodegradable"[58] and "alkylated N-atoms inactivate biodegradation" in imidazole residues[92]. The degradation mechanism of imidazole is thought to be linked to the histidine degradation by uracanase which involves an electronic rearrangement of the imidazole ring, but until now only little evidence could be found for a degradation of the N-methylimidazolium derivatives.[93] The poor biodegradability of the N-methylimidazolium head groups implies an increased hazard potential to the environmental in terms of persistency. Therefore, the risk for exposure should to be kept at low levels by responsible use, recycling and treatment. With respect to the design of inherently safer chemicals, IL cationic structures that might serve as biodegradable alternatives for N-methylimidazolium could be found, but these still need to be investigated on their technological application potential.

4.1.4 Chemical structure – overview

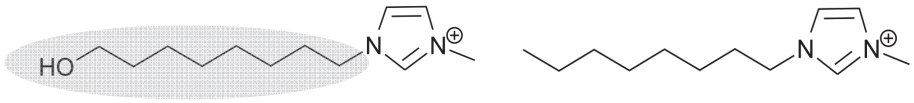
To get a better overview of the tested structures and observations, the observations made during the presented studies are summarised in Tab. 4.

Tab. 4: Observations made for the (bio)degradation of ionic liquids during the presented studies. The choice of side chains, cationic core structures and anions of ionic liquids influence the microbial degradability. Degradable structures are shaded with grey. Parameter: Ready biodegradability, measured via OECD guideline 301, unless stated otherwise

Observation	Biodegradability of ionic liquids	
	Detected	Not detected

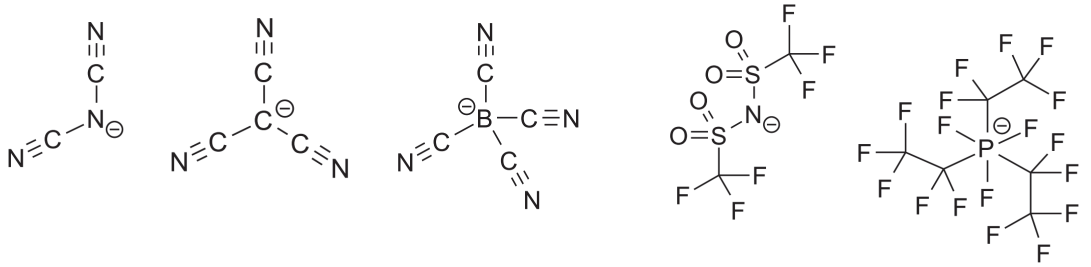
#1 – Preference of aerobic conditions – since 2010

"The presence of oxygen in the environment is essential for the biodegradation of IL alkyl side chains initialised by omega oxidation."[74]



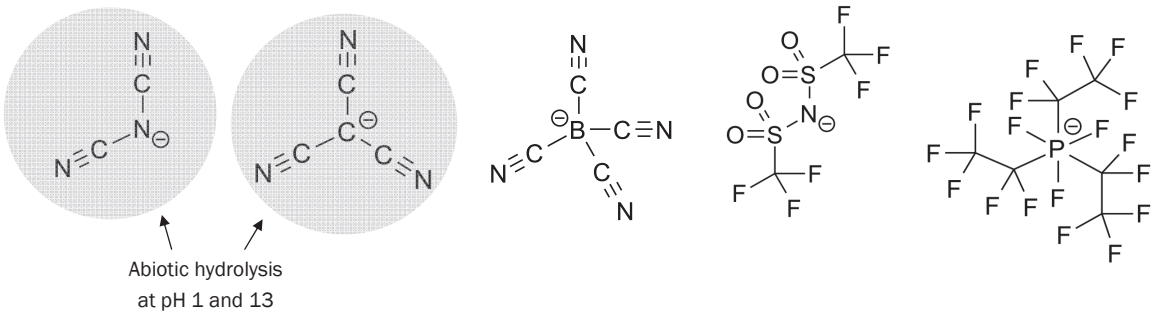
#2 – The biological stability of cyano-based and fluoroorganic anions – since 2012 & 2013

"The tested cyano-based and fluoroorganic anions are not biodegradable neither under aerobic nor under anaerobic conditions, neither by activated sludge nor by cyanide-degrading bacteria."[75,94]



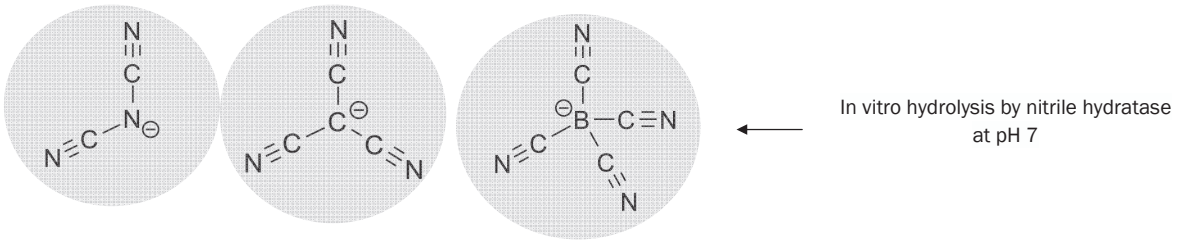
#3 – The hydrolytical stability of the anions – since 2012

"All investigated cyano-based and fluoroorganic anions are hydrolytically stable under most pH values. Under strong acidic or basic conditions $N(CN)_2^-$ and $C(CN)_3^-$ are hydrolysable without HCN production."[95]



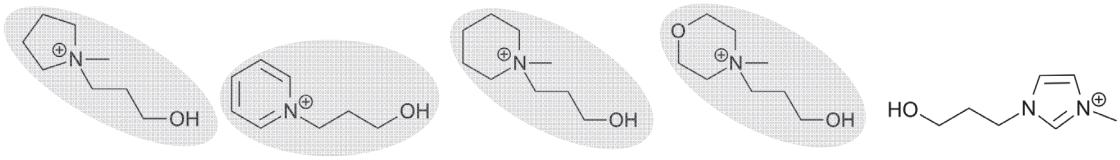
#4 – The enzymatic hydrolysis of cyano-based anions at pH 7 – since 2013

"Hydrolysis under neutral conditions canbe catalysed enzymatically in vitro by nitrilehydratase for all of the cyano-based anions."[95]



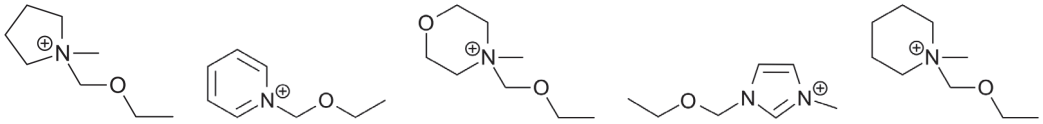
#5 – Biodegradable head group and side chain combinations – since 2013

"The investigated propyl hydroxy ILs with pyrrolidinium, pyridinium, morpholinium or piperidinium head groups are better biodegradable than the corresponding N-methylimidazolium ILs."[96]



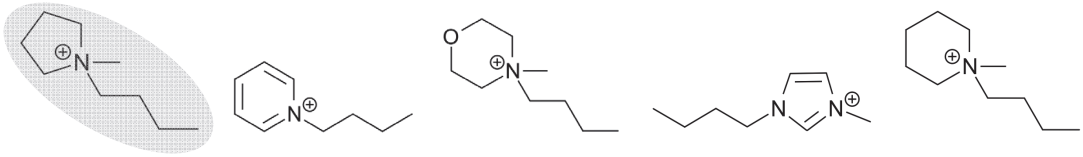
#6 – Non-biodegradable head group and side chain combinations – since 2013

"None of the head groups enhanced the biodegradability of the ether substituted IL cations."[96]



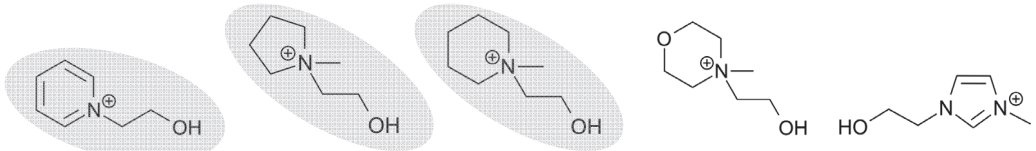
#7 – The pyrrolidinium preference – since 2013

"Among the investigated butyl substituted head groups, the pyrrolidinium one was the only biodegradable IL cation."[96]



#8 – The pyridinium preference – since 2013

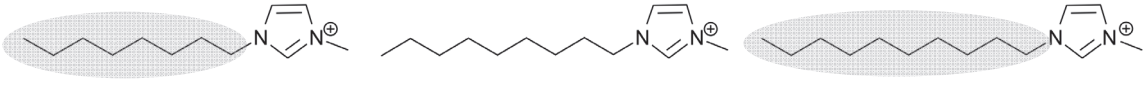
"Among the investigated ethyl hydroxy substituted head groups only the pyridinium head group could be readily biodegraded. The pyrrolidinium and piperidinium ones could also be completely mineralised, but only after prolonging the test duration to 60 d."[96]



After 28 d | After 60 d | After 60 d

#9 – The N-methylimidazolium stability – part II – since 2013

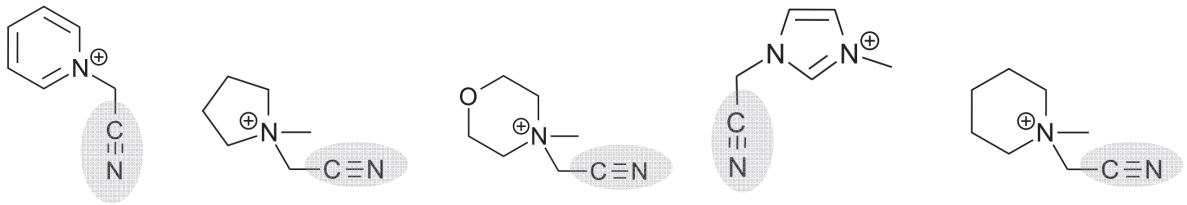
"Odd numbered side chains did not enhance the full mineralisation potential of N-methylimidazolium IL cations."[94]



Octyl | Nonyl | Decyl

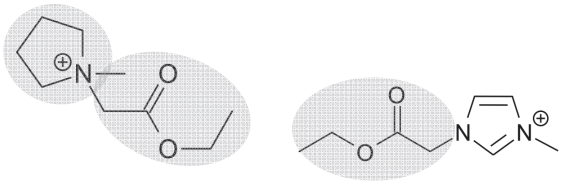
#10 – The primary degradation of cyano methyl side chains – since 2013

"Cyano methyl side chains are primarily degraded into carboxylic acids."[96]



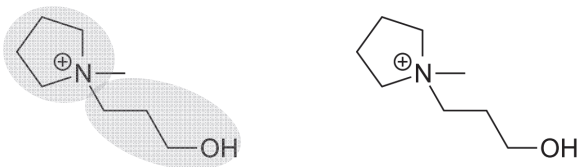
#11 – The full mineralisation of ethoxy carbonyl ILs – since 2013

"Ethoxy carbonyl side chains are primarily degraded into carboxylic acids. In combination with the pyrrolidinium head group it is fully mineralised."[53,96]



#12 – The black box in IL biodegradation studies

"Different biodegradation test runs may lead to different results due to biological variability of the inoculum and limited information on the biodegradation."[96]



100 % degradation within less than 20 d

0 % degradation within 28 d

4.2 Contribution to the structural design of ionic liquids and consequences for hazard assessment

Around ten years ago when the research on the biodegradability of ILs started, the stability of imidazolium ILs was one of the key properties for their technological application, but also one of the major concerns regarding their potential environmental persistency.[43] Over the years the knowledge of the biodegradation potential of ILs could then be developed simultaneously with the knowledge of their technological applicability, in contrast to the widespread application of DDT without consideration of the negative impact on the environment at first. Such a simultaneous approach has already been a success for the hazard assessment of chemicals and the presented studies were part of a series of biodegradation studies that were conducted all around the world. The question "what makes ILs degradable from an environmental and structural chemical point of view?" could be addressed for a range of ILs and for different environmental conditions. From these studies, it can be concluded that it is possible to lower the potential persistency by intelligent design of chemical structures that are easily accessible to biological attack and hydrolytical cleavage, and by providing the appropriate degradation conditions. The technological applicability of the biodegradable ILs as alternatives to more biologically stable ones now needs to be checked as suggested in the eco-design approach.[38] The knowledge of the environmental conditions and structures hopefully enables chemists to design ILs that do not persist and accumulate in the environment. That, in turn, would provide an opportunity for future development to generate less persistent substances.

As an example, I would like to look at one of the predominantly described ILs in scientific papers: IM14 BF_4. Although it has become very popular to study this as a green solvent, e.g. in selective separations,[97,98] enzyme stabilisation[99,100] and catalytic reactions,[101,102] with respect to its degradation potential, it is no inherently safe chemical. The cation is poorly biodegradable and the anion is hydrolytically instable, generating the toxic and corrosive acid HF. A hydrolytically stable alternative was found with fluoroorganic anions, such as $(CF_3SO_2)_2N^-$, which has now become a standard anion in IL research. However, although it has desirable effects on the physico-chemical properties of the IL for technical application, e.g. lowering the melting point and the viscosity, increasing its hydrophobicity and widening the electrochemical window,[103] this anion is only poorly biodegradable under aerobic and anaerobic conditions.[75] $B(CN)_4^-$ may therefore be used as technological alternative, since it has a similar effect to the physico-chemical properties of the IL for technological application.[104–106] Although $B(CN)_4^-$ was also biologically stable under the experimental conditions,[75] I found it to be enzymatically hydrolysed, which may lead to an enhanced biodegradability when using the appropriate microbial degraders. The poorly biodegradable cation IM14 may be substituted with Pyr13OH, which is readily biodegradable under aerobic conditions (Tab. 5).

Tab. 5: Examples of the design potential of ionic liquids. The choice of side chains, cationic core structures and anions influences the degradability of the ILs.

Cations \ Anions	BF_4^-	$(CF_3SO_2)_2N^-$	$B(CN)_4^-$
IM14	C^+ : Poorly biodegradable A^- : Hydrolytically instable	C^+ : Poorly biodegradable A^- : Hydrolytically stable and poorly biodegrad.	C^+ : Poorly biodegradable A^- : Hydrolytically stable, but potentially biodegrad.
Pyr13OH	C^+ : Readily biodegrade. A^- : Hydrolytically instable	C^+ : Readily biodegrad. A^- : Hydrolytically stable and poorly biodegrad.	C^+ : Readily biodegrad. A^- : Hydrolytically stable, but potentially biodegrad.

Looking at the cation and anion degradability individually can give a first hint regarding the overall degradability of the IL. However, how far the combination of cation and anion increases or decreases the degradability of the overall IL in microbial communities is still to be investigated. Additionally, one shall keep in mind that the biodegradability of a substance is no absolute material property, but rather is influenced by many different factors that are not only related to the structural composition of the molecule, e.g. the environmental conditions and presence or absence of the IL-degrading bacteria/enzymes may still lead to an enhanced or reduced degradation potential on the contaminated site.

With respect to future restrictions of IM14 BF_4 in the course of REACH and the evaluation of its greenness according to the Twelve Principles of Green Chemistry, the unfavourable (bio)degradation potential is not the only criterion for exclusion from the global market. The degradation potential is one important aspect in a set of factors that can lead to a more sustainable process. The overall evaluation of sustainability can only be made when considering further important parameters, e.g. toxicity, applicability, recyclability and waste production and in general the ecological, economical and social impact.

The presented studies have also shown that the sole investigation of the structural composition of the chemicals to predict the biodegradation potential may exclude a false categorisation as readily biodegradable, but cannot be used to classify the chemical as not biodegradable at all. The biodegradability of the ILs was largely dependent on the microbial composition of the inoculum. Therefore, in addition to the structural investigations, microbial and environmental

condition investigations should help to gain greater understanding of the (bio)degradation potential of ILs for a sound hazard assessment.

4.3 Need for Research

Since the beginning of the presented studies in 2008, the overall amount of biodegradation studies on ILs has been doubled and could already have been reviewed several times.[37,107–109] One could think that now the need for further research is low. However, for some commercially available ILs, e.g. guanidinium and isouronium/thiouronium based IL cations, biodegradation data is still missing. Furthermore, mixing effects of different cations and anions might also exhibit a different degradation potential than the single investigated IL ion combined only with a halide or an alkali metal ion and has not yet been investigated.

With respect to the usage of degradable ILs, we are still at the beginning of a huge amount of work. At the moment, most of the applied technologically relevant ILs, such as the short-chained imidazolium based IL cations and fluoroorganic anions, are biologically and hydrolytically stable solvents for technical applications and of concern for environmental persistency. We now need chemists who imply the results on the design for degradation in ILs in their daily work of synthesis and find technologically relevant application processes for biodegradable ILs. For instance, $B(COOH)_4^-$ could already be designed as a potential biodegradable alternative to $B(CN)_4^-$.

With respect to the hazard assessment of PBT or vPvB properties of ILs, some more experiments will be needed before REACH may consider an IL as substance of very high concern. In terms of persistency, readily biodegradable ILs will need no further investigations on their biodegradation potential. ILs that are, “not readily biodegradable” are not automatically classified as “persistent”. Further biodegradation tests need to be conducted: biodegradation tests under less stringent conditions (OECD guideline no. 302) and simulation tests of aerobic sewage treatment (OECD guideline no. 303) may enhance the biodegradation potential of poorly biodegradable ILs towards being inherently biodegradable. In the case that the IL of interest also fails to be biodegradable under less stringent test conditions and is not accessible to any abiotic transformation process, the IL has a high potential for long-term stability within the environment. It then fulfils one of the criteria to be classified as SVHC. Further criteria, such as the toxicity of the IL and the bioaccumulation potential will also need to be assessed before restrictions will be made. In addition, the potential exposure and biodegradation behaviour of ILs in situ, meaning towards different environments, e.g. rivers, lakes, soils or marine systems, should be determined when widely applied. Adequate use in closed operation systems and an adequate waste management system including recycling strategies and treatment of contaminated waste and wastewater will maintain the risk for exposure at a low level.

Concerning the assessment of biodegradation data in the presented studies, the reproducibility of the biodegradation data was lower when the IL was not readily biodegradable. According to REACH, the experimental run time may then be prolonged or the test volume/inoculum concentration may be increased to enable reliable assessment of biodegradation data. The theoretical reasoning behind such a measure is to increase the biodiversity and potential for the presence of degraders for the chemicals of interest in the inoculum. Since, at the time of writing, the used mixtures of microorganisms are a “black box” of unknown potential degraders, the knowledge on the microorganisms, enzymes and involved transport ways of ILs through the microbial cell membrane may lead to a more directed design of ILs for the biodegradation in specific environments and biological treatment processes of interest.

One aspect that has not been explicitly addressed in the presented thesis, but posed a problem for the determination of (bio)degradability of some ILs, was that there were ILs that could not be tested on their (bio)degradation potential, either because it was not possible to detect them specifically in the biological matrix (N111OH, P666-14) or they were too toxic, e.g. IM1-10 Cl, in the concentration that was needed to be able to detect them in the biological test system like WTW Oxitop ®. A valid analytical method is yet to be found.

5 Conclusion

In the presented studies, the search for factors that influence the (bio)degradability of ILs was conducted. The change of environmental conditions in terms of the redox potential, the hydrolytical stability in relation to the pH value and its catalysis by different enzymes, the use of potentially degrading bacteria and the systematic investigation of the structural composition of the ILs have been considered. This approach finally led to the identification of potential (bio)degradation pathways for ILs that are or may be of high technological relevance as green solvents and catalysts, but also to the identification of poorly biodegradable chemical structures that are already in use in the scientific and industrial community. Although the results highlight the dilemma between the current use of ILs with an increased potential for persistency and their favourable technological applicability, such a simultaneous development of technological applications and data on the environmental impact, is a first success for the prevention of potentially severe damage to the environment. If the IL of concern possesses high PBT or vPvB properties and a low overall sustainability with respect to economical and social factors, we may exchange the IL with poor (bio)degradation potential by those with higher ones. In contrast to the use of DDT, we still have the chance to prevent a widespread use and negative impact on the environment and human health.

