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Catalytically active perrhenate based ionic liquids: A preliminary ecotoxicity and biodegradability assessment

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Recently it was shown that water- and air stable perrhenate based ionic liquids (ILs) are promising catalysts for oxidation reactions. For a broader application and in context of green chemistry it is important to study in addition to their technical performance also their potential impact on environment and human health. This paper presents the first account on (eco)toxicological and biodegradation data for a set of ammonium and imidazolium based IL with perrhenate anion.

Introduction

Ionic liquids (ILs) are salts with melting points below 100 °C that are formed solely by cations and anions, one or both of which are weakly coordinating ions. In recent years ILs have attracted considerable interest, which has led to an impressive number of > 60,000 publications, including more than 9,000 patents.[1] The burgeoning number of publications and the still increasing interest in ILs is mainly due to the physicochemical properties of ILs, for instance, their often high electrochemical and thermal stability, wide range of viscosity, low vapour pressure, and favourable solvation capabilities. These properties make them interesting in different fields of research and application. They are nowadays used in small-scale to pilot-plant to large-scale industrial applications.[2] The combinability of their different components has led to a vast number of ILs: millions of structures are theoretically accessible, several thousand have been synthesised, and a few hundred are commercially available to date. The structural variability permits systematic investigations and a structural design allowing the adaptation of ILs with improved property profiles for certain applications.[3]

While ILs are very well examined and applied as reaction media, their properties as reactants or catalysts still play only a minor role. Recently, we and other groups reported the synthesis[4,5] and the application of water and air stable imidazolium-based ILs with the perrhenate anion ($[ReO_4]^-$) as surprisingly efficient, selective and reusable catalysts for the oxidation of sulfides to sulfoxides.[6] Further, it was shown that imidazolium perrhenates mediate the epoxidation of unfunctionalized olefins.[7,8]

Currently, research is directed towards the design of novel metal-containing ILs with optimised catalytic properties in compliance with the basic principles of green chemistry.[9] The optimisation of the technological properties of a substance should always be investigated together with the minimisation of its (eco)toxicological hazard potential. This approach allows focusing on environmentally benign structures already in the early steps of the research and development process.

During the past few years, more and more data on ILs' (eco)toxicology, biodegradability and environmental fate was becoming available and several hundred papers have been published on these aspects in the recent past - summarised in several review papers.[10–17] These studies have shown big variations in the hazard potential of ILs. Furthermore, it appears that structural design of ILs with a reduced hazard is feasible.

This study is the first report on the ecotoxicity and biodegradability of a set of ammonium- and imidazolium-based $[ReO_4]^-$ ILs (Figure 1) which have shown good catalytic activity[7,8]. These investigations aim to derive qualitative structure-activity relationship that can be used for the design of new ILs with improved properties both from a catalytic and a hazard point of view. For the investigations described here tests with an enzyme (acetylcholinesterase inhibition), leukaemia cells from rats (IPC-81), limnic green algae (*Senedesmus vacuolatus*) and water fleas (*Daphnia magna*) were applied. These tests have been proven useful for determining the acute toxicological hazards of ILs[18–21] Furthermore, the biodegradability of test compounds using biochemical oxygen demand (BOD) tests has been examined.

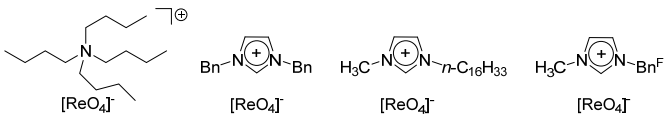

Figure 1. Illustration of the ammonium- and imidazolium perrhenates used in the study: $[NBu_4][ReO_4]$ (**1**), [BnBnIm][ReO_4] (**2**), [MeHDecIm][ReO_4] (**3**), [$MeBn^FIm$][ReO_4] (**4**) (Bn = benzyl, Me = methyl, HDec = Hexadecyl, Bn^F = 2,3,4,5,6-pentafluorobenzyl, Im = imidazolium).

Results and discussion

The results of enzyme inhibition, cytotoxicity and aquatic toxicity (Table 1) are expressed as half maximal inhibitory (IC_{50}) or effective concentrations (EC_{50}). Imidazolium-based ILs were selected as reference compounds for lower (1-Ethyl-3-methylimidazolium chloride, [MeEthylIm]Cl]) and higher (1-Methyl-3-octylimidazolium chloride, [MeOctylIm]Cl) acute cation toxicity (Table 1). To assess the influence of the ReO_4^- anion on IL toxicity the obtained data were compared with the reference compound $[NH_4][ReO_4]$.