However, the determination of the overall sustainability of ILs will need a huge amount of work on systematic data acquisition and a knowhow in interdisciplinary thinking on many different levels from chemists, microbiologists, engineers, ecologists, toxicologists, economists and so on. In the presented studies, we benefitted from cooperation between chemists, microbiologists and the chemical industry to be able to conduct the experiment and to target the most relevant substances.

Around 50 years ago, Rachel Carson set the ball rolling for the prevention of negative effects on the ecological system and this ball is still rolling in times of climate change, sustainable energy provision and loss of environmental resources. Science and technology still play a major role in providing knowledge on the “identification, avoidance and control of environmental risks”[4] and the realisation of techniques for sustainable development. The knowledge and creative ability of mankind will hopefully be used “to meet the needs of present generations without compromising the ability of future generation to meet their own needs”[11] as long as the world keeps on turning, so that at the end of the day we can sing together with Louis Armstrong about our wonderful world:

“I hear babies cryin', I watch them grow, they'll learn much more, than I'll ever know, and I think to myself, what a wonderful world.”

Bob Thiele and George David Weiss

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Annex I

Method validation

A range of challenges arose when working with the chromatographic systems and the bacteria samples from the biodegradation tests. Inline filters that were used throughout the tubes in the IC easily block and needed to be changed frequently. The autosampler tubes needed to be covered with cup covers to prevent evaporation. The sample flow direction needed to be adjusted from a drawing mechanism to a pushing flow to minimize the occurrence of gas insight the tubes. The peristaltic pump on the autosampler and the suppressor module are further weak points in the processes. Regular maintenance can prevent the peristaltic tubes from being sticky and flat and not supportable anymore. The solvent for washing the injector needle was changed to a methanol water mixture (50:50), since unlike the inorganic ions that are usually measured with ion chromatographic systems, the organic ILs can adsorb to the PVC surface of the tubes that lead to the injector, the column and the detector. Furthermore unlike the inorganic ions, most of the more lipophilic IL cation and anions measured, needed an additional organic solvent concentration (here: acetonitrile) to reduce the retention time within the chromatographic column and make a short precise detection possible. Conventional cation and anion columns could still be used. Syringe filters could be used to minimise the bacteria load on the columns and the filter system. Facing such a huge amount of uncertainties the question for reliability of the measurements arose and an excel sheet for method validation and a script for a practical course on calibrating as part of a method validation have been established for use at our institute.

Excel work sheets – example: Pyr13OH Cl

The excel sheet can help to calculate relevant analytical quality parameters summarised on work sheet 5 “Values”. All other work sheets contain further information on method validation or the underlying calculation tables. On the following pages the major work sheets are shown with the example of Pyr13OH Cl for intra-day method validation.

The work sheets:

1st worksheet "info"

How to use this template

This validation template has been created on the bases of official guidelines from DIN Deutsches Institut für Normung e.V., Eurachem - A Focus for Analytical Chemistry in Europe, IUPAC International Union of Pure and Applied Chemistry and ICH International Conference on Harmonization.

More details on relevant literature and a detailed description of the practical execution of a calibration can be found in **"Leitfaden - Kalibration"** on our server under the folder "method validation". This folder also includes the guidelines, internet sources, scientific paper and more information relevant for an understanding of the topic.

If you already know what method validation is and you would like to use this template immediately, you best follow the order of the worksheets and look out for these cells in light blue: In those light blue cells you type in the information required and in every other cell the data will be automatically generated. In the main sheet **"Values"** all relevant information is summarised.

However, if the chosen working range was too large and needs correction, you need to delete the not usable concentration levels in all of the worksheets.

The worksheets

"SOP" shall include all relevant information on the operation procedure (type of samples, sample preparation, instrument parameter, data evaluation). In **"Definitions"** the main terms are explained.

Under **"purpose"** you can select the type of analytical test for which you would like to conduct the method validation and see which analytical parameters you will finally need to determine.

The worksheet **"Values"** is the main sheet. There you find an overview of all the parameters received from the validation of the calibration curve and further parameters from additional tests on the validity of the method. It is also the sheet where you can type in the data received from your calibration.

The data is calculated with the help of tables and equations in the **dark blue worksheets** at the end of this excel worksbook. These sheets usually do not need to be altered except in the case of working range correction.

In **"presentation"** you see how the results of your analysis of real samples must finally be presented.

The **light blue worksheets** "Intermediate values", "Recovery" and "Specificity" need additional measurements….

"Intermediate values" will contain the data for the determination of the intermediate precision that is calculated from measurements over a period of six days.

"Specificity" requires additional measurements for the selectivity of the method, which is in qualitative tests given by the correct classification of a substance and in quantitative analysis comprised in the recovery rate.
The determination of the **"Recovery"** rate is conducted by standard addition method in the sample matrix. The comparison between the fortified and unfortified sample with the added sample will give you

Main guidelines used

DIN Deutsches Institut für Normung e.V.	38 402 Teil 51	"Kalibrierung von Analyseverfahren etc."	1986
	32645	"Chemical analysis – Decision limit, etc."	2008
Eurachem - A Focus for Analytical Chemistry in Europe	"The Fitness for Purpose of Analytical Methods"		1998
IUPAC International Union of Pure and Applied Chemistry	"NOMENCLATURE FOR THE PRESENTATION OF RESULTS OF CHEMICAL ANALYSIS"		1994
ICH International Conference on Harmonization.	"VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY Q2(R1)"		2005

2nd worksheet "SOP"

Standard operation procedure

Stock solution	conc. wanted	weight wanted			actual weight	actual concentration
	in µM	in g/L	in g	in mL	in g	in g/L
	13913	2.5	0.25	in 100	0.25	2.5

Ion chromatography

Metrohm (Schweiz)
881 Compact IC pro
863 Compact Autosampler

Column	Metrosep C 4 50	6.1050.450	Serial-No. 1122.5017
Precolumn	Metrosep C 4 Guard	6.1050.500	Serial-No. 1041.0468
RP Filter	Metrosep RP 2 Guard	6.1011.030	Serial-No. 1031.0805

Eluent
5 mM HNO3 25 % Acetonitrile

3rd worksheet "Definitions"

Definitions

from: http://www.analytik.de/content/category/8/182/109/

Method validation

VALIDIERUNG
Validation
Validierung (validation) ist: - die Bestätigen aufgrund einer Untersuchung und durch Führung eines Nachweises, dass die besonderen Forderungen für einen speziellen, vorgesehenen Gebrauch erfüllt worden sind (ISO 9000 und ISO 17025). - Beweisführung in Übereinstimmung mit den Grundsätzen der Guten Herstellungspraxis, dass Verfahren, Prozesse, Geräte, Anlagen, Ausrüstungsgegenstände, Materialien, Arbeitsgänge oder Systeme tatsächlich zu den erwarteten Ergebnissen führen (Qualifizierung).

VALIDITÄT
Validity
Validität ist ein qualitatives Maß der Gültigkeit eines Resultats oder einer Studie.

VALIDIERUNGSBERICHT
Validitation report
Der Validierungsbericht (validation report) protokolliert die Durchführung der Validierung, die ermittelten Daten, eventuelle Besonderheiten und die Gesamtbeurteilung und beinhaltet die Freigabe des validierten Verfahrens durch die verantwortliche Person (Herstellungs- oder Kontrollleiter).

MESSEN
Measure
Ermittlung einer quantitativen Aussage über eine physikalische Größe mittels einer Messeinrichtung. Der Vorgang des Messens heißt Messung. Die gemessene Größe ist meistens eine Eigenschaft eines Messobjektes. Das Ergebnis (quantitative Aussage) heißt Messwert, der durch ein Produkt aus Zahlenwert und Einheit angegeben wird.

MESSGERÄT
Measuring device
Gerät, das allein oder in Verbindung mit anderen Einrichtungen für die Messung einer Messgröße vorgesehen ist.

ANALYSENPROBE
Analysis sample
Eine Analysenprobe (auch Messprobe) ist diejenige Probe, deren Gehalt an einem zu bestimmenden Stoff unmittelbar gemessen werden kann. Sie wird aus der (Labor)probe durch Aufarbeitung und ggf. Zusätze von Reagenzien erzeugt.

MESSERGEBNIS
Result of determination
Das Messergebnis (result of determination) ist der durch die Anwendung einer Messmethode festgestellte Merkmalswert.

MESSUNSICHERHEIT
Uncertainty of determination
Nach der klassischen Definition ist die Messunsicherheit (uncertainty of measurement) ein aus Messungen gewonnener Schätzbetrag zur Kennzeichnung eines Wertebereichs, innerhalb dessen der Bezugswert der Messgröße mit einer vorgegebenen Wahrscheinlichkeit liegt, wobei der Bezugswert je nach Festlegung oder Vereinbarung der wahre Wert, der richtige Wert oder der Erwartungswert sein kann. Nach dem internationalen Wörterbuch der Metrologie und dem GUM (Guide to the expression of Uncertainty in Measurement, Leitfaden zur Angabe der Unsicherheit beim Messen) ist sie ein dem Messergebnis zugeordneter Parameter, der die Streuung der Werte kennzeichnet, die vernünftigerweise der Messgröße zugeordnet werden können.

WAHRER WERT
True value

Der Wahre Wert (true value) ist der tatsächliche Merkmalswert, der sich ist in der Regel praktisch durch Messen (stetige Messwertverteilung) nicht ermitteln lässt und somit unbekannt ist. Selbst wenn die Grundgesamtheit zur Prüfung zur Verfügung stehen würde, ist es theoretisch nicht möglich, alle möglichen Werte zu messen. Die wahren Werte können nur angenähert werden.Bei diskreten Werteverteilungen können wahre Werte ermittelt werden wie z.B. durch Zählen der Anzahl der in einer Grundgesamtheit enthaltenen Elemente.

RICHTIGER WERT
Conventional true value

Der richtige Wert (conventional true value) ist ein anstelle des wahren Wertes akzeptierter Wert zu Vergleichszwecken, dessen Abweichung vom wahren Wert als vernachlässigbar betrachtet wird.

ERWARTUNGSWERT
Expected value

Der Erwartungswert (expectation, expected value) einer Zufallsvariablen ist der Wert, von dem man "erwartet", dass er sich bei einer häufigen Wiederholung des Experiments im Durchschnitt ergibt. Z.B. das arithmetische Mittel aus den Werten von unendlich vielen "Ziehungen" einer Zufallsvariablen. Er bestimmt die Lage einer Verteilung und ist vergleichbar mit dem empirischen arithmetischen Mittel einer Häufigkeitsverteilung. Das Gesetz der großen Zahlen sichert in vielen Fällen zu, dass der Stichprobenmittelwert bei wachsender Stichprobengröße gegen den Erwartungswert konvergiert, d.h., der berechnete Mittelwert xquer nähert sich dem wahren Wert μ (Mittelwert der Grundgesamtheit). Der aus (vielen) Messwerten berechnete Mittelwert ist die beste Schätzung für den Erwartungswert.

ABWEICHUNG
Deviation, error

Allgemein: Nichterfüllung einer Forderung, z.B. in einem Audit festgestellt Nichtkonformität (nonconformity). - Im metrologischen Sinn: Abweichung (deviation, Error) einer Messung vom wahren Wert, wobei zwischen zufälligen und systematischen Abweichungen unterschieden wird. Der ältere Begriff "Fehler" sollte nicht mehr verwendet werden, da er eine falsche Handhabung suggeriert. Fehler sind vermeidbar, Messabweichungen sind aber prinzipiell unvermeidbar.

MESSABWEICHUNG
Measuring deviation

Messabweichung ist ein bei einer Messung auftretende Differenz zum Erwartungswert, die auf zufällige oder systematische Fehlerursachen zurückgeht.

RELATIVE MESSUNSICHERHEIT
Relative uncertainty of measurement

Relative Messunsicherheit (relative uncertainty of measurement) ist die Messunsicherheit dividiert durch den Betrag des (berichtigten) Messergebnisses, sofern dieser Betrag verschieden von Null ist.

MEHRFACHINJEKTION
Multiple injections

Mehrfache Injektion einer messfertigen Lösung, so dass sich zwar mehrere Messwerte ergeben, die aber alle aus derselben Probenlösung stammen. Die resultierende Streuung ist also eine Streuung des Messvorganges, nicht aber des kompletten Verfahrens. Man spricht manchmal auch von unechten Mehrfachbestimmungen bzw. Gerätepräzision.

MEHRFACHBESTIMMUNG
Multiple determination

Mehrfache Bestimmung einer Komponente durch komplette Wiederholung des Analysengangs.

Values

KALIBRIERUNG
Calibration

Unter Kalibrierung versteht man - in der Analytik die Messung von Kalibrierlösungen, festen oder gasförmigen Standards mit als bekannt vorausgesetzten Gehalten und anschließendem Aufstellen der Kalibrierfunktion. Die Kalibrierlösungen können durch Aufstocken der Leerprobe mit dem gesuchten Bestandteil erzeugt werden. - Ermittlung der systematischen Messabweichung einer Messeinrichtung unter vorgegebenen Anwendungsbedingungen ohne verändernden Eingriff in die Messeinrichtung - Arbeitsgänge, durch die unter festgelegten Bedingungen die Beziehungen zwischen den durch ein Messgerät oder ein Messsystem angezeigten oder den sich aus einer Materialmessung ergebenden Werten und den entsprechenden bekannten Werten eines Referenzstandards bestimmt werden.

KALIBRIERFUNKTION
Calibration function

Die Kalibrierfunktion (calibration function) ist der funktionale Zusammenhang zwischen dem Erwartungswert einer Messgröße (z. B. Extinktion) und dem Gehalt (z. B. Massenkonzentration). Die Modelle der Kalibrierfunktion werden berechnet und durch statistische Tests betätigt. Die am häufigsten verwendete Kalibrierfunktion ist die Kalibriergerade. Bei bestimmten Analysengeräten und Prüfmethoden ist es empfehlenswert, mit Kalibrierfunktionen 2. und 3. Grades zu arbeiten, die zusätzlich quadratische oder kubische Glieder enthalten.

LEERPROBE

Die Leerprobe ist unter Idealbedingungen eine Probe, die den zu bestimmenden oder nachzuweisenden Bestandteil nicht enthält, sonst aber mit der Analysenprobe übereinstimmt. Beide Forderungen sind häufig nicht erfüllbar. Die Messung der Leerprobe (Probenmatrix ohne den Analyten) ergibt Messwerte, deren Dichtemittel als Leerwert bezeichnet wird. Bei einer symmetrischen Verteilung ist dies der arithmetische Mittelwert. Diesem Leerwert entspricht der Ordinatenabschnitt einer Kalibriergeraden. Der Leerwert kann also auch Null sein (leerwertfreies Verfahren, Proportionalität). Unter realen Bedingungen ist die Leerprobe eine Probe, die nur einen sehr geringen Gehalt des gesuchten Bestandteils aufweist und in der restlichen Zusammensetzung der Analysenprobe möglichst nahe kommt.

ARITHMETISCHES MITTEL
Mean value

Der arithmetische Mittelwert (mean value, xquer) ist die Summe aller Merkmalswerte dividiert durch die Anzahl der Merkmalswerte. Der Mittelwert fasst die Messergebnisse von Messreihen bzw. Analysenserien zusammen. Der Mittelwert ist der am häufigsten verwendete Lageparameter. Der aus den Messergebnissen berechnete Mittelwert ist ein Schätzwert für den wahren Wert μ (der Grundgesamtheit).

ORDINATENABSCHNITT
Axis intercept

Der Abschnitt auf der Ordinate (Ordinatenabschnitt, Achsenabschnitt), der gebildet wird zwischen dem Schnittpunkt einer Funktion bei dem Abszissenwert x = 0 und dem y-Wert y = 0. Dieser Abschnitt ist immer vorhanden, wenn die Funktion nicht durch den 0-Punkt geht. Dieser Abschnitt kann als Leerwert gedeutet werden, allerdings nur dann, wenn statistisch geprüft wurde, dass dieser Wert nicht mit 0 vereinbar ist. Der Vertrauensbereich darf den 0-Punkt nicht einschließen.

KALIBRIERPLAN
Calibration schedule

Aufstellung aller durchzuführenden Kalibriermaßnahmen (was, wann, wer, wo, wie)

KALIBRIERMATERIAL
Calibration material

Alle Chemikalien und Materialien, die zur Kalibration einer Prüfmethode dienen

KALIBRIEREN
Calibrate

Das Kalibrieren eines Systems ist die Ermittlung und Festlegung eines funktionalen Zusammenhangs zwischen einer zähl- bzw. messbaren Größe und einer bestimmenden Konzentration (Objekteigenschaft) aus Daten, die im allgemeinen mit zufälligen Abweichungen behaftet sind. (DIN 1319 Teil 1)

KALIBRIERKURVENVERFAHREN
Calibration curve method

Das Kalibrierkurvenverfahren (calibration curve method) dient zur Berechnung der Nachweis- und Bestimmungsgrenze und ist auf kalibrierfähige Verfahren anwendbar. Es ist in DIN 32645 beschrieben. Dazu wird eine Leerprobe mit möglichst 6 unterschiedlich konzentrierten Kalibrierlösungen aufgestockt. Die Abstände der Konzentrationen sollten möglichst gleich sein und die höchste Konzentration soll die vermutete Nachweisgrenze nicht um mehr als den Faktor 10 überschreiten. Aus der Messreihe wird zunächst auf Linearität geprüft und anschließend die Nachweis- und Bestimmungsgrenze berechnet. Das Kalibrierkurvenverfahren wird auch als indirektes Verfahren (oder indirekte Methode) bezeichnet.

NACHWEISGRENZE
Detection limit

Die Nachweisgrenze (detection limit) ist der kleinste, mit einer festzulegenden statistischen Aussagewahrscheinlichkeit erkennbare Gehalt eines Stoffes, der bei einmaliger Messung qualitativ nachgewiesen werden kann. Die Nachweisgrenze ist eine Entscheidungsgrenze für das Vorhandensein eines Bestandteils. An der Nachweisgrenze besteht eine Wahrscheinlichkeit von 50%, den Bestandteil zu finden bzw. nicht zu finden. Die Ermittlung kann über das Signal/Rausch-Verhältnis oder gemäß DIN 32645 nach dem Leerwert- oder dem Kalibrierkurvenverfahren erfolgen. Nach der ICH-Richtlinie kann die Nachweis- und Bestimmungsgrenze basierend auf der Standardabweichung des Signals und der Kalibrierfunktion (Steigung) ermittelt werden.

ERFASSUNGSGRENZE
Limit of identification

Die Erfassungsgrenze gibt den Mindestgehalt an, der mit hoher vorgegebener Wahrscheinlichkeit nachgewiesen werden kann. An der Erfassungsgrenze sind der Fehler 1. Art (alpha-Fehler) und der Fehler 2. Art (beta-Fehler) gleich groß. Die Erfassungsgrenze ist dann etwa doppelt so groß wie die Nachweisgrenze, aber kleiner als die Bestimmungsgrenze. Nach DIN 32645 darf als Qualitätsgarantie für den Höchstgehalt eines Bestandteils im untersuchten Stoff nur die Erfassungsgrenze herangezogen werden.

BESTIMMUNGSGRENZE
Limit of quantification

Die Bestimmungsgrenze (quantitation limit, determination limit) ist der kleinste, mit einer festzulegenden statistischen Wahrscheinlichkeit erkennbare Gehalt eines Stoffes, der bei einmaliger Analyse quantitativ nachgewiesen werden kann. Nach der ICH-Richtlinie kann die Nachweis- und Bestimmungsgrenze basierend auf der Standardabweichung des Signals und der Kalibrierfunktion (Steigung) ermittelt werden. Die Ermittlung kann über das Signal/Rausch-Verhältnis oder gemäß DIN 32645 nach dem Leerwert- oder dem Kalibrierkurvenverfahren erfolgen. Die Bestimmungsgrenze liegt naturgemäß über der Nachweisgrenze. Als Faustregel gilt: die Bestimmungsgrenze ist 3 mal die Nachweisgrenze.

FEHLER 1. ART
Alpha error - "Fehlalarm" - falsch positiv

Fehler 1. Art, Alpha-Fehler oder Alpha-Risiko ist da Risiko, mit dem ein statistische Hypothesentest auf einen Sachverhalt hindeutet, der in Wahrheit nicht vorhanden ist. Allerdings ist dieser Sachverhalt nicht erkennbar und somit auch nicht bewusst. Z.B. würde in einer Produktion ein Alarm wegen schlechter Qualität ausgelöst, obwohl keine Verschlechterung vorliegt (Fehlalarm). Das Risiko wird über alpha festgelegt. Ein alpha-Wert von 5% bedeutet dann, dass von 100 Entscheidungen auch 5 falsch sein können. In der Analytik wird eine Komponente "gefunden", obwohl sie abwesend ist (bei Nachweis- und Erfassungsgrenze).Siehe auch Fehler 2. Art

FEHLER 2. ART
Beta error - "Acceptance error" - falsch negativ

Fehler 2. Art, Beta-Fehler oder Beta-Risiko ist das Risiko, mit dem der statistische Hypothesentest auf keinen Sachverhalt hindeutet, obwohl in Wahrheit einer vorhanden ist. Allerdings ist dieser Sachverhalt nicht erkennbar und somit auch nicht bewusst. Z.B. würde in einer Produktion das Produkt als in Ordnung eingestuft, obwohl eine Verschlechterung vorliegt (unterlassener Alarm). In der Medizin: Eine tatsächlich vorhandene Krankheit wird nicht erkannt (falsch negativ). In der Analytik wird eine Komponente "nicht gefunden", obwohl sie enthalten ist (bei Nachweis- und Erfassungsgrenze).Siehe auch Fehler 1. Art

ALPHA
Alpha

a: Allgemein in der Statistik Symbol für die Irrtumswahrscheinlichkeit. Bei den statistischen Testmethoden Symbol für das Signifikanzniveau.

SIGNIFIKANZNIVEAU
Significance level

Testniveau zur Prüfung einer statistischen Hypothese. Das Signifikanzniveau (level of significance) alpha (Irrtumswahrscheinlichkeit) eines statistischen Tests bestimmt die maximale Eintrittswahrscheinlichkeit für das Eintreten eines Fehlers 1. Art (H0 wird verworfen, obwohl H0 wahr ist). Ein Signifikanzniveau von 5% würde bedeuten, dass im Durchschnitt von 100 Entscheidungen 5 falsch sind.Auf der Basis der vorgegebenen Signifikanzniveaus werden die Grenzen einer Shewhart-Karte berechnet.

BLINDWERT
Blind value

Der Blindwert ergibt sich aus dem Analysengang ohne Probe (also ohne Analyt und ohne Matrix). Beispiel: Reagentienblindwerte in der Photometrie. Nicht jedes Prüfverfahren ergibt einen Blindwert. Der Blindwert ist vom Leerwert zu unterscheiden.

ERGEBNISUNSICHERHEIT
Uncertainty

Die Ergebnisunsicherheit (uncertainty {of result}) ist ein Kennwert, der aus Messungen und/oder Schätzungen gewonnen und zusammen mit dem Messergebnis zur Kennzeichnung eines Wertebereiches für den wahren Wert der Messgröße dient. Die Ergebnisunsicherheit ist ein quantitatives Maß für den qualitativ zu verwendenden Begriff der Genauigkeit, der allgemein die Annäherung des Messergebnisses an den wahren Wert der Messgröße bezeichnet. Die Begriffe Messunsicherheit und Ergebnisunsicherheit sowie Unsicherheit werden häufig synonym verwendet.

ARBEITSKALIBRIERUNG
Working calibration

Die Arbeitskalibrierung (working calibration) sollte in regelmäßigen Abständen durchgeführt werden. Die Vorgehensweise zur Arbeitskalibrierung ist in der Prüfmethode oder in der Vorschrift zur Kalibrierung des Prüfgerätes zu beschreiben. Darin sind Häufigkeit und Mindestanzahl der Messwertpaare für die Arbeitskalibrierung festzulegen. Mindestanforderungen an eine Arbeitskalibrierung sind: 1 Kalibrierstützpunkt bei Proportionalität, 2 Kalibrierstützpunkte bei Linearität, 3 Kalibrierstützpunkte bei Kalibrierfunktion 2. Grades, 4 Kalibrierstützpunkte bei Kalibrierfunktion 3. Grades.

GRUNDKALIBRIERUNG
Basic calibration

Die Grundkalibrierung (basic calibration) dient zur einmaligen Festlegung der Kalibrierfunktion. Mindestanforderungen an eine Grundkalibrierung sind: 6 Kalibrierstützpunkte bei Proportionalität, 6 Kalibrierstützpunkte bei Linearität, 8 Kalibrierstützpunkte bei Kalibrierfunktion 2. Grades, 10 Kalibrierstützpunkte bei Kalibrierfunktion 3. Grades. Hinweise zur Durchführung der Grundkalibrierung: mehr Messpunkte bei unterschiedlichen Konzentrationen erhöhen die statistische Sicherheit, Mehrfachmessungen bei einer Konzentration sind zur Modellfestlegung weniger effektiv als die Erhöhung der Zahl der Messwerte, die Messwerte sollten gleichmäßig über den Arbeitsbereich verteilt sein, eine Häufung der Messwerte in einem Teil des Arbeitsbereiches wirkt sich ungünstig aus, Doppeleinspritzungen bei Chromatographen sind keine statistisch unabhängigen Werte, sie sind zu mitteln.

ARBEITSBEREICH
Working range

Der Arbeitsbereich (working range) wird definiert über den kleinsten und größten Abszissenwert bei der Grundkalibrierung. Vor Beginn der Messungen ist der vorläufige Arbeitsbereich der Methode festzulegen. Der vorläufige Arbeitsbereich wird nach Durchführung der Messungen und statistischen Tests entweder bestätigt oder eingeengt. Der Arbeitsbereich umfasst den mit akzeptabler Genauigkeit abgedeckten Konzentrationsbereich, an dessen Enden idealerweise Varianzenhomogenität nachweisbar ist.

PARAMETER
Parameter

Frei wählbarer, aber während der Anwendungsdauer festgehaltener Wert. Die charakteristischen Größe einer Grundgesamtheit wie Mittelwert oder Standardabweichung (die wahren aber unbekannten Werte) werden ebenfalls Parameter genannt. Parameter werden im Allgemeinen mit griechischen Buchstaben bezeichnet. Sie sind die Werte, die eigentlich bestimmt werden sollen, aber nur mit Hilfe der Stichproben durch „Kennwerte" abgeschätzt werden können.

EMPFINDLICHKEIT
Sensitivity

Die Steigung der Kalibrierfunktion wird als Empfindlichkeit (sensitivity) der Methode bezeichnet. Bei linearen Funktionen ist die Empfindlichkeit (= Steigung) konstant, bei Funktionen 2. oder 3. Grades ist sie abhängig vom gewählten Wert xi.

REGRESSION
Regression

Regression (regression) ist ein mathematische Methode zur Aufstellung einer funktionalen Beziehung zwischen empirisch gefundenen Wertepaaren. Der Regressionsgrad einer Kalibrierfunktion ist abhängig von der Präzision der Messdaten und vom Arbeitsbereich: je höher die Präzision der Messdaten, desto größer ist die Wahrscheinlichkeit für eine höhere Ordnung, je kleiner der Arbeitsbereich, desto wahrscheinlicher ist eine lineare Beziehung.

REGRESSIONSKOEFFIZIENT
Regression coefficient

Regressionskoeffizienten (regression coefficient) werden die Kennwerte der Regressionsgleichung, z.B. a, b bei y = a + bx, bezeichnet.

STANDARDABWEICHUNG
Standard deviation

Die Standardabweichung (standard deviation) ist die zweite, (positive) Wurzel der Varianz. Am häufigsten gebrauchtes Streuungsmaß. Bei der Berechnung der Standardabweichung ist zu unterscheiden zwischen einer Stichprobe und einer Grundgesamtheit.Siehe auch Varianz

RESTSTANDARDABWEICHUNG sy
Residual standard deviation

Die Reststandardabweichung (rest standard deviation) ist ein Maß für die Streuung der Messwerte um die Regressionskurve.

VERFAHRENSSTANDARDABWEICHUNG sx0
Process standard deviation

Aus der Reststandardabweichung und der Empfindlichkeit wird die Verfahrensstandardabweichung (standard deviation of the procedure) in der Mitte des Arbeitsbereichs berechnet. Die Verfahrensstandardabweichung ist ein Gütemaß für die Streuung einer Prüfmethode. Da sie Die Einheit der x-Achse besitzt, können mit ihr Methoden direkt verglichen werden. Aus ihr lassen sich auch Nachweis- und Bestimmungsgrenze abschätzen.

VERFAHRENSSTANDARDABWEICHUNG, RELATIVE
Coefficient of variation Vx0

Die relative Verfahrensstandardabweichung (coefficient of variation of the procedure) ergibt sich aus der Division der Verfahrensstandardabweichung durch die Mitte des Kalibrierbereichs mal 100 und wird in % angegeben. Mit der relativen Verfahrensstandardabweichung kann die Streuung der Prüfmethode in verschiedenen Labors oder von unterschiedlichen Prüfmethoden verglichen werden.

STANDARDABWEICHUNG, RELATIVE
Coefficient of Variation Vx0

Die relative Standardabweichung (relative standard deviation) ist die Standardabweichung bezogen auf den Mittelwert. Sie wird normalerweise in % angegeben und ist damit identisch mit dem Variationskoeffizienten VK.

Varianzkoeffizient VK
Variation coefficient Vx0

Der Variationskoeffizient VK ist identisch mit der relativen Standardabweichung. Er ist die Standardabweichung bezogen auf den Mittelwert und wird in % angegeben.

VERTRAUENSBEREICH
Confidence intervall

Der Vertrauensbereich (Vertrauensintervall, Konfidenzintervall, Konfidenzbereich) ist der Bereich, indem mit einer bestimmten Wahrscheinlichkeit der wahre Wert liegt. Aus Stichproben ermittelte Kennwerte sind grundsätzlich mit Unsicherheiten behaftet. Sie sind lediglich (wenn auch die besten) Schätzwerte für die unbekannten Parameter der Grundgesamtheit, denen das eigentliche Interesse gilt. Der Vorteil, die Messresultate mit Hilfe von Vertrauensintervallen anzugeben, liegt darin, dass die Verlässlichkeit der Resultate quantifiziert werden kann. Korrekterweise ist also immer die Kenngröße mit ihrem entsprechenden Vertrauensintervall zu einer definierten Wahrscheinlichkeit (z.B.95%) anzugeben. Diese Wahrscheinlichkeit wird auch statistische Sicherheit genannt.

ADDITIONAL MEASUREMENTS

SYSTEMATISCHE ABWEICHUNG
Systematic deviation

Systematische Abweichung (systematischer Fehler, systematic deviation, systematic error, Bias): Abweichung des unberichtigten Messergebnisses vom Erwartungswert. Systematische Messabweichungen treten einseitig auf, so dass sie durch Wiederholmessungen nicht erfassbar sind (Beispiele: Dejustierung des Messgerätes, Analytverluste bei Extraktion, Zersetzung einer Lösung). Es werden konstante und proportionale Messabweichungen unterschieden. Der Nachweis der Abwesenheit systematischer Messabweichungen ist unter dem Prüfpunkt Richtigkeit zu führen.

BIAS
Bias (Verzerrung)

Bias oder Verzerrung ist die systematische Abweichung einer Schätzfunktion vom Erwartungswert des gesuchten Parameters (systematischer Fehler).

RICHTIGKEIT
Trueness

Richtigkeit (trueness, accuracy {of the mean}) ist ein qualitativer Begriff und beschreibt das Ausmaß der Annäherung des Erwartungswertes an den richtigen Wert. Die Richtigkeit wird von systematischen Abweichungen beeinflusst. Man unterscheidet zwischen konstanten und proportionalen systematischen Abweichungen. Konstante systematische Abweichungen liegen vor, wenn die Abweichungen unabhängig von der Konzentration des zu bestimmenden Stoffes sind (z.B. Blindwerte). Liegt eine (proportionale) Abhängigkeit von der Konzentration vor, spricht man von proportionalen systematischen Abweichungen (z.B. Matrixeffekte). Als quantitatives Maß für die Richtigkeit kann die systematische

REFERENZMATERIAL
Reference material

Alle Chemikalien und Materialien, die Kalibration herangezogen werden. Wenn technisch und wirtschaftlich vertretbar, sollen Kalibriermaterial und Kontrollmaterial nicht identisch sein.

SPEZIFITÄT
Specificity

Spezifität (specifity) ist die Fähigkeit einer Analysenmethode, den Analyten ohne Störung durch in der Probe enthaltene Substanzen oder Matrixbestandteile zu bestimmen. Eine niedrige "Querempfindlichkeit" entspricht einer hohen Spezifität. Häufig werden die Begriffe Spezifität und Selektivität bedeutungsgleich verwendet.

SELEKTIVITÄT
Selectivity

Selektivität (selectivity) ist die Fähigkeit eines Analysenverfahrens, den zu bestimmenden Stoff von anderen in der Probe zu unterscheiden. In der Chromatographie kann die Selektivität über eine relative Retention von Analyt und Störsubstanzen beschrieben werden. Je höher die Selektivität ist, desto größer ist auch die Auflösung R (bei konstanter Peakbreite). sein. Häufig werden die Begriffe Spezifität und Selektivität bedeutungsgleich verwendet.

WIEDERFINDUNG
Recovery

Die Wiederfindung (recovery) oder Wiederfindungsrate (recovery rate) W gibt das Verhältnis des unter Wiederholbedingungen gemessenen Mittelwertes zum richtigen Wert eines Analytgehaltes in Prozent an. Ihr Idealwert ist 100%. Sie wird durch den Vergleich der Messergebnisse eines Analyten mit einer Analytprobe in Gegenwart der Matrix (Wirkstoff: andere Verunreinigungen, Präparat: Hilfsstoffe) sichergestellt. Es ist sinnvoll, die Wiederfindung für unterschiedliche Konzentrationsniveaus sicherzustellen (siehe Wiederfindungsfunktion). In der Regel decken 3 Niveaus, z.B. 70, 100 und 130% des zu erwartenden Wertes den Arbeitsbereich ausreichend ab. Die Wiederfindungsrate ist lediglich ein anderes Maß zur Charakterisierung der Richtigkeit einer Methode. Die Wiederfindungsrate macht Aussagen über Verluste durch Probenvorbereitungsschritte oder Matrixeinfluss und ist in der Spurenanalytik besonders wichtig. Bei einer Wiederfindungsrate, die ungleich 100 Prozent und unabhängig vom Gehalt des Analyten ist, liegt ein proportionaler systematischer Fehler vor. Bei einer zu kleinen Wiederfindungsrate empfiehlt es sich die Messsignale des Analyten auf eine dem Analyten möglichst ähnliche Hilfskalibriersubstanz (interner Standard) zu beziehen. Der interne Standard sollte in einem möglichst frühen Aufarbeitungsschritt der Probe in definierter Menge zugegeben werden.

MATRIX
Matrix

Alle Bestandteile der Probe mit Ausnahme des oder der zu bestimmenden Analyten. Die Matrix kann durch physikalisch-chemische Effekte wie z.B. Adsorption zu Fehlbefunden führen. Ihr Einfluss auf die Ergebnisse muss daher insbesondere bei Spurenbestimmungen und/oder variabler Matrix geprüft werden. Dies kann insbesondere die Prüfpunkte Kalibrierung, Spezifität, Richtigkeit (einschließlich Wiederfindung) betreffen.

STANDARDADDITIONSVERFAHREN
Standar addition method

Das Standardadditionsverfahren (standard addition method) wird in der Analytik angewendet, wenn eine Aufnahme einer normalen Kalibrierkurve auf Grund von z.B. Matrixeffekten nicht möglich oder der Aufwand nicht gerechtfertigt ist. Insbesondere bei spurenanalytischen Bestimmungen können sich Steigung und Linearität einer mit matrixfreien Kalibrierproben erstellten Kalibrierfunktion von denen einer mit matrixhaltigen Kalibrierproben erstellten Kalibrierfunktion unterscheiden. Solche Effekte lassen sich durch Standardaddition des Analyten zu einer matrixbehafteten Realprobe erkennen. Konstante systematische Abweichungen können so allerdings nicht erkannt werden. Man erstellt die Aufstockkalibrierfunktion und die matrixfreie Kalibrierfunktion mit jeweils 6 Kalibrierlösungen. Zur Erstellung der Aufstockkalibrierfunktion (Auftragung der Messwerte der nicht aufgestockten sowie der 5 aufgestockten Proben gegen die zugegebene Menge Analyt) wird der Analyt schrittweise zu einer Probe bekannten Gehaltes dosiert, wobei sich nach 5 Standardadditionen der Gehalt des Analyten in der Probe verdoppelt bis verdreifacht haben sollte. Wenn sich die Aufstockkalibrierfunktion als linear erweist (bei Nichtlinearität ist das Standardadditionsverfahren ungeeignet), vergleicht man deren Steigung mit der Steigung der (matrixfreien) Kalibrierfunktion (Mittelwert-t-Test) auf signifikante Unterschiede. Bei einem signifikanten Unterschied zwischen beiden Steigungen ist die mit matrixfreien Proben erstellte Kalibrierfunktion für die untersuchte Probe nicht geeignet. Das Verfahren wird auch als Aufstockverfahren bezeichnet.

RICHTIGKEITSKONTROLLE
Verification

Verfahren zur Qualitätskontrolle zur Feststellung der Validität von Messungen, z.B. im medizinischen Laboratorium. Es werden Untersuchungen von definierten Referenzmaterialien durchgeführt und diese Resultate mit den zuvor mit einer Referenzmethode ermittelten Sollwerten verglichen.

REFERENZ
Reference

Der Referenzbereich ist dasjenige Intervall, innerhalb dessen die Ausprägung eines Qualitätsindikators als „unauffällig“ definiert wird. Ein Referenzwert ist ein Referenzbereich, dessen Unter- und Obergrenze zusammenfallen. Referenzbereiche bzw. -werte müssen im Rahmen der Qualitätsforderung festgelegt werden. Diese Festlegung kann entweder empirisch (statistisch) oder normativ (Expertenkonsens) erfolgen.

PRECISION

PRÄZISION
Precision

Präzision (precision) ist ein qualitativer Begriff und beschreibt das Ausmaß an Übereinstimmung zwischen Ergebnissen, wie sie bei wiederholter Anwendung eines festgelegten Mess- oder Analyseverfahrens unter vorgegebenen Bedingungen gewonnen werden. Die Präzision erfasst zufällige Abweichungen (z.B. Detektorrauschen, Probeninhomogenitäten). Als quantitatives Maß für die Präzision wird meist die Standardabweichung (unter Wiederhol-, Zwischen- oder Vergleichsbedingungen) angegeben.

ZUFÄLLIGE ABWEICHUNG
Random deviation

Abweichung des unberichtigten Messergebnisses vom Erwartungswert. Zufällige Messabweichungen (random deviation, random error) treten zweiseitig auf, so dass sie durch Wiederholmessungen erfassbar sind (Beispiele: Temperaturschwankungen im Labor, Ableseungenauigkeit, Rundungsfehler).

GENAUIGKEIT
Accuracy

Die Genauigkeit (accuracy) ist die qualitative Bezeichnung für das Ausmaß der Annäherung von Prüfergebnissen an den Erwartungswert oder den richtigen Wert. Sie wird beeinflusst von systematischen und von zufälligen Abweichungen und ist daher ein übergeordneter Begriff zu den Begriffen Präzision und Richtigkeit. Als quantitative summarische Kenngröße für die (Un)Genauigkeit wird zunehmend der Begriff Mess- bzw. Ergebnisunsicherheit verwendet.

WIEDERHOLPRÄZISION
Repeatability

Die Wiederholpräzision (repeatability) ist die Präzision unter Wiederholbedingungen. Sie erfasst nur die laborinternen Kurzzeitschwankungen (Bestfallbetrachtung) und wird quantitativ als Wiederholstandardabweichung oder Wiederholgrenze angegeben.

WIEDERHOLBEDINGUNGEN
Repeatability conditions

Wiederholbedingungen (repeatability conditions) liegen vor, wenn die gleiche Prüfmethode auf identische Proben vom selben Mitarbeiter mit demselben Gerät im selben Labor innerhalb einer kurzen Zeitspanne angewendet wird. Wiederholbedingungen führen zur geringstmöglichen Streuung der Messwerte (best case).