Comparison of test systems and effect levels

The EC_{50} and IC_{50} values (Table 1) generally span five orders of magnitude ranging between 0.034 µM (11.6 µg L^{-1}) found for [MeHDecIm]Cl towards *Daphnia magna* and 7400 µM (1.99 g L^{-1}) determined for $[NH_4][ReO_4]$ with green algae. Comparison of the results of the four different bioassays shows that the water flea *Daphnia magna* and the liminc green algae *Scenedesmus vacuolatus* are equally sensitive, whereas the cytotoxicity assay with IPC-81 is the least sensitive test in most of the cases. These differences in sensitivities are in good agreement with observations made in recent studies on ILs.[20,22,23] For an efficient screening of novel ILs the algae assays appears to be most suitable in terms of time and costs.

Effects of the IL cations

The halides (chloride and bromide) - tested as alkali salts - do not show an intrinsic anion effect in the tested concentration range of up 10 mM (data not shown). Thus the observed toxic activities of ILs with halides as counter ion can be assumed to be attributed to the cation alone. The enzyme inhibition test with acetylcholinesterase (AChE) is an important biological marker in (eco)toxicology for evaluating the influence of toxicants on the central nervous system of organisms. It is known that IL cations can strongly inhibit this enzyme and it was found that a delocalized electron-deficient aromatic system as well as a certain lipophilicity are the key features defining their inhibitory potential.[24] Accordingly, the here investigated cations (associated with bromide anions) show relatively strong inhibition potential ranging mostly between [MeEthylIm]Cl and [MeOctylIm]Cl. For $[NBu_4]^+$ a slightly reduced inhibition was observed probably due to the lack of an aromatic ring system. For [MeHDecIm]Cl an increased inhibition was observed most likely originating from the high hydrophobicity of the -hexadecyl side chain. The general interdependence of hydrophobicity and toxicity - also termed baseline toxicity or narcosis - of IL cations has been reported for several biological test systems inter alia IPC-81 cells and *Scenedesmus vacuolatus*.[25,26] This has led to the rule of thumb that the more pronounced the hydrophobicity of a cation, the higher the observed acute toxic effect. The underlying mode of toxic action is called baseline toxicity and relies on non-specific interactions with membrane systems.[27] In this context experimentally determined (HPLC)[25] or computationally derived hydrophobicity parameter ($logk_0$)[28] have been proven useful to estimate the toxicity of an IL cation.

Table 1. List of IC_{50}, EC_{50} and $logk_0$ values

Substance	AChE[1] IC_{50} [µM]	IPC-81	Algae EC_{50} [µM] (confidence intervals)	Daphnia	hydropobicity parameter ($logk_0$) of the cation
[$MeBn^FIm$][ReO_4] (4)	134 (126-142)	1009 (818-1457)	146 (77-317)	199 (159-221)	1.5[8]
[$MeBn^FIm$]Br	128 (124-133)	6714 (6173-7350)	1490 (871-2940)	254 (233-276)	
[BnBnIm][ReO_4] (2)	38 (36-39)	112.2 (101-129)	6.3 (3.6-11.2)	21 (19-24)	2.0[8]
[BnBnIm]Br	32 (30-34)	95 (88-103)	18 (7-51)	27 (24-30)	
$[NBu_4][ReO_4]$ (1)	124 (119-129)	194 (171-219)	59 (28-145)	41 (33-45)	2.28[9]
$[NBu_4]$Br	197 (176-221)	154 (142-166)	69 (35-132)	23 (19-26)	
[MeHDecIm][ReO_4] (3)	9.5 (9.0-10.2)	2.5 (2.4-2.7)	0.096 (0.055-0.167)	0.045	3.77[9]
[MeHDecIm]Cl	4.8[2] (4.5-5.1)	0.57 (0.52-0.64)	0.098 (0.05-0.197)	0.034	
$[NH_4][ReO_4]$	>2358[3]	>5145[3]	7400 (5100-12000)	>2500[3]	
[MeEthylIm]Cl	115[4] (106 - 125)	9900 (6000 - 23000)	602[6] (531 - 685)	770[3] (720 - 810)	< 0.42[10]
[MeOctylImMeOctyllIm]Cl	39[4] (37 - 42)	102[5] (91 - 115)	0.0021 (0.00087- 0.00479)[6]	0.0025[7]	1.85[10]