WIEDERHOLUNGSMESSUNG
Np

Wiederholte Messung - an der selben Probe. Das Ergebnis kann nur zur Verringerung der zufälligen Streuung der Messung selbst dienen. - an einer neuen Probe. Das Ergebnis kann zur Verbesserung der Präzision der Beurteilung der Probe verwendet werden.

WIEDERHOLBARKEIT
--> Wiederholgrenze
Repeatability

Der Begriff Wiederholbarkeit (repeatability) wird in DIN-Normen nicht mehr verwendet und sollte durch Wiederholgrenze ersetzt werden. Dadurch werden auch Unklarheiten mit dem englischen Begriff repeatability vermieden, der für Wiederholbarkeit und Wiederholpräzision steht.

WIEDERHOLGRENZE
Repeatability limit

Die Wiederholgrenze (repeatability limit) ist der kritische Wiederholdifferenzbetrag für zwei einzelne Ermittlungsergebnisse und für eine vorgegebene Wahrscheinlichkeit von 95%. (DIN 55350 Teil 13)

WIEDERHOLDIFFERENZBETRAG, KRITISCHER
Repeatability critical difference

Der kritische Wiederholdifferenzbetrag (repeatability critical difference) ist der Betrag, unter dem oder höchstens gleich dem der Absolutwert der Differenz zwischen zwei unter Wiederholbedingungen gewonnenen Ergebnissen, von denen jedes eine Serie von Ermittlungsergebnissen repräsentiert, mit einer vorgegebenen Wahrscheinlichkeit erwartet werden kann. Beispiele für solche Ergebnisse sind der arithmetische Mittelwert oder der Median einer Serie von Ermittlungsergebnissen, wobei die Serie aus nur einem Ermittlungsergebnis bestehen kann. (DIN 55350 Teil 13)

HORWITZ KRITERIUM
Horwitz criteria

Horwitz et al. haben Mitte der 80er Jahre Ergebnisse publizierter Ringversuche ausgewertet und daraus einen empirischen Zusammenhang zwischen der relativen Standardabweichung und der Konzentration des Analyten abgeleitet und als Formel angegeben. Die Erfüllung des Horwitz-Kriteriums (Horwitz criterion) ist ein Hinweis auf eine akzeptable Streuung. Das Horwitz-Kriteriums sollte aber mit Vorsicht verwendet werden, denn es spiegelt den technologischen Stand vor 1985 wieder und es unterscheidet nicht nach Prüfarten (wie z.B. Titration, HPLC, AAS...)

INTERMEDIATE PRECISION

ZWISCHENPRÄZISION
Intermediat precision

Unter Zwischenbedingungen gemessene Präzision (intermediate precision).

ZWISCHENBEDINGUNGEN
Intermediate conditions

Zwischenbedingungen (intermediate conditions) liegen vor, wenn identische Proben im selben Labor mit der gleichen Prüfmethode in größeren Zeitabständen und/oder von unterschiedlichen Personen und/oder mit unterschiedlichen Geräten untersucht werden. Sie liegen damit zwischen Wiederhol- und Vergleichsbedingungen. Idealerweise sollten sie die übliche Laborroutine widerspiegeln und somit eine gute Abschätzung für das Streuverhalten in Qualitätsregelkarten liefern.

REPRODUZIERBARKEIT
Reproducibility

Als Reproduzierbarkeit (reproducibilty) wird die Präzision unter Vergleichsbedingungen (Vergleichspräzision) bezeichnet. Sie wird aber oft fälschlicherweise für die Präzision unter Wiederholbedingungen verwendet. Daher sollte der Begriff im Validierungsumfeld nicht verwendet werden, um Unklarheiten auszuschließen.

OUTLIERS

AUSREISSER
Outlier

In Messreihen (Stichproben) kann es vorkommen, dass der Maximalwert oder Minimalwert auffällig von den übrigen Werten abweicht. Es kann vermutet werden, dass diese Werte verfälscht sind und deshalb nicht repräsentativ für die zu prüfenden Grundgesamtheit sind. Solche Werte sollten überprüft werden, ob nicht Gründe für die Abweichung zu finden sind, z.B. Schreibfehler, Rechenfehler, unbegründete Verfahrensänderungen usw. Solche Werte sind zu korrigieren, besser zu wiederholen. Ist das nicht möglich, ist es besser, diese Werte aus der Auswertung herauszunehmen.Kann kein plausibler Grund für eine Abweichung gefunden werden, so führt man mit dem verdächtigen Wert einen Ausreißertest durch.Ergibt der Test, dass die Abweichung des Wertes nicht zufällig ist, so wird er als Ausreißer (outlier) bezeichnet und nicht in die folgende Auswertung einbezogen.Werden Werte als Ausreißer aus einer Auswertung herausgenommen, so sollte dies im Prüfbericht vermerkt werden (Anzahl, Methode).Der Test auf Ausreißer setzt immer ein bestimmtes Prüfmodell voraus. Ähnlich große Werte auf einer Seite der Verteilung können sich gegenseitig maskieren, so dass sie als Ausreißer nicht mehr erkennbar sind.

AUSREISSERTEST
Outlier test

Ausreißertest (outlier test) ist ein statistischer Test auf nicht plausible Werte (sogenannte Ausreißer). Es gibt mehrere Ausreißertests wie z.B. den Dean-Dixon-Test, der Grubbs-Test oder den Nalimov-Test.

F-TEST
F-Test

F-Test ist ein Test auf Unterschied der Varianzen zweier normalverteilter Stichproben. Progresse ist der F-Wert, d.h., der Quotient aus den beiden Varianzen der beiden Messreihen. Die größere Varianz muss dabei im Zähler stehen. Aus den beiden Varianzen ergeben sich die beiden Freiheitsgrade, wobei der Zähler f1 und der Nenner f2 liefern. Die kritische Größe F(f1, f2) ist tabelliert.

FREIHEITSGRAD
Degree of freedom

Die Anzahl der Freiheitsgrade (FG, f, n) einer Zufallsgröße ist definiert als die Zahl „frei" verfügbarer Beobachtungen. Sie ergibt sich aus dem Stichprobenumfang n minus der Anzahl a, der aus der Stichprobe geschätzten Parameter mit f = n - a. Z.B. nach der Berechnung des Mittelwertes sind von den n Einzelwerten nur noch n-1 frei wählbar. Der Ausdruck "n-1" wird deshalb Freiheitsgrad genannt.
(Die Berechnung ist nicht immer n-1!).

VARIANCE HOMOGENEITY

VARIANZ
Variance

Die Varianz ein Streuungsmaß, d.h. ein Maß für die "mittlere" Abweichung einer Zufallsvariable X (z.B. Messwert) von ihrem Erwartungswert (z.B. Mittelwert). Die Varianz der Zufallsvariable X wird üblicherweise als ?2 (Grundgesamtheit) oder s2 (Stichprobe) geschrieben. Sie wird berechnet, indem man die Abweichungen vom Erwartungswert quadriert, addiert und durch den Freiheitsgrad f teilt. Der Unterschied zwischen der Varianz der Grundgesamtheit und der Stichprobe ist zu beachten.Ihr Nachteil für die Praxis ist, dass sie eine andere Einheit als die Daten besitzt. Dieser Nachteil kann behoben werden, indem man statt der Varianz die Standardabweichung benutzt. Die Standardabweichung ist die Quadratwurzel der Varianz.Die Varianz wird auch mittlere Quadratesumme (MQS) genannt.

VARIANZENHOMOGENITÄT
Variance homogeneity

Unter Varianzenhomogenität versteht man die Gleichheit der Varianzen. Bei Anwendung varianzanalytischer Methoden (ANOVA, Kleinste Quadrate Methode) ist die Varianzenhomogenität eine vorher sicherzustellende Eigenschaft des Datenmaterials. Z.B. ist die Homogenität der Restvarianz (Homoskedastizität) über den gesamten Kalibrierbereich die Voraussetzung für eine ungewichtete Regression. Die Prüfung der Varianzenhomogenität erfolgt mit dem F-Test.

VARIANZENHOMOGENITÄTSTEST
Variance homogeneity test

Die Prüfung der Varianzenhomogenität erfolgt mit dem F-Test. Siehe dort.

GEWICHTETE REGRESSION
Weighted regression

Bei Varianzeninhomogenität kann die Regression gewichtet durchgeführt werden, um eventuell einen größeren Arbeitsbereich zu nutzen. Jedem Messwert wird ein bestimmtes Gewicht zu geordnet. Als Wichtungsfaktor werden die Varianz an jedem Kalibrierpunkt oder als Hilfsgrößen die x- oder x2-Wert genutzt.

LINEARITY

LINEARITÄT
Linearity

Unter Linearität (linearity) wird die Anwendung einer linearen Kalibrierfunktion bezeichnet nach: y = a + bx. Die Beziehung zwischen Messgröße und Gehalt des zu bestimmenden Stoffes kann also innerhalb des in der Methode genannten Mess- oder Arbeitsbereiches als Geradengleichung dargestellt werden. Die Linearität muss nachgewiesen werden z.B. mit Hilfe desLinearitätstest nach Mandel. Die Mehrzahl der Prüfmethoden beruht auf einer linearen Kalibrierbeziehung. Es ist zu beachten, dass von einigen Normen und Auswerteverfahren die Linearität vorausgesetzt wird, z.B. bei der Bestimmung der Nachweis- und Bestimmungsgrenze nach dem Kalibrierkurvenverfahren. Selbstverständlich sind auch nicht lineare Kalibrierfunktionen validierbar.Eine lineare Regression kann (ohne Test) auch erzwungen (festgelegt) werden, was nicht unbedingt zur besten Anpassung (geringste Abweichungen) führt.

PROPORTIONALITÄT
Proportionality

Die Proportionalität (proportionality) ist ein Spezialfall der Linearität. Bei der Proportionalität erfolgt die Kalibrierung nach einem linearen Modell, bei dem definiert wird, dass die Kalibriergerade durch den Nullpunkt geht, der Ordinatenabschnitt a also gleich Null wird. Im unteren Arbeitsbereich einer proportionalen Kalibrierfunktion kann ein Proportionalitätsfehler auftreten, da häufig ein geringfügiger und durch den Test nicht nachgewiesener positiver oder negativer y-Achsenabschnitt existiert, der im Spurenbereich zu einer systematischen Abweichung führen kann, die bei größeren Konzentrationen vernachlässigbar klein ist.

LINEARITÄTSTEST NACH MANDEL
Linearity test according to Mandel

Der Linearitätstest nach Mandel prüft, ob die Regression 2. Ordnung (quadratische Regression) signifikant besser ist als die Regression 1. Ordnung (lineare Regression). Zur Prüfung werden die jeweiligen Quadrate der Reststandardabweichungen aus linearer und quadratischer Regression verwendet. Der Test selbst ist ein F-Test.

KORRELATION
Correlation

Die Korrelation ist eine statistische Beziehung zwischen zwei oder mehr statistischen Variablen. Wenn sie besteht, ist noch nicht gesagt, ob eine Größe die andere kausal beeinflusst, ob beide von einer dritten Größe kausal abhängen oder ob sich überhaupt ein Kausalzusammenhang folgern lässt.

VALIDATION OF THE LOWEST VALUE

T-VERTEILUNG
Student's t-distribution

Die t-Verteilung (Student-t-Verteilung) ist stetig, symmetrisch und glockenförmig und hat einen Variationsbereich von -? bis +?. Sie ist der Standardnormalverteilung ähnlich. Die Form der t-Verteilung ist aber unabhängig von µ und s, sie wird nur durch den Freiheitsgrad (FG) bestimmt. Bei kleiner Werteanzahl (kleine Stichprobe) ist sie flacher und breiter als die Standardnormalverteilung. Je kleiner der FG, um so größer sind die Abweichungen von der Standardnormalverteilung. Bei großem FG geht die t-Verteilung in die Standardnormalverteilung über. Die t-Verteilung ist die Anpassung der Normalverteilung an kleine Stichproben mit unbekannter Standardabweichung s (nur s ist bekannt).

EINSEITIGER TEST
One-sided test

Ein einseitiger Test ist die Prüfung einer einseitigen statistischen Hypothese.

KRITISCHER WERT DER MESSGRÖSSE YK
Critical value yk

Der kritische Wert der Messgröße ist der Messwert, bei dessen Überschreitung unter Zugrundelegung einer festgelegten Irrtumswahrscheinlichkeit erkannt wird, dass der Gehalt des Bestandteils in der Analysenprobe größer als derjenige in der Leerprobe ist. Beim Leerwert- und Kalibrierkurvenverfahren ist der kritische Wert der Messgröße die Summe aus Leerwert bzw. Ordinatenabschnitt der Kalibrierfunktion und der Breite des einseitigen Prognoseintervalls.

4th worksheet "Purpose"

Purpose

"The purpose of validation is to test the suitability of methods, as well as the capacity of the staff and the laboratory. The validation is based on statistical parameters of the procedure. The procedures and scope of validation are not always the same and must be established individually."

Rambla-Alegre et al. 2011

Type of assay **Quantitative impurity test**

Testing for impurities can be either a quantitative test or a (qualitative) limit test for the impurity in a sample. Either test is intended to accurately reflect the purity characteristics of the sample.

ICH Harmonised Tripartite Guideline

Parameter	Required?
Specificity	Yes
Outliers check	Yes
Repeatability	Yes
Intermediate precision	Yes
Trueness check	Yes
Variance homogenicity	Yes
Linearity check	Yes
Lowest value xu valid	Yes
Sensitivity b	Yes
Blind value a	Yes
Standard deviation sy	Yes
Standard deviation sx0	Yes
Variation coefficient Vx0	Yes
Critical value yk	Yes
Limit of detection xLOD	Yes
Limit of identification xLOI	Yes
Limit of quantification xLOQ	Yes

Table 1. Criteria to establish for different categories of methods of analysis.

Method-performance	Type of assay				
	Identification test	Impurity test		Assay test	
		Limit impurity test	Quantitative impurity test		Dropdown menu
Specificity (a)	Yes	Yes	Yes	Yes	Identification test
Repeatability	No	No	Yes	Yes	Limit impurity test
Intermediate precision	No	No	Yes	Yes	Quantitative impurity test
Accuracy	No	No	Yes	Yes	Assay test
Reproducibility	No	No	Yes	Yes	
Range	No	No	Yes	Yes	
Linearity	No	No	Yes	Yes	
Limit of detection	No	Yes	No	No	
Limit of quantitation	No	No	Yes	No	

(a) Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).
(b) In cases where reproducibility has been performed, intermediate precision is not needed.
(c) May be needed in some cases.

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5th worksheet "Values"

First entry

Ion chromatography, 1-(3-hydroxypropyl)-1-methylpyrrolidinium chloride		Molecular weight	Stock solution		Solution used for calibration		Dilution				Operator:	Jen & Rehana
IC Methrom		in µg/µmol	in µM	in mg/L	in µM	in mg/L					Date:	29/10/2012
min retention time tr	Pyr13OH Cl	nm	179.688	13,913	2500	13913	2500	1.0				

No.	Dilution	Concentration		mean value	RSDr	VBxi	measuring signal									
i	in x mL in 25mL	in mg/L	in µM	AU	in %	in mg/L	1	2	3	4	5	6	7	8	9	10
1	0.05	5	27.83	0.10	5.68	4.04	0.0980	0.09845	0.10342	0.0997	0.09832	0.10257	0.1113	0.11321	0.10648	0.09648
2	0.125	13	69.57	0.25	0.77	3.91	0.2446	0.24767	0.24357	0.24362	0.24372	0.24735	0.24416	0.2427	0.24767	0.24548
3	0.25	25	139.13	0.48	1.39	3.72	0.4689	0.47204	0.47089	0.46951	0.46913	0.48534	0.48021	0.47923	0.48494	0.47994
4	0.5	50	278.26	0.95	0.23	3.38	0.9498	0.95199	0.94726	0.94582	0.95086	0.94702	0.9462	0.95033	0.9472	0.94642
5	0.75	75	417.39	1.42	0.31	3.14	1.4074	1.40949	1.4157	1.41386	1.41944	1.41552	1.42021	1.4144	1.41565	1.42115
6	1	100	556.52	1.89	0.19	2.99	1.8853	1.88743	1.89349	1.88671	1.89129	1.89446	1.89461	1.88779	1.88976	1.89369
7	1.25	125	695.65	2.36	0.25	2.98	2.3562	2.36444	2.36127	2.3631	2.36518	2.35317	2.36064	2.36632	2.35147	2.3701
8	1.5	150	834.78	2.81	0.45	3.08	2.8122	2.78307	2.81267	2.81766	2.8102	2.8092	2.83453	2.81026	2.81642	2.80675
9	1.75	175	973.91	3.27	0.37	3.30	3.2498	3.27282	3.27288	3.27601	3.27079	3.25187	3.28665	3.28325	3.27647	3.27534
10	2	200	1113.04	3.79	0.37	3.62	3.7995	3.79237	3.80132	3.79315	3.76691	3.76794	3.77027	3.79711	3.7995	3.78997
11	2.25	225	1252.17	4.22	0.54	0.00	4.22612	4.22981	4.2611	4.22395	4.22331	4.18974	4.19276	4.22272	4.23057	4.18773
12	2.5	250	1391.30	4.48	0.38	0.00	4.4845	4.48789	4.48754	4.44775	4.49477	4.49515	4.46197	4.48576	4.50508	4.49178
	Sum	1392.5	7749.5	17												

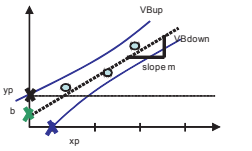
Number of measurements			
1.	Np	10	number of parallel measurements (default 6)
2.	N	12	numbers of measurements (default 10)

Raw data check					N° of conc. in range	
3.	Outliers check	positive	**No outliers**			
4.	Repeatability	positive	**Acceptable precision**			
7.	Variance homogenicity	NEGATIVE	**No variance homogenicity! Use range to decrease LOD.**	homogeneous Range	8	10
8.	Linearity check	positive	**Linear**			
9.	Lowest value xu valid	NEGATIVE	**xu is NOT valid! Use values which are higher than xp.**	xp 6.70	Lowest value valid:	12.50

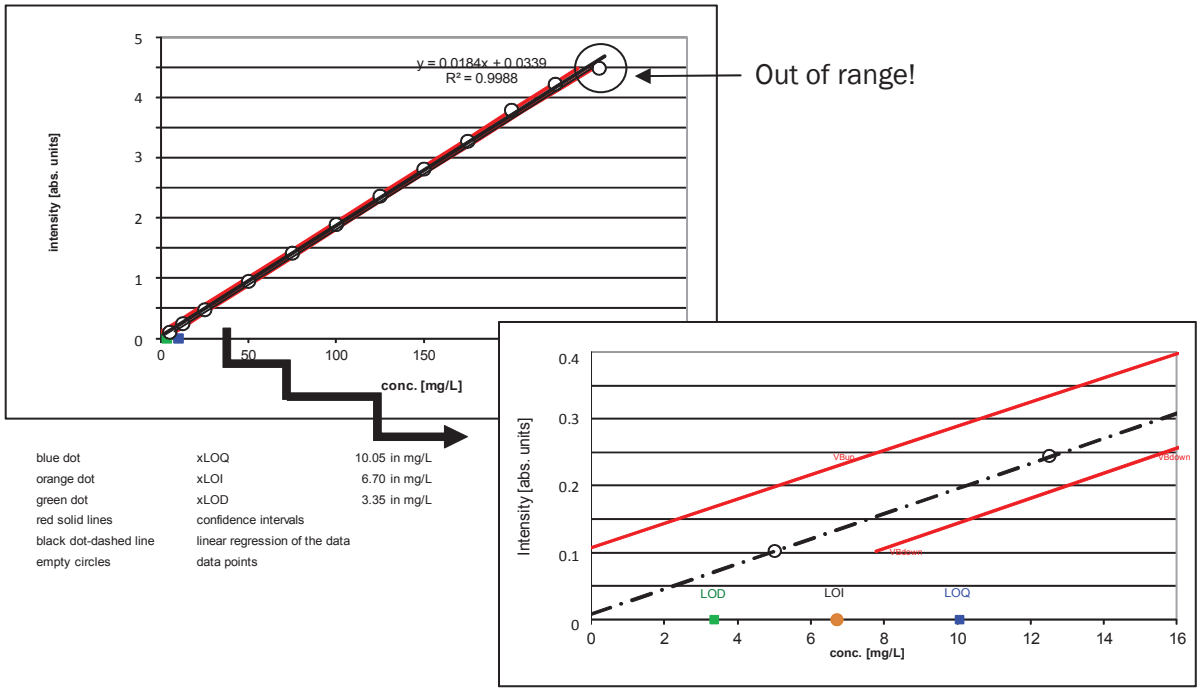
Calibration curve characteristics			
10.	Sensitivity b	0.018 AU	slope m of the 1st order linear regression
11.	Blind value a	0.034 AU	intercept b of the 1st order linear regression
12.	Standard deviation sy	0.057 AU	(Precision)
13.	Standard deviation sx0	3.11	(Performance)
14.	Variation coefficient Vx0	2.68 %	(Comparable Performance)
15.	Critical value yk	0.095	yk (y-intercept) The analyte is present above this value.

Limits	for α=β=5 %			
16.	Limit of detection x_{LOD}	3.35 in mg/L	18.65	in µM
17.	Limit of identification x_{LOI}	6.70 in mg/L	37.29	in µM
18.	Limit of quantification x_{LOQ}	10.05 in mg/L	55.94	in µM

Further parameters from method validation			
19.	Intermediate precision	not tested	**Acceptable intermediate precision**
20.	Trueness check	not tested	**Unacceptable recovery rate!**
21.	Specificity	not tested	**Acceptable recovery rate of samples, in matrix or spiked with possible interferants**



1st order linear regression



blue dot	xLOQ	10.05 in mg/L
orange dot	xLOI	6.70 in mg/L
green dot	xLOD	3.35 in mg/L
red solid lines	confidence intervals	
black dot-dashed line	linear regression of the data	
empty circles	data points	

Correction

Ion chromatography, 1-(3-hydroxypropyl)-1-methylpyrrolidinium chloride — Operator: Jen & Rehana

IC Methrom — Date: 29/10/2012

	Molecular weight	Stock solution		Solution used for calibration		Dilution
	in µg/µmol	in µM	in mg/L	in µM	in mg/L	
min retention time tr	179.688	13,913	2500	13913	2500	1.0
Pyr13OH Cl						
nm						

No.	Dilution	Concentration		mean value	RSDr	VBxi	measuring signal									
i	in x mL in 25mL	in mg/L	in µM	AU	in %	in mg/L	1	2	3	4	5	6	7	8	9	10
1	0.05	5	27.83	0.10	5.68	4.04	0.0980	0.09845	0.10342	0.0997	0.09832	0.10257	0.1113	0.11321	0.10648	0.09648
2	0.125	13	70	0.25	0.77	3.91	0.2446	0.24767	0.24357	0.24362	0.24372	0.24735	0.24416	0.2427	0.24767	0.24548
3	0.25	25	139	0.48	1.39	0.8	0.4689	0.47204	0.47089	0.46951	0.46913	0.48534	0.48021	0.47923	0.48494	0.47994
4	0.5	50	278	0.95	0.23	0.8	0.9498	0.95199	0.94726	0.94582	0.95086	0.94702	0.9462	0.95033	0.9472	0.94642
5	0.75	75	417	1.42	0.31	0.7	1.4074	1.40949	1.4157	1.41386	1.41944	1.41552	1.42021	1.4144	1.41565	1.42115
6	1	100	557	1.89	0.19	0.7	1.8853	1.88743	1.89349	1.88671	1.89129	1.89446	1.89461	1.88779	1.88976	1.89369
7	1.25	125	696	2.36	0.25	0.7	2.3562	2.36444	2.36127	2.3631	2.36518	2.35317	2.36064	2.36632	2.35147	2.3701
8	1.5	150	835	2.81	0.45	0.7	2.8122	2.78307	2.81267	2.81766	2.8102	2.8092	2.83453	2.81026	2.81642	2.80675
9	1.75	175	974	3.27	0.37	0.8	3.2498	3.27282	3.27288	3.27601	3.27079	3.25187	3.28665	3.28325	3.27647	3.27534
10	2	200	1113	3.79	0.37	0.9	3.7995	3.79237	3.80132	3.79315	3.76691	3.76794	3.77027	3.79711	3.7995	3.78997
11	2.25	225	1252	4.22	0.54	0.0	4.22612	4.22981	4.2611	4.22395	4.22331	4.18974	4.19276	4.22272	4.23057	4.18773
	Sum	1142.5	6358.2	17												

Number of measurements

1.	Np	10	number of parallel measurements (default 6)
2.	N	11	numbers of measurements (default 10)

Raw data check

					N° of conc. in range	
3.	Outliers check	positive	**No outliers**			
4.	Repeatability	positive	**Acceptable precision**			
7.	Variance homogenicity	NEGATIVE	**No variance homogeneity! Use range to decrease LOD.**	homogeneous Range	8	11
8.	Linearity check	positive	**Linear**			
9.	Lowest value xu valid	positive	**xu is valid**	xp 1.53	Lowest value valid:	5.00

Calibration curve characteristics

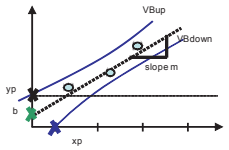
10.	Sensitivity b	0.019 AU	slope m of the 1st order linear regression
11.	Blind value a	0.010 AU	intercept b of the 1st order linear regression
12.	Standard deviation sy	0.013 AU	(Precision)
13.	Standard deviation sx0	0.68	(Performance)
14.	Variation coefficient Vx0	0.66 %	(Comparable Performance)
15.	Critical value yk	0.024	yk (y-intercept) The analyte is present above this value.

Limits for α=β=5 %

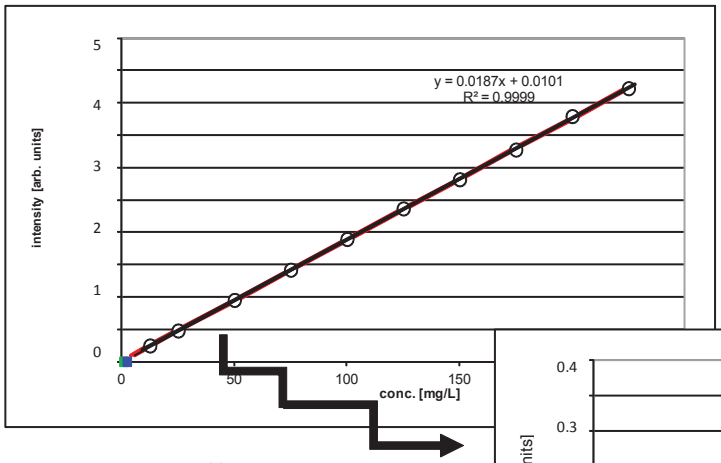
16.	Limit of detection x_{LOD}	0.76 in mg/L	4.25	in µM
17.	Limit of identification x_{LOI}	1.53 in mg/L	8.50	in µM
18.	Limit of quantification x_{LOQ}	2.29 in mg/L	12.75	in µM

Further parameters from method validation

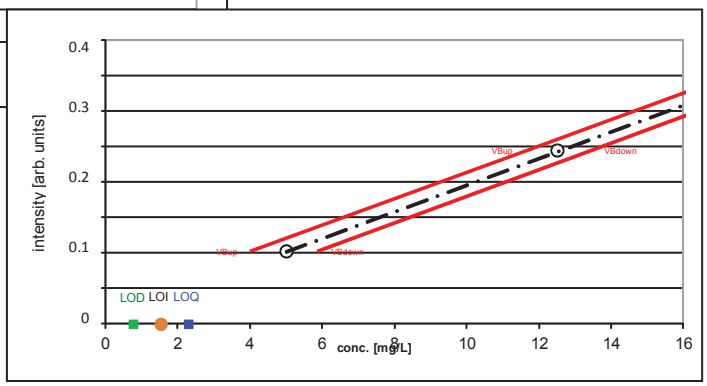
19.	Intermediate precision	not tested
20.	Trueness check	not tested
21.	Specificity	not tested



1st order linear regression



blue dot	xLOQ	2.29 in mg/L
orange dot	xLOI	1.53 in mg/L
green dot	xLOD	0.76 in mg/L
red solid lines	confidence intervals	
black dot-dashed line	linear regression of the data	
empty circles	data points	



6th worksheet "Presentation"

Np - Analysis	3		Valid working range		
f	1		min.	max.	
t	12.7		5.00	200.00	in mg/L

No.	Signal1	Signal2	Signal3	Signal mean	Result in mg/L	Vbxi
1	0.0980	0.09845	0.10342	0.09994	4.8	6.66
2	0.0434	0.058	0.0424	0.04793333	2.0	6.72
3	0.0194	0.0289	0.0128	0.02036667	0.6	6.74
4						
5						
6						
7						
8						
9						
10						

Presentation			
4.8	±	6.7	in mg/L
detected			
not detected	max.	1.5	in mg/L

Tabelle 4 — Angabe von Analyseergebnissen und Entscheidungsgrenzen

Ergebnis	Angabe	Zusatzangabe
$x \geq x_{BG}$	Gehalt	Vertrauensbereich[a]
$x_{NG} \leq x$	nachgewiesen	
$x < x_{NG}$	nicht nachgewiesen	Höchstgehalt x_{EG}

[a] Falls der Vertrauensbereich in der Analysenvorschrift angegeben ist, kann diese Zusatzangabe entfallen.

7th worksheet "Precision"

Precision check

Acceptable precisions are given if the TV value is lower than the HW.

Acceptable precision

Repeatability positive Horwitz-Value $HW = 2^{(1-0.5 \cdot \log 10(c))}$

Hw_i

No.	in mg/L	in µM	Conc. in kg/kg	theoretical	practical	RSDr	check	positive
1	5.0	27.8	0.000005	12.6	6.3	5.7	good	1
2	12.5	69.6	0.000013	10.9	5.5	0.8	good	1
3	25.0	139.1	0.000025	9.9	4.9	1.4	good	1
4	50	278	0.000050	8.9	4.4	0.2	good	1
5	75	417	0.000075	8.4	4.2	0.3	good	1
6	100	557	0.000100	8.0	4.0	0.2	good	1
7	125	696	0.000125	7.7	3.9	0.3	good	1
8	150	835	0.000150	7.5	3.8	0.4	good	1
9	175	974	0.000175	7.4	3.7	0.4	good	1
10	200	1113	0.000200	7.2	3.6	0.4	good	1
11	225	1252	0.000225	7.1	3.5	0.5	good	1

Acceptable intermediate precision

Intermediate precision positive Horwitz-Value **not tested; example only**

Hw_i

No.	in mg/L	in µM	Conc. in kg/kg	theoretical	practical	RSDR%	check	positive
1	5	27.82600953	0.000005	12.6	6.3	4.2	good	1
5	75	417.3901429	0.000075	8.4	4.2	0.5	good	1
10	200	1113.040381	0.000200	7.2	3.6	0.8	good	1

8th worksheet "Residual analysis - precision"

Residual check

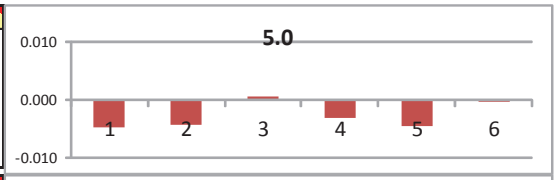
Check whether the residuals ui are randomly distributed or not.

Random pattern --> ok
Non-random pattern --> bias, systematic error

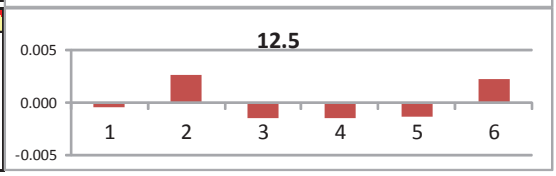
RESULT: Random pattern

Np

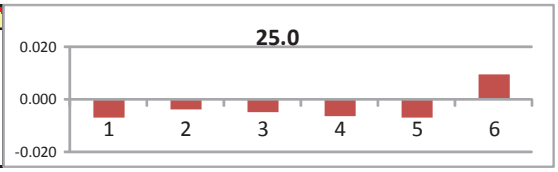
No.	in mg/L	in µM	measured signal	mean value	ui
1	5.0	27.8	0.098	0.103	-0.005
			0.098	0.103	-0.004
			0.103	0.103	0.001
			0.100	0.103	-0.003
			0.098	0.103	-0.004
			0.103	0.103	0.000



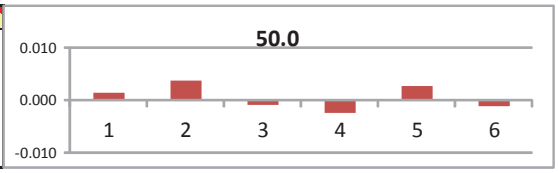
No.	in mg/L	in µM	signal	value	ui
2	12.5	69.6	0.245	0.245	0.000
			0.248	0.245	0.003
			0.244	0.245	-0.001
			0.244	0.245	-0.001
			0.244	0.245	-0.001
			0.247	0.245	0.002



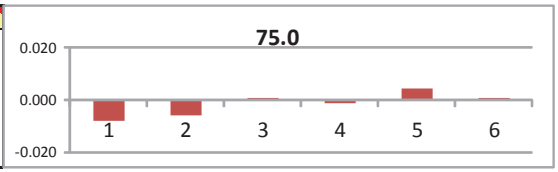
No.	in mg/L	in µM	signal	value	ui
3	25.0	139.1	0.469	0.476	-0.007
			0.472	0.476	-0.004
			0.471	0.476	-0.005
			0.470	0.476	-0.006
			0.469	0.476	-0.007
			0.485	0.476	0.009



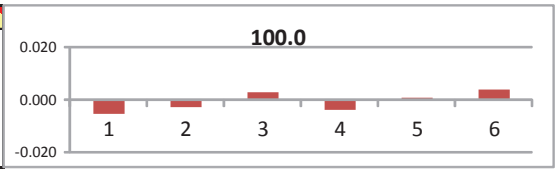
No.	in mg/L	in µM	signal	value	ui
4	50.0	278.3	0.950	0.948	0.002
			0.952	0.948	0.004
			0.947	0.948	-0.001
			0.946	0.948	-0.002
			0.951	0.948	0.003
			0.947	0.948	-0.001



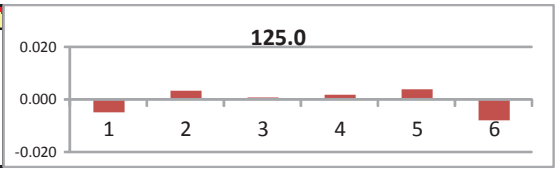
No.	in mg/L	in µM	signal	value	ui
5	75.0	417.4	1.407	1.415	-0.008
			1.409	1.415	-0.006
			1.416	1.415	0.000
			1.414	1.415	-0.001
			1.419	1.415	0.004
			1.416	1.415	0.000



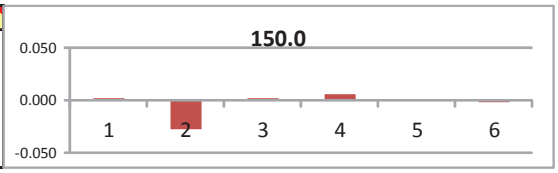
No.	in mg/L	in µM	signal	value	ui
6	100.0	556.5	1.885	1.890	-0.005
			1.887	1.890	-0.003
			1.893	1.890	0.003
			1.887	1.890	-0.004
			1.891	1.890	0.001
			1.894	1.890	0.004



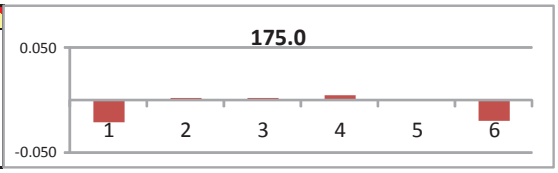
No.	in mg/L	in µM	signal	value	ui
7	125.0	695.7	2.356	2.361	-0.005
			2.364	2.361	0.003
			2.361	2.361	0.000
			2.363	2.361	0.002
			2.365	2.361	0.004
			2.353	2.361	-0.008



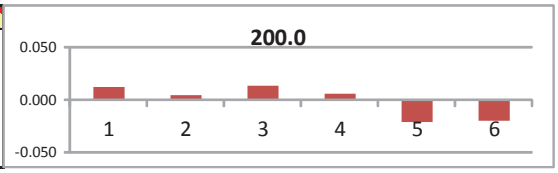
No.	in mg/L	in µM	signal	value	ui
8	150.0	834.8	2.812	2.811	0.001
			2.783	2.811	-0.028
			2.813	2.811	0.001
			2.818	2.811	0.006
			2.810	2.811	-0.001
			2.809	2.811	-0.002



No.	in mg/L	in µM	signal	value	ui
9	175.0	973.9	3.250	3.272	-0.022
			3.273	3.272	0.001
			3.273	3.272	0.001
			3.276	3.272	0.004
			3.271	3.272	-0.001
			3.252	3.272	-0.020



No.	in mg/L	in µM	signal	value	ui
10	200.0	#####	3.799	3.788	0.012
			3.792	3.788	0.005
			3.801	3.788	0.014
			3.793	3.788	0.005
			3.767	3.788	-0.021
			3.768	3.788	-0.020



9th worksheet "Outliers"

Outlier check

positive

No outliers

Np1	Np2		Max TV	11.59	<	12.25	F-value
10	9						

Maxima TV#

		5.03	11.59	1.05	1.39	2.87	3.71	5.23	4.73	3.59	2.82
N°	TV 0	TV 1	TV 2	TV 3	TV 4	TV 5	TV 6	TV 7	TV 8	TV 9	TV 10
1	1	0.77	0.63	0.12	0.37	0.67	0.11	2.64	4.73	0.48	1.31
2	1	0.16	2.35	0.71	0.67	0.59	1.72	0.32	1.83	2.35	0.16
3	1	1.31	0.44	0.68	1.08	1.21	2.43	0.48	0.33	2.18	0.44
4	1	0.54	3.79	0.30	1.39	1.51	0.41	0.98	0.93	0.33	0.79
5	1	4.71	2.05	0.12	0.20	0.99	0.11	1.41	0.15	0.12	2.11
6	1	2.72	0.82	0.83	1.26	0.16	1.47	1.60	0.65	0.14	0.94
7	1	0.78	0.38	0.11	0.20	0.53	2.16	0.12	0.83	3.59	2.82
8	1	0.12	**11.59**	0.12	0.34	0.12	0.14	**5.23**	0.12	0.26	0.23
9	1	5.03	0.12	0.12	0.23	0.12	3.71	1.84	1.06	0.26	0.20
10	1	0.79	0.21	1.05	0.24	2.87	2.52	1.85	0.53	0.79	0.13
11	1	0.20	0.32	5.43	0.16	0.15	1.90	1.48	0.14	0.36	2.24

Variances

No.	including outliers	excluding Np#1	excluding Np#2	excluding Np#3	excluding Np#4	excluding Np#5	excluding Np#6	excluding Np#7	excluding Np#8	excluding Np#9	excluding Np#10
1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0

F = F(f1;f2;α)

F-value 12.25

f1 = 1
f2 = Np2-2
P = 99 %

n	1
m	7

$$TV = \frac{(N_{p1} - 2)s_1^2 - (N_{p2} - 2)s_2^2}{s_2^2}$$

f n = 1 m = Np2-2	F-value
1	4052
2	98.5
3	34.12
4	21.2
5	16.26
6	13.75
7	12.25
8	11.26
9	10.56

10th worksheet "Variance homogeneity"

Variance check

NEGATIVE

No variance homogeneity! Use range to decrease LOD.

Variance homogenicity is given if the TV value is lower than the F-Value.