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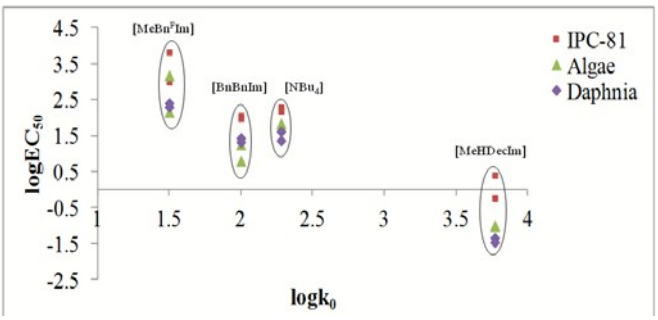
[1] AChE: acetylcholinesterase; data taken from: [2] Arning et al. 2008; [3] Steudte et al. 2014;
[4] Stock et al. 2007; [5] Ranke et al 2007; [6] Stolte et al 2007; [7] Couling et al. 2005; [8] calculated according to Cho et al. 2013; [10] Ranke et al. 2009

This tendency can be confirmed by plotting the logEC_{50} (Table 1) towards IPC-81, algae and daphnia against the logk_0 values of investigated cations (Figure 2). The tetrabutylammonium cation, however, appears to exhibit a slightly lower toxicity than expected from its hydrophobicity. This might be due to steric effects, since the positive charge is shielded by four butyl side chains. This might reduce the intercalation into membranes in comparison to e.g. linear surfactant-like 1-methyl-3-octylimidazolium cations (one hydrophobic tail and a polar head group). If the results obtained for tested cations are compared to those of the reference ILs, it can be noted that the toxicities towards IPC-81, algae and daphnia are in most cases distinctly higher than the corresponding toxicity found for [MeEthylIm]Cl and lower than or similar to those of the long-chain [MeOctylIm]Cl. According to GHS regulations,[29] the results from tests with water fleas (Table 2) demonstrate a very high acute aquatic toxicity of [MeHDecIm]Cl (category I). The other halides:[BnBnIm]Br and [NBu_4]Br are in category II and [$MeBn^FIm$]Br in III - indicating a certain hazard for aquatic organisms.

Figure 2. The correlation between toxicity (log EC_{50}) and lipophilicity (logk_0) for all test compounds and different species.



Effects of the $[ReO_4]^-$ anion

The influence of the $[ReO_4]^-$ anion on toxicity is identified by comparing the IC_{50}s and EC_{50}s of ILs with the same cations, but varying anions ($[ReO_4]^-$ vs. chloride/bromide). Taking the confidence intervals into account, the determined values (Table 1) were similar or in the same order of magnitude in most of the cases indicating a minor contribution of the $[ReO_4]^-$ anion on the ILs' toxicity. These results are supported by the (recently demonstrated) low acute (eco)toxicity of our reference compound NH_4ReO_4 that shows high IC_{50}s/EC_{55}s of > 2.5 mM (Table 1).[30] However, larger deviations were found for IL **4**, i.e. higher toxicity towards IPC-81, green algae and the water fleas than the corresponding bromide. In the case of the algae the EC_{50} of the perrhenate was one order of magnitude smaller than that of [$MeBn^FIm$]Br. This example shows that the approach, which considers ILs as two separately acting species - the cation and the anion - is not valid for all IL combinations. Such an approximation does not consider interactions between cation and anion.

Table 2. Categories of acute aquatic toxicity.

Substance	*Daphnia magna*		GHS* category of acute aquatic toxicity
	EC_{50} [µM]	EC_{50} [mg L^{-1}]	
[$MeBn^FIm$][ReO_4] (**4**)	199	102	without
[$MeBn^FIm$]Br	254	87	III
[BnBnIm][ReO_4] (**2**)	21	11	III
[BnBnIm]Br	27	9	II
[NBu_4][ReO_4] (**1**)	41	20	III
[NBu_4]Br	23	7	II
[MeHDecIm][ReO_4] (**3**)	0.045	0.017	I
[MeHDecIm]Cl	0.034	0.011	I
[NH_4][ReO_4]	> 2500	> 670	without
[MeEthylIm]Cl	770	113	without
[MeOctylIm]Cl	0.0025	0.006	I

*Acute Cat. I $EC_{50} \leq 1.00$ mg L^{-1}, Acute Cat. II 10.0 mg $L^{-1} \geq EC_{50} > 1.00$, Acute Cat. III 100.0 mg $L^{-1} \geq EC_{50} \geq 10.0$ mg L^{-1}

In a recent parallel study, we have shown that the perrhenate anion exhibits a significant degree of contact to protons of organic cations via O–H bonds in both solution and the solid state, in some cases forming supramolecular ion pairs.[31] In the case of the imidazolium perrhenates investigated here, perrhenate forms the strongest contact with the proton at the C2 position of the imidazolium ring. Further, in the case of fluorinated benzyl groups perrhenate also interacts both with acidic methylene protons and the fluorine atoms at the phenyl ring. It is assumed that due to these interactions between cation and anion their bioavailability and hence their biological effects might be influenced. In a recent study it was shown that the toxicological effects of cations and anions, described in terms of mixture toxicity and distinct cation–anion combinations, led to either synergistic[19,32] or antagonistic effects.[33]

However, the results point to a low intrinsic acute ecotoxicity of the perrhenate anion. Due to this and the relatively high molecular mass (250 g mol^{-1}) in comparison to halides also the categories of acute aquatic toxicity (based on mg L^{-1}) are shifted by one category for the same cation. However, combined with $[ReO_4]^-$ instead of Cl^-/Br^- only IL **3** remains in category I - here the pronounced effects of the cation overrules

by far changes in classification that are only based on differences in molecular masses.

Biodegradability of cations

The biodegradability is a key parameter in the hazard assessment of chemicals and is of the high concern for environmental considerations. A high biodegradability of chemicals implies a reduced tendency to bio-accumulate or to persist in the environment. Moreover, exposure and long-term adverse effects of chemicals on biota is largely influenced by their biodegradability.

For the first time the aerobic biodegradability of these cations with halide was investigated over a period of 28 days using an inoculum from a domestic wastewater treatment plant (WWTP). For none of the cations significant degradation was determined (all < 5%; table 1). Hence, the rapid biodegradation of these chemicals in WWTP cannot generally be assumed. The recalcitrance of the imidazolium core substituted with short alkyl side chains[34,35] or aromatic substituents was shown before.[36] 1-alky-3-methyl-imidazolium compounds with long alkyl side chains (e.g. -octyl) show a certain degradability of the chain, but the core itself remains resistant towards biodegradation.[37] In this context the hexadecyl side chain of IL **3** was expected to be biodegradable. In order to determine if the lack of biodegradation was due to inherent properties of the structure and not a result of toxic inhibition we examined the degradability of readily biodegradable benzoic acid in presence of each IL. The positive control with only benzoic acid showed high degree of mineralisation (over 50% of BOD reduction after 5 days) whereas in presence of 15 mg L^{-1} [MeHDecIm]Cl it was completely inhibited indicating the high toxicity of this compound towards the microbial community. The strong inhibition potential of long chained imidazolium compounds towards WWTP microorganism was recently described.[38] None of the other test substances inhibited significantly the biodegradability of the reference compound (benzoic acid), indicating that other ILs are not toxic towards the micro-organisms in the concentration range tested.

Table 3. Biodegradation and inhibition of microbial community

Substance	Biodegradation in %	Inhibition of benzoic acid degradation
[MeBn^{F}Im]Br	<5	not significant
[BnBnIm]Br	<5	not significant
[NBu_4]Br	<5	not significant
[MeHDecIm]Cl	<5	complete

Conclusions

This paper describes the first assessment of the (eco)toxicity and biodegradability of a set of perrhenate based ionic liquids. The initial evaluation of the hazard potential of these ionic liquids has shown that the perrhenate anion does not contribute significantly to their toxicity and that effects are mainly driven by the cation. In general the $[ReO_4]^-$ (combined with a none toxic cation) exhibits a low intrinsic acute toxicity, much lower than effects found for other established IL anions such as $(CF_3SO_2)_2N^-$ or $[(C_2F_5)_3PF_3^-$ [39] and similar to e.g. octyl sulfate.[20]