Range up

No.	s²	shigh²	slow²	TV	check	NEGATIVE	range	
1	0.00003	0.00052	0.00000	147	bad!	0	-	
2	0.00000	0.00052	0.00000	147	bad!	0	-	
3	0.00004	0.00052	0.00000	104	bad!	0	-	
4	0.00000	0.00052	0.00000	104	bad!	0	-	
5	0.00002	0.00052	0.00001	42	bad!	0	-	
6	0.00001	0.00052	0.00001	42	bad!	0	-	
7	0.00004	0.00052	0.00004	15	bad!	0	-	
8	0.00016	0.00052	0.00014	4	good	1	8	
9	0.00014	0.00052	0.00014	4	good	1	9	
10	0.00019	0.00052	0.00019	3	good	1	10	
11	0.00052	0.00052	0.00052	1	good	1	11	

Working range

Delete values from the top to adjust range.

lowest N°	8
highest N°	11
valid measurements	4

Range down

No.	s²	shigh²	slow²	TV	check	NEGATIVE	range	
1	0.00003	0.00003	0.00003	1	good	1	1	
2	0.00000	0.00003	0.00000	10	bad!	0	-	
3	0.00004	0.00004	0.00000	12	bad!	0	-	
4	0.00000	0.00004	0.00000	12	bad!	0	-	
5	0.00002	0.00004	0.00000	12	bad!	0	-	
6	0.00001	0.00004	0.00000	12	bad!	0	-	
7	0.00004	0.00004	0.00000	12	bad!	0	-	
8	0.00016	0.00016	0.00000	45	bad!	0	-	
9	0.00014	0.00016	0.00000	45	bad!	0	-	
10	0.00019	0.00019	0.00000	55	bad!	0	-	
11	0.00052	0.00052	0.00000	147	bad!	0	-	

Working range

lete values from the top to adjust rang

lowest N°	1
highest N°	1
valid measurements	1
max. valid measurements	4

F = F(f1;f2;α **F-value** 5.35

n	9
m	9

f1 = f2 = Np-1
P = 99 %

$$TV = \frac{s_{high}^2}{s_{low}^2}$$

f n = m m = Np-1	F-value
1	4052
2	99
3	29.46
4	15.98
5	10.97
6	8.47
7	6.99
8	6.03
9	5.35

		largest range
Range up	8	11
Range down	-	-
Range	8	11

11th worksheet "Linearity check"

Linearity check positive

Linear

Testing value **0.0023** < **11.26** F-Value

F = F(f1;f2;α)

f1 = 1
f2 = N - 3
P = 99 %

F-value 11.26

n	1
m	8

f n = 1 / m = N-3	F-value
1	4052
2	98.5
3	34.12
4	21.2
5	16.26
6	13.75
7	12.25
8	11.26
9	10.56

N	Np	x*	y*
11	10	104	2

Calibration 1st order
y = a + bx

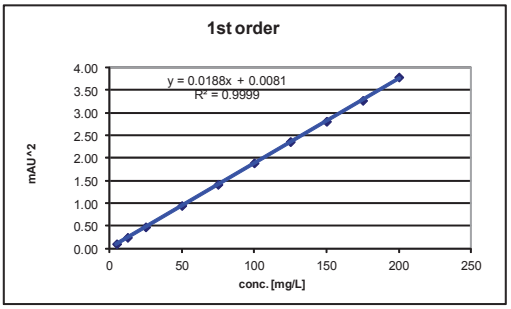
a	0.0098
b	0.0187

Sum of squares

Qxx	59642
Qyy	21
Qxy	1118

s1² 0.0002

$$s_1^2 = \frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N-2}$$



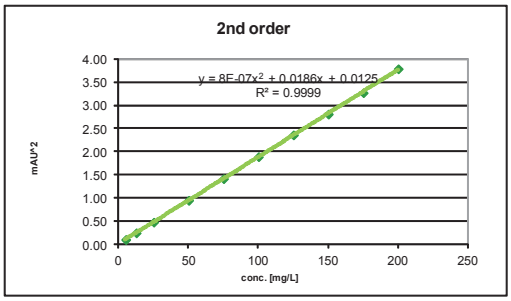
Calibration 2nd order
y = a + bx + cx²

a	0.0095
b	0.0188
c	0.0000

Quadratsummen s2² 0.0002

Qx3	13123168
Qx4	3099194638
Qx2y	246032.2619

$$s_2^2 = \frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N-3}$$



$$TV = \frac{(N-2)s_1^2 - (N-3)s_2^2}{s_2^2}$$

Auxiliary calculations:

Sum of square

No.	x	x²	y	y²	xy	x³	x^4	x²y
1	5.00	25	0	0	1	125	625	3
2	12.50	156	0	0	3	1,953	24,414	38
3	25.00	625	0	0	12	15,625	390,625	298
4	50.00	2500	1	1	47	125,000	6,250,000	2,371
5	75.00	5625	1	2	106	421,875	31,640,625	7,961
6	100.00	10000	2	4	189	1,000,000	100,000,000	18,904
7	125.00	15625	2	6	295	1,953,125	244,140,625	36,894
8	150.00	22500	3	8	422	3,375,000	506,250,000	63,254
9	175.00	30625	3	11	573	5,359,375	937,890,625	100,192
10	200.00	40000	4	14	758	8,000,000	1,600,000,000	151,512
11	225.00	50625	4	18	949	11,390,625	2,562,890,625	213,576
Sum	1143	178306	22	63	3354	31642703	5989478164	595003

Variances

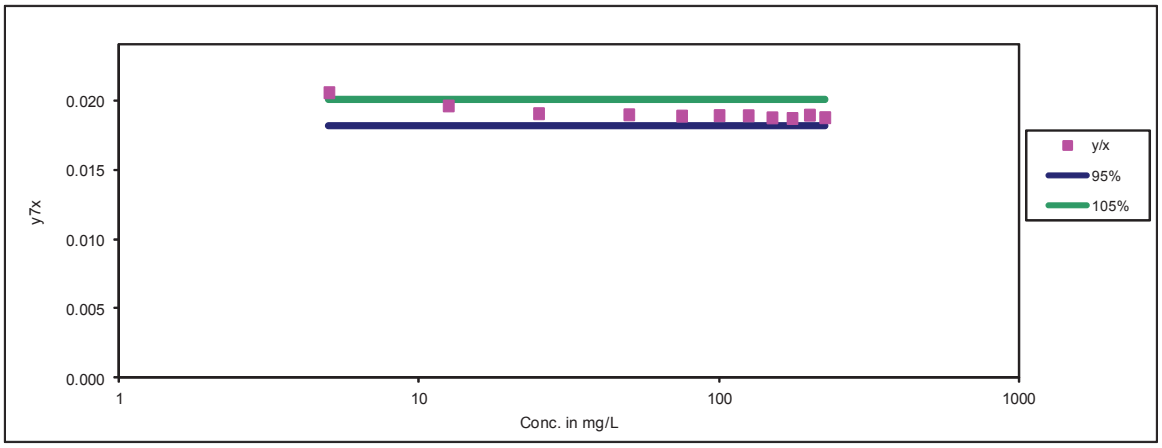
y1	y2	(y-y1)²	(y-y2)²
0	0	0	0
0	0	0	0
0	0	0	0
1	1	0	0
1	1	0	0
2	2	0	0
2	2	0	0
3	3	0	0
3	3	0	0
4	4	0	0
4	4	0	0
22	22	0	0

12th worksheet "Linearity - graphically"

Linearity **according to Umweltbundesamt** should be a straight line log scale

Concentration draw horizontal lines at 95 % and 105 %

N°	in mg/L	mean value	RSDr	y/x	Stdabw.	95%	105%
1	5.0	0.1028	0.0058	0.0206		0.0181	0.0200
2	12.5	0.2451	0.0019	0.0196		0.0181	0.0200
3	25.0	0.4760	0.0066	0.0190		0.0181	0.0200
4	50.0	0.9483	0.0022	0.0190		0.0181	0.0200
5	75.0	1.4153	0.0044	0.0189		0.0181	0.0200
6	100.0	1.8904	0.0035	0.0189		0.0181	0.0200
7	125.0	2.3612	0.0060	0.0189		0.0181	0.0200
8	150.0	2.8113	0.0126	0.0187		0.0181	0.0200
9	175.0	3.2716	0.0120	0.0187		0.0181	0.0200
10	200.0	3.7878	0.0139	0.0189		0.0181	0.0200
11	225.0	4.2188	0.0227	0.0188		0.0181	0.0200
Sum	1143	22		0.210		0.019	Mean value y/x



13th worksheet "Residual analysis - linearity"

Residual check

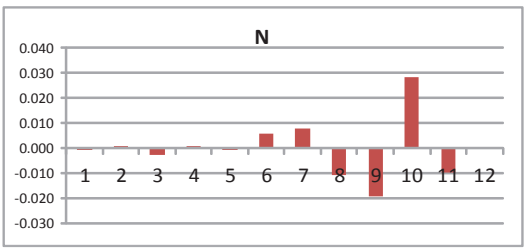
Check whether the residuals ui are randomly distributed or not.

N

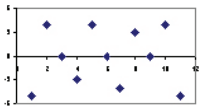
Sum ui 0 RESULT Random pattern --> linear

Mean value ui 0 **linear** Non-random pattern --> non-linear

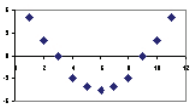
No.	in mg/L	in µM	measured y	predicted yi	ui
1	5.0	27.8	0.103	0.104	-0.001
2	12.5	69.6	0.245	0.244	0.001
3	25.0	139.1	0.476	0.479	-0.003
4	50	278	0.948	0.947	0.001
5	75	417	1.415	1.416	-0.001
6	100	557	1.890	1.885	0.006
7	125	696	2.361	2.353	0.008
8	150	835	2.811	2.822	-0.011
9	175	974	3.272	3.291	-0.019
10	200	1113	3.788	3.760	0.028
11	225	1252	4.219	4.228	-0.010



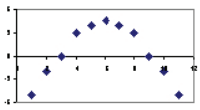
Compare:



Random pattern



Non-random: U-shaped



Non-random: Inverted U

http://stattrek.com/regression/residual-analysis.aspx

14th worksheet "Lowest valid value & limits"

Validating the lowest value

xu > xp = positive

lowest value valid = 5.00

xu is valid

Test value xp 1.53 < 5.00 lowest value xu of working range

critical value yk 0

N°	Conc. in mg/L	mean value	xi	yi	(yi-y*)²	VBxi	VB_{up}	VB_{down}	value > xp	check	Limits in mg/L
1	5.00	0	5.0	0	3	0.92	4.0	5.9	5.00	good	LOD
2	12.50	0	12.5	0	3	0.89	11.7	13.4	12.50	good	0.76
3	25.00	0	24.9	0	2	0.84	24.0	25.7	25.00	good	
4	50.00	1	50.1	1	1	0.76	49.3	50.8	50.00	good	LOI
5	75.00	1	75.0	1	0	0.70	74.3	75.7	75.00	good	1.53
6	100.00	2	100.3	2	0	0.68	99.6	101.0	100.00	good	
7	125.00	2	125.4	2	0	0.69	124.7	126.1	125.00	good	LOQ
8	150.00	3	149.4	3	1	0.74	148.7	150.2	150.00	good	2.29
9	175.00	3	174.0	3	2	0.81	173.2	174.8	175.00	good	
10	200.00	4	201.5	4	3	0.91	200.6	202.4	200.00	good	
11	225.00	4	224.5	4	5	1.02	223.5	225.5	225.00	good	
MW	91.8	1.7	Summe	17	16			Min value	5.00		

Quantil der Studentschen t-Verteilung
(einseitige Fragestellung)

f	α = 0,05; P = 95 %
1	6.314
2	2.92
3	2.353
4	2.132
5	2.015
6	1.943
7	1.895
8	1.86
9	1.833
10	1.812

f = N - 2

t = 1.833

f 9

Quantil der Studentschen t-Verteilung
(zweiseitige Fragestellung)

f	α = 0,05; P = 95 %
1	12.706
2	4.303
3	3.182
4	2.776
5	2.571
6	2.447
7	2.365
8	2.306
9	2.262
10	2.228

f = N - 2

t = 2.262

f 9

Intermediate values

The results of the inter-day precision is exemplified for IL cations that were used in the first study on the anaerobic biodegradability of IL cations under denitrifying conditions.

Summary Nov-09 CC125/4 Nucleosil 100-5SA

in deionised water

	repeatability intra-day precision		stability inter-day prec.	stability inter-day prec.				
	sy, n=6	r², n=6	sy6, n=3 (best)	sy6, n=3 (worst)	LOD	LOQ	LOD	LOQ
Substance	in %		in %	in %	in mg L-1	in mg L-1	in µmole L-1	in µmole L-1
IM12 Cl	0.20	0.9997	2.88	3.14	0.9	2.5	7.79	22.10
IM18 Cl	0.2	0.9998	2.54	3.41	1.3	3.8	6.9	19.7
IM18OH Br	0.45	0.9997	7.28	12.33	2.46	6.80	11.6	32.2
Py2 Cl	0.30	0.9998	3.06	4.50	0.59	1.70	5.41	15.7
Py1-4NMe2 I	0.44	0.9996	3.30	5.00	1.12	3.19	8.16	23.3
Py2-4NMe2 Br	0.05	0.999995	6.39	8.04	0.14	0.42	0.92	2.7
Py4-4NMe2 Cl	0.29	0.9999	4.64	6.20	1.32	3.78	7.4	21.1
Py6-4NMe2 Cl	0.52	0.9996	2.84	4.66	2.82	7.70	13.62	37.1
Py8 Cl	0.26	0.9999	0.23	0.58	0.88	2.57	4.59	13.4

Paper B.6 - Leitfaden zur Quantifizierung mit Hilfe einer validierten Kalibrationsgeraden nach DIN 38 402 Teil 51

Neumann J (2012) Leitfaden zur Quantifizierung mit Hilfe einer validierten Kalibrationsgeraden nach DIN 38 402 Teil 51.

Chromatographie

Praktikum zur Vorlesung

„Einführung in die Chromatographie“

Leitfaden zur Quantifizierung mit Hilfe einer validierten Kalibrationsgeraden nach DIN 38 402 Teil 51

Jennifer Neumann

Bachelorstudiengang Chemie - 5. Fachsemester WS 2011/2012

Stand: 06.07.2012

Einleitung

Ziel und Aufgabenstellung

Die quantitative Analyse von Coffein und Theobromin in verschiedenen Lebensmitteln soll mit Hilfe einer standardisierten Kalibrationsgeraden erfolgen unter Einbeziehung der DIN 38 402 Teil 51 und DIN 32 645 des Deutschen Instituts für Normung e.V. Im Folgenden sollen die Grundideen und die Herangehensweise an solch ein Vorhaben erklärt und im Labor dann von Ihnen praktisch durchgeführt werden. Im Versuchsprotokoll werden die analytischen Qualitätsparameter in einem Analyseprotokoll zusammengefasst dargestellt und bewertet. Eine graphische Darstellung der Kalibrationsgeraden, der Vertrauensbänder und der analytischen Grenzen soll ebenfalls erarbeitet werden. Abweichungen von den Erwartungen sollen gegebenenfalls diskutiert werden. Die Ergebnisse der quantitativen Analyse werden wie hier beschrieben dargestellt.

Im Versuchsprotokoll sollen folgende Parameter berechnet und gegebenenfalls erklärt werden:

- Berechnung der Konzentrationswerte $\hat{x}$ der realen Proben
- Wiederfindungsrate WFR als Maß für die Richtigkeit
- Angabe des Analyseergebnisses nach DIN 32 645
- Nachweis-, Erfassungs- und Bestimmungsgrenzen $x_{NG,}\ x_{EG,}\ x_{BG}$
- Kritischer Wert y_k

- Arbeitsbereich, Anzahl der Konzentrationsstufen N und Parallelmessungen N_p
- Ausreißer, Varianzhomogenität
- Linearität, Absicherung des untersten Wertes

- Geradengleichung $y = a + bx$
- Quadratsummen $Q_{xx}, Q_{yy}, Q_{xy},$
- Steigung b als Maß für die Empfindlichkeit der Methode
- Ordinatenabschnitt a als Maß für den Blindwert
- Arithmetisches Mittel der Konzentrationen $\bar{x}$ und der Messsignale $\bar{y}$
- Standardabweichung der Messwerte s_i
- Reststandardabweichung der Kalibriergeraden s_{yi} als Maß für die Präzision
  (*i* steht hier für die Funktionsordnung; linear → 1. Ordnung: quadratisch → 2. Ordnung)
- Verfahrensstandardabweichung s_{x0} als Maß für die Güte
- Relative Verfahrensstandardabweichung V_{x0} als Maß für die relative Güte

Weitere Symbole

- *i* laufende Nummer der Konzentrationsstufen
- *j* laufende Nummer der Analysen je Messwert
- $\hat{}$ Variable bezogen auf Analysenprobe, sonst immer bezogen auf Kalibrierexperiment
- $\hat{=}$ Variabel bezogen auf den Messmittelwert
- $_{inkl}$ bzw. $_{ohne}$ mit Ausreißer bzw. ohne Ausreißer
- $_o$ bzw. $_u$ obere bzw. untere Ende des Arbeitsbereichs oder der Streuung
- $y = a + bx + cx^2$ Geradengleichung 2.Ordnung

Theoretischer Hintergrund

Um eine quantitative Aussage über den Gehalt einer Substanz in einer Probe treffen zu können, wird im Allgemeinen zunächst die Beziehung zwischen Messsignal und Konzentrationswert analysiert. Ist diese Beziehung bekannt, können Messsignale der zu untersuchenden Probe mit unbekannter Konzentration den Messsignalen von Proben bekannter Konzentration (Referenzproben) verglichen werden und so die gesuchte Konzentration abgeschätzt werden. Innerhalb des erwarteten Konzentrationsbereich und dem dazugehörigen Arbeitsbereiches werden dazu verschiedene Konzentrationen des Analyten äquidistant in einer Verdünnungsreihe angesetzt. Im Falle eines proportionalen Zusammenhanges zwischen Konzentration und Messsignal, können die unbekannten Konzentrationen des Analyten über eine lineare Kalibrierfunktion aus der Messung der Referenzproben berechnet werden. Eine statistische Absicherung über die Auswertung von Mehrfachmessungen ein und derselben Konzentrationsstufe ermöglicht eine Eingrenzung des berechneten Analytgehalts durch die Einbeziehung der statistischen Unsicherheit.

Die Erstellung einer linearen Kalibrierfunktion nach DIN 38 402 Teil 51 mit Hilfe eines statistischen Analyseverfahrens (linearen Regression), die Bestimmung ihrer Präzision und Güte, sowie die anschließende Probenauswertung unter Einbeziehung eines Prognoseintervalls sollen im Folgenden erläutert werden. Außerdem werden die Berechnung der Nachweis-, Erfassungs- und Bestimmungsgrenzen nach DIN 32 645, sowie Wiederholbarkeit und Richtigkeit der Messmethode erklärt. Andere statistische Tests können angewendet werden und weitere analytische Parameter je nach Ziel der Kalibrierung hinzugefügt werden.

Die lineare Kalibrierfunktion

Ein linearer Zusammenhang zwischen einer Konzentration x und einem Messsignal y besteht dann, wenn sich die beiden Werte proportional zueinander verändern, ausgedrückt durch die Geradengleichung:

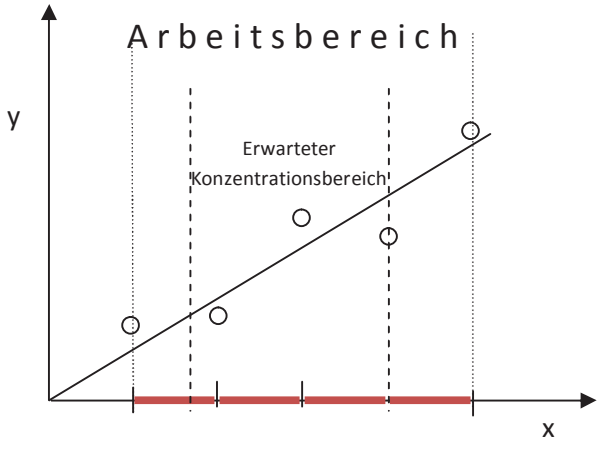
$$y = a + bx$$

Das Wissen um diesen Zusammenhang ermöglicht die Bestimmung der Konzentration unbekannter Proben $\hat{x}$ aus einem Messsignal $\hat{y}$.

$$\hat{x} = \frac{\hat{y} - a}{b}$$

Die dazugehörige lineare Kalibrierfunktion, in der die Steigung b und der Achsenabschnitt a bekannt sind, kann über eine lineare Regression „die Methode der kleinsten Quadrate“, berechnet und durch die so generierte Ausgleichsgerade oder auch Regressionsgerade in einem Diagramm visualisiert werden.

Dazu wird das Verhältnis von Messsignalen unterschiedlicher Konzentrationsstufen zueinander analysiert. Mindestens fünf Konzentrationsstufen des Analyten werden äquidistant (gleicher Konzentrationsunterschied zwischen den einzelnen Stufen) im gewünschten Arbeitsbereich angesetzt und gemessen.



Das Ansetzen der Kalibrierproben erfolgt dabei zur Fehlerminimierung möglichst ohne großen Aufwand aus einem Standard (keine sequentielle Verdünnung) und der Arbeitsbereich sollte so gewählt werden, dass die erwartete Konzentration der realen Probe im mittleren Konzentrationsbereich des Arbeitsbereichs liegt, der untere Konzentrationswert sich signifikant von Null unterscheidet, also sich oberhalb der Nachweisgrenze befindet, und die Präzision über den gesamten Bereich den Erfordernissen entspricht und konstant ist (Varianzhomogenität).

Die Durchführung einer solchen Kalibration soll hier dargestellt werden und umfasst im Wesentlichen folgende Schritte:

I. Praktische Durchführung und Messung der Kalibrierproben

Herstellen der Kalibrierlösung

Zehn Kalibrierlösungen werden in einem Konzentrationsbereich von 10 – 100 mg/L aus einer Stammlösung (c = 500 mg/L) angesetzt. In 10 mL Messkolben sollen in 200 µL-Schritten von 200 µL bis 2000 µL der Stammlösung mit deionisiertem Wasser/Methanol verdünnt und für eine chromatographische Analyse in Autosamplervials überführt werden. Anschließend soll mit der ausgewählten Methode mindestens 5-fach gemessen werden.

II. Statistische Auswertung

a. Absicherung der Messwerte und des Arbeitsbereichs

Teil 1 – Ausreißertest, Präzision, Varianzhomogenität

Damit eine lineare Regression der Kalibration durchgeführt werden kann, sollen Ausreißer erkannt werden und die Präzision über den gesamten Bereich den Erfordernissen entsprechen und konstant sein (Varianzhomogenität).

Um die Präzision zu berechnen, werden mindestens drei Messsignale jeder Konzentrationsstufe benötigt.

Nr.	Konzentration c in mg/L	Peakfläche 1 in mAu*min	Peakfläche 2 in mAu*min	Peakfläche 3 in mAu*min	Mittelwert
i	x_i	y_{i1}	y_{i2}	y_{i3}	$\bar{y}_i$
1	10				
…	…				
10	100				

Ausreißertest

Nachdem man die Messwerte der Parallelmessungen erhalten hat, werden die Werte zunächst auf eventuelle Ausreißer überprüft. Ein Ausreißer ist ein Messwert dann, wenn er signifikant von den anderen Werten abweicht. Um die Signifikanz zu bestimmen, wird häufig ein statistischer Ausreißertest mit Hilfe der F-Verteilung durchgeführt „F-Test“. Dazu wird zuerst die Varianz ${s_i}^2$ als Maß der Streuung über die Summe der Differenzen zwischen den Messsignalen der Parallelmessungen y_i und deren arithmetischen Mittel $\bar{y}_i$ und der Anzahl der Parallelmessungen N_p bestimmt.

$$ {s_i}^2 = \frac{\sum_{j=1}^{N_p}(y_{ij} - \bar{y}_i)^2}{(N_p - 1)} $$

Und der Mittelwert durch

$$ \bar{y}_i = \frac{\sum_{j=1}^{N_p}(y_{ij})}{N_p} $$

Um einen Ausreißer zu erkennen, wird nun die Varianz s_{inkl}^2 aller Parallelmessungen $N_{p_{inkl}}$ einer Konzentration inklusive des vermuteten Ausreißers und einmal die Varianz s_{ohne}^2 ohne den vermuteten Ausreißer berechnet. Der dazugehörige Prüfwert PW sollte innerhalb der statistischen Grenzen, gegeben durch die F-Verteilung, liegen. Ist dies der Fall, also PW kleiner als der F-Wert, so handelt es sich mit einer bestimmten statistischer Sicherheit (hier: 99%) um eine statistisch normale Streuung des Wertes.

$$PW = \frac{(N_{p_{inkl}} - 2)s_{inkl}^2 - (N_{p_{ohne}} - 2)s_{ohne}^2}{s_{ohne}^2}$$

Der F-Wert der F-Verteilung wird einer Tabelle entnommen. Er ist abhängig von der gewählten statistischen Sicherheit und der Anzahl der Parallelmessungen. Für drei Parallelmessungen liegt er bei 4052, weshalb mindestens eine Fünffachmessung empfohlen wird. Der F-Wert läge dann bei 21,20. Weitere Werte für unterschiedliche Parallelmessungen stehen in untenstehender Tabelle. *m* und *n* bezeichnen die jeweiligen Freiheitsgrade der F-Verteilung - Faktoren, die für unterschiedliche F-Tests frei gewählt werden können. Sie beziehen sich auf den Zähler *n* und den Nenner *m* des Prüfwertes und sind daher abhängig vom jeweiligen Test, weshalb ihre Berechnung im Test immer mitangegeben wird. In dem Fall des Ausreißertests ist *n* immer 1 und *m* ist abhängig von der Anzahl der Parallelmessungen ohne Ausreißer $N_{p_{ohne}}$ angezeigt durch s_{ohne}^2 (siehe Prüfwert).

Freiheitsgrade n, m n = 1 m = $N_{p_{ohne}}$ - 2	Irrtumswahrscheinlichkeit α Statistische Sicherheit P α = 0,01; P = 99 %
1	4052
2	98,50
3	34,12
4	21,20
5	16,26
6	13,75
7	12,25
8	11,26
9	10,56

Präzisionsprüfung

Um entscheiden zu können, ob die unerwünschte Streuung der Messwerte nun ausreichend gering ist, wird die Präzision der Parallelmessungen ermittelt und bewertet. Die Präzision der Messsignale wird durch die Standardabweichung s_i angegeben. Sie ist die Wurzel der jeweiligen Varianz ${s_i}^2$. Da die akzeptierte Präzision einer Messung abhängig von der Konzentration des Analyten in der Probe ist und unabhängig von der Art des Analyten, der Matrix und der Methode sein soll, können akzeptable Werte über einen allgemeinen konzentrationsabhängigen Wert, dem sogenannten Horwitz-Wert HW, ermittelt werden. Der theoretische Wert wird über folgende Gleichung bestimmt:

$$HW = 2^{(1-0{,}5\,*log10(c))}$$

Dieser Wert kann nun mit der Standardabweichung s_i in % verglichen werden.

$$s_i in\,\% = \frac{s_i}{\bar{y}_i} * 100$$

Ist sie kleiner als der akzeptierte Wert, gegeben durch Horwitz, sind die Messungen ausreichend präzise. Wenn nicht soll die Präzision durch Verringerung systematischer und zufälliger Fehler erhöht werden.

In der Praxis wird oft für die akzeptierte Präzision ein Wert von 0,5 mal dem HW angenommen. Dabei ist darauf zu achten, dass die Konzentration *c* hier als dimensionslose Einheit mit $\frac{kg}{kg}$ eingefügt wird. (1 L = 1 dm³ = 0,001 m³; Dichte von Wasser bei 20 °C: 998,20 $\frac{kg}{m^3}$).

Nr. i	Konzentration in mg/L x_i	Mittelwert $\bar{y}_i$	Konzentration in kg/kg c_i	Horwitz-Wert HW_i Theoretisch	Horwitz-Wert HW_i Praktisch	Standardabweichung s_i in %	Bewertung S_i < HW = OK; S_i ≥ HW = nicht OK
1	10		ca. 0,00001***				
…	…						
10	100						

*** 10 mg/L = 0,010 g/L = 0,000010 kg/L = 0,00001 kg/0,001 m^3 = 0,01 kg/m^3 / 998,20 kg/m^3 ≈ 0,00001 - Kurz: x mg/L = 10^-6 kg/kg

Varianzhomogenität

Da die Varianzen homogen in dem gewählten Arbeitsbereich sein sollen, empfiehlt es sich den untersten und obersten Konzentrationswert zunächst häufiger zu messen (von uns aus Zeitgründen nicht durchgeführt), im Idealfall mindestens zehn Mal, um eine empfindlichere statistische Aussage treffen zu können. Sollte dabei die Streuung der beiden Werte ${s_o}^2$ und ${s_u}^2$ signifikant voneinander abweichen, also die Varianzen nicht homogen sein, kann eine weitere statistische Analyse durch lineare Regression in dem gewählten Arbeitsbereich nicht mehr durchgeführt werden und der Arbeitsbereich wird entsprechend verkleinert. Die statistische Überprüfung der Varianzen erfolgt durch den Vergleich eines Quotienten (Prüfwert PW) aus den Varianzen der oberen und unteren Grenze des Arbeitsbereichs und des kleinsten Wertes mit einem statistischen Grenzwert, dem F-Wert.

$$PW = \frac{{s_o}^2}{{s_u}^2}$$

Liegt dieser Prüfwert PW innerhalb der gewählten statistischen Grenzen, d.h. ist kleiner als der F-Wert, so liegt eine homogene, vergleichbare Streuung über den gesamten Arbeitsbereich vor. Die statistischen Werte sind dabei abhängig von der Anzahl der Parallelmessungen und der gewünschten statistischen Sicherheit. Eine gängige statistische Sicherheit P im Test auf Varianzhomogenität liegt bei 99 %, d.h. dass das Ergebnis mit einer Irrtumswahrscheinlichkeit α von 1 % fehlerhaft sein kann. Die Anzahl der Parallelmessungen in dem vorliegenden theoretischen Fall wäre 10, wodurch sich ein Prüfwert von 5,35 ergäbe. Aus praktischen Gründen kann eine andere Anzahl von Mehrfachmessungen sinnvoller sein. Die dazugehörigen Prüfwerte können aus folgender Tabelle entnommen werden, wobei m und n die Anzahl der Freiheitsgrade angibt. In diesem Fall sind die Freiheitsgrade m Nenner und n Zähler gleich und werden wie bei der Berechnung der Varianz s_i^2 durch N_p - 1 dargestellt.

Freiheitsgrade n, m n = m = N_p - 1	Irrtumswahrscheinlichkeit α Statistische Sicherheit P α = 0,01; P = 99 %
1	4052
2	99
3	29,46
4	15,98
5	10,97
6	8,47
7	6,99
8	6,03
9	5,35

Teil 2 – Erstellen einer Kalibrierfunktion durch lineare Regression – die Methode der kleinsten Quadrate

Kalibrierfunktion 1.Ordnung

Ist der Arbeitsbereich soweit festgelegt, dass Ausreißer bestimmt sind, die Präzision akzeptabel ist und die Varianzen homogen verteilt sind, kann nun ein linearer Zusammenhang zwischen den Messwerten der Kalibration und der Konzentration des Analyten hergestellt werden. Ob es sich schließlich wirklich um einen linearen Zusammenhang handelt und sich auch der untere Wert des Arbeitsbereichs signifikant von Null unterscheidet, wird anschließend mit Hilfe des F-Tests geklärt.

Um nun die Geradengleichung der Kalibrierfunktion ($y = a + bx$) zu ermitteln, werden zunächst Hilfsgrößen, die Quadratsummen, berechnet. Ein solches Vorgehen ist sinnvoll, da die Geradengleichung nicht nur eine unbekannte Variable, sondern zwei enthält, die durch mehrere Datenpunkte (x_i, y_i) beschrieben werden. Außerdem ist mit den Quadratsummen eine einfache Vorgehensweise verfügbar, die zur Not ohne Computer nur mit Taschenrechner bewältigt werden kann, sofern man sich nicht zu viel vertippt.

$$Q_{xx} = \sum_{i=1}^{N} {x_i}^2 - \frac{(\sum_{i=1}^{N} x_i)^2}{N}$$

$$Q_{yy} = \sum_{i=1}^{N} {y_i}^2 - \frac{(\sum_{i=1}^{N} y_i)^2}{N}$$

$$Q_{xy} = \sum_{i=1}^{N} (x_i * \bar{y}_i) - \frac{\sum_{i=1}^{N} x_i * \sum_{i=1}^{N} \bar{y}_i}{N}$$

Nr.	Konzentration in mg/L	Mittelwert	Für Q_{xx}	Für Q_{yy}	Für Q_{xy}
i	x_i	$\bar{y}_i$	${x_i}^2$	${y_i}^2$	$(x_i * \bar{y}_i)$
1	10				
…	…				
10	100				
N = 10	$\sum_{i=1}^{N} x_i$	$\sum_{i=1}^{N} y_i$	$\sum_{i=1}^{N} {x_i}^2$	$\sum_{i=1}^{N} {y_i}^2$	$\sum_{i=1}^{N} (x_i * y_i)$

Die Steigung b als Maß für die Empfindlichkeit der Methode ergibt sich dann leicht aus

$$b = \frac{Q_{xy}}{Q_{xx}}$$

Nun kann der Ordinatenabschnitt a als Maß für den Blindwert aus der Geradengleichung berechnet werden.

$$a = \bar{y} - b\bar{x}$$

mit dem arithmetischen Mittel der Konzentrations-und Signalwerte $\bar{x} = \frac{\sum_{i=1}^{N} x_i}{N}$ und $\bar{y} = \frac{\sum_{i=1}^{N} \bar{y}_i}{N}$

Präzision und Güte des Verfahrens

Da solch eine Kalibrierfunktion immer nur eine Abschätzung der Wirklichkeit ist, soll im Folgenden geklärt werden wie präzise die durchgeführte lineare Regression war und von welcher Güte die Kalibrierung ist. Wie präzise die aus den Quadratsummen zuvor berechnete Steigung b und der Achsenabschnitt a sind, wird durch die Reststandardabweichung s_y beschrieben. Sie gibt die Streuung der Messsignale y_i in y-Richtung um die berechnete Kalibriergerade (Regressionsgerade) an. Ein solcher Restfehler ergibt sich aus der Differenz des gemessenen Signals zu dem berechneten Wert aus der Regressionsgeraden. Wäre s_y gleich 0, so lägen alle Messwerte auf der berechneten Kalibriergeraden (1 = 1. Ordnung).

$$s_{y1} = \sqrt{\frac{\sum_{i=1}^{N}[y_i - (a + b * x_i)]^2}{N - 2}}$$

Der Einfachheit halber wird die Reststandardabweichung s_y meist über die Quadratsummen berechnet.

$$s_{y1} = \sqrt{\frac{Q_{yy} - \frac{{Q_{xy}}^2}{Q_{xx}}}{N - 2}}$$

Die Güte der Kalibration ist das Resultat aus einer Kombination aus Reststandardabweichung und der Empfindlichkeit. Dies wird mit Hilfe der Verfahrensstandardabweichung s_{x0} ausgedrückt. Eine geringe Reststandardabweichung und eine hohe Empfindlichkeit sind dabei erwünscht (s_{x0} gegen 0).

$$s_{x0} = \frac{s_y}{b}$$

Um nun die Anpassung durch die Kalibriergeraden mit anderen Verfahren und Arbeitsbereichen vergleichen zu können, wird die Verfahrensstandardabweichung s_{x0} in Relation zum mittleren Konzentrationsbereich wie folgt gesetzt.

$$V_{x0} = \frac{s_{x0}}{\bar{x}} * 100$$

Dabei gilt immer: Je besser das Verfahren, desto kleiner die Standardabweichung.

Linearitätsüberprüfung

Obwohl die Beziehung zwischen Messsignal und Konzentration oft durch eine lineare Kalibrierfunktion (1.Ordnung) dargestellt wird, kann es sein, dass die quadratische Kalibrierfunktion einer Parabel (2.Ordnung), eine bessere Abschätzung des Messverfahrens liefert.

$$y = a + bx + cx^2$$

Um das zu überprüfen, gibt es wieder mehrere Verfahren, wobei hier wieder mittels F-Test untersucht werden soll, ob es einen signifikanten Unterschied zwischen der Reststandardabweichung einer Kalibrierfunktion 1.Ordnung und einer der 2.Ordnung gibt. Ist dies der Fall, sollte eine Kalibrierung 2.Ordnung bevorzugt bzw. der Arbeitsbereich zum linearen Bereich hin verkleinert werden.

Um die Reststandardabweichung der Kalibrierfunktion 2.Ordnung zu berechnen, wird zunächst über die Quadratsummen eine solche Funktion berechnet. Die Quadratsummen aus der Berechnung der Kalibrierfunktion 1.Ordnung und folgende weitere werden dazu benötigt:

$$Q_{x^2y} = \sum_{i=1}^{N} ({x_i}^2 * y_i) - \frac{(\sum_{i=1}^{N} {x_i}^2) * (\sum_{i=1}^{N} y_i)}{N}$$

$$Q_{x^3} = \sum_{i=1}^{N} {x_i}^3 - \frac{(\sum_{i=1}^{N} x_i) * (\sum_{i=1}^{N} {x_i}^2)}{N}$$

$$Q_{x^4} = \sum_{i=1}^{N} {x_i}^4 - \frac{(\sum_{i=1}^{N} {x_i}^2)^2}{N}$$

Nr.		Für Q_{x^3}	Für Q_{x^4}	Für Q_{x^4}
i	${x_i}^2$	${x_i}^3$	${x_i}^4$	$({x_i}^2 * y_i)$
1				
…				
10				
N = 10	$\sum_{i=1}^{N} {x_i}^2$	$\sum_{i=1}^{N} {x_i}^3$	$\sum_{i=1}^{N} {x_i}^4$	$\sum_{i=1}^{N} ({x_i}^2 * y_i)$

Die Variablen a, b und c der quadratischen Funktion werden wie folgt berechnet.

$$c = \frac{Q_{xy} * Q_{x^3} - Q_{x^2y} * Q_{xx}}{{Q_{x^3}}^2 - Q_{xx} * Q_{x^4}}$$

$$b = \frac{Q_{xy} - cQ_{x^3}}{Q_{xx}}$$

$$a = \frac{(\sum_{i=1}^{N} y_i) - (b * \sum_{i=1}^{N} x_i) - (c * \sum_{i=1}^{N} {x_i}^2)}{N}$$

Mit Hilfe der Kalibrierfunktion kann nun der Signalwert $\hat{y}_i$ der quadratischen Abschätzung berechnet werden.

$$\hat{y}_i = a + bx_i + cx_i^2$$

Die Unterschiede zu dem gemessenen Messsignal fließen wie folgt in die Berechnung der Reststandardabweichung der Kalibrierfunktion 2. Ordnung ein:

$$s_{y2} = \sqrt{\frac{\sum_{i=1}^{N}(y_i - \hat{y}_i)^2}{N-3}}$$

Weichen nun die Reststandardabweichungen 1. Ordnung s_{y1} und die der 2. Ordnung s_{y2} signifikant voneinander ab, so besteht kein linearer Zusammenhang zwischen Konzentration und Messwert. Die Signifikanz der Abweichung kann wieder durch einen F-Test berechnet werden mit folgendem Prüfwert:

$$PW = \frac{(N-2)s_{y1}^2 - (N-3)s_{y2}^2}{s_{y2}^2}$$

Der F-Wert wird untenstehender Tabelle entnommen, in unserem Fall beträgt er 12,25.

Freiheitsgrade n, m n = 1 m = N - 3	Irrtumswahrscheinlichkeit α Statistische Sicherheit P α = 0,01; P = 99 %
1	4052
2	98,50
3	34,12
4	21,20
5	16,26
6	13,75
7	12,25
8	11,26
9	10,56

Teil 3 – Probenauswertung, Prognoseintervall und Absicherung des unteren Arbeitsbereichs

Probenauswertung und Prognoseintervall

Nachdem nun die Abschätzung durch die lineare Kalibrierfunktion erfolgt ist, kann die reale Konzentration der Probe berechnet werden.

$$\hat{x} = \frac{\hat{y} - a}{b}$$

Aus den Berechnungen für die Reststandardabweichung lässt sich schon erahnen, dass auch der berechnete Wert fehlerbehaftet ist und um die Kalibriergerade streut. Der Bereich, in dem der wahre Konzentrationswert einer Probe liegen könnte. wird durch Prognosebänder oder auch Vertrauensbänder einer Kalibriergeraden wiedergegeben und ist unter anderem abhängig von der Reststandardabweichung der Kalibriergeraden und der Anzahl der Konzentrationsstufen N, sowie der Anzahl der Kalibriermessungen N_p. $y_{iu,o}$ sind dabei die Messsignale, die man durch die Streuung an einer bestimmten Konzentration x_i des Analyten erhalten kann, nach u unten und o oben gestreut in vertikaler Richtung.

$$y_{iu,o} = \hat{y}_i \pm s_y * t_{f,\alpha} * \sqrt{\frac{1}{N} + \frac{1}{\widehat{N_p}} + \frac{(x_i - \bar{x})^2}{Q_{xx}}}$$

Durch das Quantil der Studentschen t-Verteilung für eine zweiseitig Fragestellung $t_{f,\alpha}$ soll eine übliche statistische Unsicherheit von 5 % miteingerechnet werden. Der Wert ist einer Tabelle zu entnehmen und mit dem Freiheitsgrad N-2. Hier: Für N=10 liegt er bei 2,306.

Freiheitsgrad N - 2	Irrtumswahrscheinlichkeit α Statistische Sicherheit P α = 0,05; P = 95 %
1	12,706
2	4,303
3	3,182
4	2,776
5	2,571
6	2,447
7	2,365
8	2,306
9	2,262
10	2,228

Der wahre unbekannte Konzentrationswert liegt dann mit einer Sicherheit von 95 % in diesem Prognoseintervall oder auch Vertrauensband VB.

$$\hat{\hat{x}} \pm VB_x$$

$$VB_x = \frac{s_y}{b} * t_{f\alpha} * \sqrt{\frac{1}{N} + \frac{1}{\hat{N}_p} + \frac{(\hat{\bar{y}} - \bar{y})^2}{b^2 Q_{xx}}}$$

Nr. i	Berechneter Signalmittelwert $\hat{\hat{y}}_i$	Unterster Signalwert y_{iu}	Oberster Signalwert y_{io}	Berechneter Konzentrationsmittelwert $\hat{\hat{x}}_i$	Vertrauensbereich VB
1					
…					
10					
N = 10					

Absicherung des unteren Arbeitsbereichs

Aufgrund dieser Streuung der Werte kann es sein, dass der unterste Wert des Arbeitsbereiches x_u so stark fehlerbehaftet ist, dass er nicht mehr von einem Konzentrationswert von Null unterschieden werden kann. Man sagt deshalb, dass die unterste Konzentration des Arbeitsbereichs mindestens doppelt so groß sein soll wie das Vertrauensband. x_p ist dabei der Prüfwert zu der Konzentration, die mit einer Wahrscheinlichkeit von 95 % von einer Probe ohne Analyten unterschieden werden kann.

$$x_u > x_p = 2\,VB_x$$

Interessant ist auch der kritische Wert des Messsignals y_k, der noch von Null unterschieden werden kann, denn dieser Wert fließt in die Bestimmung der Nachweis, Erfassungs- und Bestimmungsgrenzen mit ein. Es ist das Vertrauensband über dem Achsenabschnitt *a*, der den Blindwert der Kalibration angibt.

$$y_k = a + s_y * t_{f\alpha} * \sqrt{\frac{1}{N} + \frac{1}{N_p} + \frac{\bar{x}^2}{\sum_{i=1}^{N}(x_i - \bar{x})^2}}$$

Hier wird die t-Verteilung für eine einseitige Fragestellung herangezogen, die aus der folgenden Tabelle entnommen werden kann.

Freiheitsgrad N - 2	Irrtumswahrscheinlichkeit α Statistische Sicherheit P α = 0,05; P = 95 %
1	6,314
2	2,92
3	2,353
4	2,132
5	2,015
6	1,943
7	1,895
8	1,86
9	1,833
10	1,812

b. Nachweis-, Erfassungs- und Bestimmungsgrenzen

Die Nachweisgrenze den Konzentrationswert x_{NG} an, der mit einer üblichen Wahrscheinlichkeit von 95 % von Null unterschieden werden kann. Wie oben schon erwähnt, wird dazu der kritische Wert genutzt.

$$x_{NG} = \frac{y_p - a}{b}$$

Durch die t-Verteilung wurde eine statistische Absicherung eingerechnet, die vermeidet, dass ein Wert verworfen wird, obwohl er wahr ist (α, Fehler 1.Art). Nun kann es aber auch passieren, dass ein Wert nicht verworfen wird, obwohl er falsch ist (β, Fehler 2.Art). Um das sicherzustellen, wird im Falle einer Detektion unterhalb der Nachweisgrenze neben dem Vermerk „n.d." für „nicht detektierbar" eine Konzentration angegeben, bis zu der man Nullwerte als „n.d." bezeichnet, obwohl dennoch der Analyt in der Probe vorhanden sein könnte. Diese Grenze wird Erfassungsgrenze x_{EG} genannt und ist die Nachweisgrenze zuzüglich eines Vertrauensintervalls, gegeben durch:

$$x_{EG} = x_{NG} + \frac{s_y}{b} * t_{f,\beta} * \sqrt{\frac{1}{N} + \frac{1}{N_p} + \frac{\bar{x}^2}{Q_{xx}}}$$

Ist α genauso groß wie β, so kann die Gleichung wie folgt vereinfacht werden:

$$x_{EG} = 2 * x_{NG}$$

Die Bestimmungsgrenze gibt nun an, ab welcher Konzentration der Analyt in einer Probe quantifiziert werden kann und berechnet sich aus folgender Gleichung, wobei die relative Ergebnisunsicherheit k einen üblichen Wert von 3 annimmt.

$$x_{BG} = k * \frac{s_y}{b} * t_{f,\alpha} * \sqrt{\frac{1}{N} + \frac{1}{N_p} + \frac{(k * x_{NG} \quad \bar{x})^2}{Q_{xx}}}$$

Die Analyseergebnisse werden nach DIN 32 645 folgendermaßen angegeben:

Tabelle 4 — Angabe von Analyseergebnissen und Entscheidungsgrenzen

Ergebnis	Angabe	Zusatzangabe
$x \geq x_{BG}$	Gehalt	Vertrauensbereich[a]
$x_{NG} \leq x$	nachgewiesen	
$x < x_{NG}$	nicht nachgewiesen	Höchstgehalt x_{EG}

[a] Falls der Vertrauensbereich in der Analysenvorschrift angegeben ist, kann diese Zusatzangabe entfallen.

c. Richtigkeit

Um Matrixeinflüsse auf das Messverfahren zu beurteilen, wird zusätzlich die Wiederfindungsrate bestimmt. Dabei wird eine reale Probe gemessen und die Konzentration $\hat{x}_0$ berechnet. Danach wird die Probe um ungefähr den gleichen Betrag aufgestockt, gemessen und die Konzentration $\hat{x}_A$ bestimmt. Der Quotient aus der Differenz der berechneten Werte und der eigentlich hinzugefügten Konzentration Δx bildet die Wiederfindung.

$$WFR = \frac{\hat{x}_A - \hat{x}_0}{\Delta x} * 100$$

Idealerweise ist sie 100 %, d.h., dass durch die Probenaufbereitung oder die Matrix der Analyt nicht verloren gegangen ist.

Akzeptierte Werte für die Wiederfindung sind konzentrationsabhängig und zum Beispiel durch die AOAC vorgegeben. (AOAC international – The Scientific Association Dedicated to Analytical Excellence®)

Table 5. Acceptable recovery percentages as a function of the analyte concentration [8]

Analyte %	Analyte ratio	Unit	Mean recovery (%)
100	1	100%	98–102
10	1.00E – 01	10%	98–102
1	1.00E – 02	1%	97–103
0.1	1.00E – 03	0.10%	95–105
0.01	1.00E – 04	100 ppm	90–107
0.001	1.00E – 05	10 ppm	80–110
0.0001	1.00E – 06	1 ppm	80–110
0.00001	1.00E – 07	100 ppb	80–110
0.000001	1.00E – 08	10 ppb	60–115
0.0000001	1.00E – 09	1 ppb	40–120

Tabelle kopiert aus:

Tavernier et al., 2004: *Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance*, Trends in Analytical Chemistry, Vol. 23 (8), pp. 535-552.
Cites: L. Huber (Ed.), Validation and Qualification in Analytical Laboratories, Interpharm Press, East Englewood, CO, USA, 1998.

III. Messung der realen Proben

a. Berechnung des Konzentrationswertes und des Vertrauensbereichs

Die Berechnung erfolgt wie im Kapitel „II.a Teil 3 – Probenauswertung, Prognoseintervall und Absicherung des unteren Arbeitsbereichs“ beschrieben

b. Dokumentation der Ergebnisse

Auswertungsbogen

Datum:……

Informationen über das Messverfahren: Probenursprung und -aufbereitung, Messinstrument, Detektion, Auswertung, Operator

Nr.	Probe	Inhalt	Sonstiges	gemessene Konzentration
				(inkl. VB, falls nötig; Angabe siehe oben „Tabelle 4“)

Wichtige Verfahrenskenngrößen		Wert
Wiederfindungsrate *WFR* Nachweisgrenze *NG* Erfassungsgrenze *EG* Bestimmungsgrenze *BG* Kritischer Wert y_k		
Reststandardabweichung s_y	(Präzision)	
Verfahrensstandardabweichung s_{x0}	(Leistungsfähigkeit des Verfahrens)	
Variationskoeffizient V_{x0}	(Relative Leistungsfähigkeit)	
Empfindlichkeit *b* Blindwert *a*		
Arbeitsbereichsüberprüfung - lineare Kalibrierfunktion $y = a + bx$ - varianzhomogen - Standardabweichung s_i innerhalb der akzeptierten Grenzen (Horwitz) - Anzahl der Ausreißer - Anzahl der Messungen *N* - Anzahl der Parallelmessungen N_p		

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Anhang

- f-Verteilung (aus: http://www.mathematik.uni-kl.de/~stockis/Tabelle6.pdf, zuletzt abgerufen am 09.Juli 2012)
- t-Verteilung (aus: Praktikum Analytische Chemie Im Bachelor-Studiengang *Wasser: Chemie, Analytik, Mikrobiologie,* Praktikumsleiter: Dr. Denkhaus, Dr. Krohn, Dr. Telgheder,2003 http://lims.uni-duisburg.de/Lehre/Material/PrAnalytik/Statistik.pdf, zuletzt abgerufen am 09.Juli 2012)
- DIN 38 402 und DIN 32 645

Tabelle 6 b: **0.99-Quantile der F-Verteilung mit (n,m) Freiheitsgraden**

	n:	1	2	3	4	5	6	7	8	9
m:										
1		4052	4999.5	5403	5625	5764	5859	5928	5981	6022
2		98.50	99.00	99.17	99.25	99.30	99.33	99.36	99.37	99.39
3		34.12	30.82	29.46	28.71	28.24	27.91	27.67	27.49	27.35
4		21.20	18.00	16.69	15.98	15.52	15.21	14.98	14.80	14.66
5		16.26	13.27	12.06	11.39	10.97	10.67	10.46	10.29	10.16
6		13.75	10.92	9.78	9.15	8.75	8.47	8.26	8.10	7.98
7		12.25	9.55	8.45	7.85	7.46	7.19	6.99	6.84	6.72
8		11.26	8.65	7.59	7.01	6.63	6.37	6.18	6.03	5.91
9		10.56	8.02	6.99	6.42	6.06	5.80	5.61	5.47	5.35
10		10.04	7.56	6.55	5.99	5.64	5.39	5.20	5.06	4.94

Quantil der Studentschen t-Verteilung (einseitige Fragestellung)

f	$\alpha = 0{,}10$ P = 90 %	$\alpha = 0{,}05$ P = 95 %	$\alpha = 0{,}01$ P = 99 %	$\alpha = 0{,}005$ P = 99,5 %	$\alpha = 0{,}001$ P = 99,9 %
1	3,078	6,314	31,821	63,656	318,289
2	1,886	2,920	6,965	9,925	22,328
3	1,638	2,353	4,541	5,841	10,214
4	1,533	2,132	3,747	4,604	7,173
5	1,476	2,015	3,365	4,032	5,894
6	1,440	1,943	3,143	3,707	5,208
7	1,415	1,895	2,998	3,499	4,785
8	1,397	1,860	2,896	3,355	4,501
9	1,383	1,833	2,821	3,250	4,297
10	1,372	1,812	2,764	3,169	4,144
11	1,363	1,796	2,718	3,106	4,025
12	1,356	1,782	2,681	3,055	3,930
13	1,350	1,771	2,650	3,012	3,852
14	1,345	1,761	2,624	2,977	3,787
15	1,341	1,753	2,602	2,947	3,733
16	1,337	1,746	2,583	2,921	3,686
17	1,333	1,740	2,567	2,898	3,646
18	1,330	1,734	2,552	2,878	3,610
19	1,328	1,729	2,539	2,861	3,579
20	1,325	1,725	2,528	2,845	3,552
21	1,323	1,721	2,518	2,831	3,527
22	1,321	1,717	2,508	2,819	3,505
23	1,319	1,714	2,500	2,807	3,485
24	1,318	1,711	2,492	2,797	3,467
25	1,316	1,708	2,485	2,787	3,450
26	1,315	1,706	2,479	2,779	3,435
27	1,314	1,703	2,473	2,771	3,421
28	1,313	1,701	2,467	2,763	3,408
29	1,311	1,699	2,462	2,756	3,396
30	1,310	1,697	2,457	2,750	3,385
40	1,303	1,684	2,423	2,704	3,307
50	1,299	1,676	2,403	2,678	3,261
60	1,296	1,671	2,390	2,660	3,232
70	1,294	1,667	2,381	2,648	3,211
80	1,292	1,664	2,374	2,639	3,195
90	1,291	1,662	2,368	2,632	3,183
100	1,290	1,660	2,364	2,626	3,174
200	1,286	1,653	2,345	2,601	3,131
300	1,284	1,650	2,339	2,592	3,118
400	1,284	1,649	2,336	2,588	3,111
500	1,283	1,648	2,334	2,586	3,107
1000	1,282	1,646	2,330	2,581	3,098
∞	1,282	1,645	2,326	2,576	3,090

Quantil der Studentschen t-Verteilung (zweiseitige Fragestellung)

f	$\alpha = 0{,}10$ P = 90 %	$\alpha = 0{,}05$ P = 95 %	$\alpha = 0{,}01$ P = 99 %	$\alpha = 0{,}005$ P = 99,5 %	$\alpha = 0{,}001$ P = 99,9 %
1	6,314	12,706	63,656	127,321	636,578
2	2,920	4,303	9,925	14,089	31,600
3	2,353	3,182	5,841	7,453	12,924
4	2,132	2,776	4,604	5,598	8,610
5	2,015	2,571	4,032	4,773	6,869
6	1,943	2,447	3,707	4,317	5,959
7	1,895	2,365	3,499	4,029	5,408
8	1,860	2,306	3,355	3,833	5,041
9	1,833	2,262	3,250	3,690	4,781
10	1,812	2,228	3,169	3,581	4,587
11	1,796	2,201	3,106	3,497	4,437
12	1,782	2,179	3,055	3,428	4,318
13	1,771	2,160	3,012	3,372	4,221
14	1,761	2,145	2,977	3,326	4,140
15	1,753	2,131	2,947	3,286	4,073
16	1,746	2,120	2,921	3,252	4,015
17	1,740	2,110	2,898	3,222	3,965
18	1,734	2,101	2,878	3,197	3,922
19	1,729	2,093	2,861	3,174	3,883
20	1,725	2,086	2,845	3,153	3,850
21	1,721	2,080	2,831	3,135	3,819
22	1,717	2,074	2,819	3,119	3,792
23	1,714	2,069	2,807	3,104	3,768
24	1,711	2,064	2,797	3,091	3,745
25	1,708	2,060	2,787	3,078	3,725
26	1,706	2,056	2,779	3,067	3,707
27	1,703	2,052	2,771	3,057	3,689
28	1,701	2,048	2,763	3,047	3,674
29	1,699	2,045	2,756	3,038	3,660
30	1,697	2,042	2,750	3,030	3,646
40	1,684	2,021	2,704	2,971	3,551
50	1,676	2,009	2,678	2,937	3,496
60	1,671	2,000	2,660	2,915	3,460
70	1,667	1,994	2,648	2,899	3,435
80	1,664	1,990	2,639	2,887	3,416
90	1,662	1,987	2,632	2,878	3,402
100	1,660	1,984	2,626	2,871	3,390
200	1,653	1,972	2,601	2,838	3,340
300	1,650	1,968	2,592	2,828	3,323
400	1,649	1,966	2,588	2,823	3,315
500	1,648	1,965	2,586	2,820	3,310
1000	1,646	1,962	2,581	2,813	3,300
∞	1,645	1,960	2,576	2,807	3,290

Annex II

Curriculum Vitae

Since 2012	Start of the research project "Photocatalytic degradation of environmentally harmful solvents from bio-butanol" in cooperation with prosys° Gesellschaft für Produktionsintegrierte Umweltsystemtechnologien und -management mbH and Merck KGaA. This project is funded by the German federal state of Bremen in the programme Angewandte Umweltforschung (AUF)
2008 - 2011	Research fellowship from the Universität Bremen on "Structural optimisation of ionic liquids by systematic investigations on their biological degradability and development of an applied regeneration process" and the beginning of the presented studies at the centre for environmental research and sustainable technologies (UFT) at the Universität Bremen (Germany)
2006 - 2008	International Master of Science Programme (M.Sc.): WASTE "WASTE - Air Quality Control, Solid Waste and Waste Water Process Engineering" Universität Stuttgart (Germany) Master thesis: "*Analytical Determination and Characterisation of Potential Metabolites generated by the Biodegradation of Poly(ε-Caprolactone) (PCL) under Denitrifying Conditions*"; Department of Biology, Chair of Hydrochemistry and Hydrobiology, Institute for Sanitary Engineering, Water Quality and Solid Waste Management (ISWA), Stuttgart (Germany); supervisor: Dr.-Ing. W.-R. Müller
2005	Work placement at the Max Planck Institute of Biochemistry, Martinsried (Germany); examination of the viability of cells after cryofixation (culturing, dyeing and light microscopy of E. coli); department of molecular structural biology; director: Prof. Dr. W. Baumeister; supervisor: Dr. A. Leis
2002-2005	International Bachelor of Science Programme (B.Sc.): Water "Water: Chemistry, Analytics, Microbiology" Universität Duisburg-Essen (Germany) Bachelor thesis: "*Assembly and Production of the Shell of Euglypha Rotunda (Testacea: Protozoa)*"; School of Biosciences, University of Birmingham (UK); supervisors: Dr. BSC Leadbeater (UK) and Prof. Dr. H.-C. Flemming (Germany); funding: German Academic Exchange Service (DAAD)
2001-2002	*European Voluntary Service (EVS)/Diakonisches Jahr im Ausland (DJiA)* in La Glanerie (Belgium); educational work at *Dimension 7 asbl*, an asylum for children from disfavoured environments
1992-2001	Secondary education and upper school: Sophie-Scholl Gymnasium in Oberhausen (Germany). Major subjects: English and Maths
1988-1992	Primary school: Grundschule Schwarze Heide in Oberhausen (Germany)
19.05.1982	Born in Oberhausen, Rheinland, (Germany) as the second daughter of Wolfgang and Marianne Schmeier

Korrekturen:

Zusätzliches Titelblatt wurde entfernt, wodurch die Seitenzahlen um zwei niedriger sind als in der vorherigen Version.

Die Nennung der Erst- und Zweitgutachter und das Datum des Kolloquiums wurden hinzugefügt.

Seite IX:

Zeile 7 von unten – Streichung des Wortes „zeigten“

Zeile 6 von unten: Statt „Nitrilhydratase“ „Nitril-Hydratase“

Angaben zu den Publikationen wurden wie folgt geändert:

Seite 67

“Neumann, J., *Pawlik, M., Bryniok, D., Thöming, J. & Stolte, S. Biodegradation potential of cyano-based ionic liquid anions in a culture of Cupriavidus spp. and their in vitro enzymatic hydrolysis by nitrile hydratase. Environ. Sci. Pollut. Res. Int. (2013). doi:10.1007/s11356-013-2341-2*

December 2013:
The final publication is now available at the SpringerLink platform:
http://link.springer.com/article/10.1007/s11356-013-2341-2.

This thesis includes the version that was accepted for publication in Environmental Science and Pollution Research in October 2013.”

Seite 109

“A revised version of the article was accepted for publication in Green Chemistry in January 2014. The revised article will finally be found on the RSC website.

This thesis includes the version that was submitted to Green Chemistry in October 2013.”