The cations investigated and used so far for oxidation of sulfides[6] as well as for the epoxidation of olefins[7,8] are from (eco)toxicological point of view less favorable. In particular ILs with a hydrophobic cation such as 1-hexadecyl-3-methylimidazolium showed strong effects towards the investigated aquatic organisms (EC_{50} towards daphnids 0.034 µM). Other cations exhibited moderate toxic effects, however, none of them was found to be readily biodegradable. According to the benign by design approach less toxic and more biodegradable cations should be used, among them ammonium based compounds[40–42] or pyrrolidinium, pyridinium, piperidinium and morpholinium cations with short and polar side chains – favorable in terms of ecotoxicity[22] – that have been proven to be readily or at least inherently biodegradable (low or reduced risk of persistence).[35] If hydrophobic (and possibly more toxic) cations might be needed from application point of view at least compounds with better biodegradation potential such as pyridinium based ILs[43] would be preferred over the less biodegradable imidazolium head group.

The proposed design criteria may lead to ILs with improved biodegradability and ecotoxicological properties, but they may also limit the applicability for catalysis purposes. Further examinations in these directions are currently under way in our laboratories.

Experimental

Chemicals and reagents

The preparation of ammonium- and imidazolium bromides used as precursors for the respective perrhenate salts was performed according to literature procedures.[44] The general synthesis of ammonium-, 1-methyl-3-alkylimidazolium perrhenates and benzyl-substituted imidazolium perrhenates was published elsewhere.[5] In a standard procedure the bromide salt (0.08 mmol) solution in water (10 ml) is slowly added on an ion exchange resin (pH = 7). The product is washed slowly with water off the column until the pH of the eluent remains equal to 7, affording the desired product (c=0.26 mol/L). Subsequently, 1.2 equiv. of $[NH_4][ReO_4]$ are added and the solution is heated to 70 °C for 24 h, followed by a complete removal of the residual water under reduced pressure. The excess of $[NH_4][ReO_4]$ is removed by washing the ionic liquid with acetone (30 ml) to afford the pure product.

Cell culture media, sera, and phosphate buffer were purchased from GIBCO BRL Life Technologies (Eggenstein, Germany). *Daphnia magna* were purchased from MicroBioTest Incorporation (Gent, Belgium). Antibiotics and glutamine were obtained from PAA Laboratories (Cölbe, Germany), and the WST-1 reagent was purchased from Roche Diagnostics (Mannheim, Germany). NaCl, KNO_3, $Ca(NO_3)_2$*$4H_2O$, KH_2PO_4, K_2HPO_4, MgSO4*$7H_2O$, H_3BO_3, $ZnSO_4$*$7H_2O$, Na_2MoO_4*$2H_2O$, $MnCl_2$*$4H_2O$, $FeCl_3$*$6H_2O$, Tritiplex III, NaH_2PO_4*H_2O, Na_2HPO_4*$2H_2O$, $MgSO_4$*$7H_2O$,

$CaCl_2$*$2H_2O$, $FeSO_4$*$7H_2O$, (Na_2–EDTA) and $(NH_4)_2Mo_7O_{24}$*$4H_2O$ used for the culturing media were purchased from Sigma–Aldrich (Germany).

Toxicity Data

Acetylcholinesterase inhibition assay

The inhibition of acetylcholinesterase (AChE) was determined by a colorimetric assay based on the reduction of the dye 5,5 -dithio-bis-(2 nitrobenzoic acid) (DTNB) by the thiocholine moiety enzymatically formed from the AChE substrate acetylthiocholine iodide. [18] Basically, dilution solutions of the test substances in phosphate buffer (0.02 M, pH 8.0) composing of max. 1% methanol was prepared directly in the wells of a 96-well microwell plate. DTNB (2 mM, 0.185 mg $mL^{-1}NaHCO_3$ in phosphate buffer pH 8.0) and the enzyme (0.2 U mL^{-1}, 0.25 mg mL^{-1} bovine serum albumin in phos-phate buffer pH 8.0) were added to each well. The reaction was started by the addition of acetylthiocholine iodide (2 mM in phosphate buffer). The final test concentrations were 0.5 mM of DTNB and acetylthiocholine iodide and 0.05 U mL^{-1} acetylcholinesterase respectively. Each plate contained blanks (no enzyme) and controls (no toxicant). Enzyme kinetics were determined at 405 nm at 30s intervals in a microplate-reader (MRX Dynatech) for 5 min. The enzyme activity was measured as the slope of optical density (in OD min^{-1}) from a linear regression.

Cell viability assay

The cytotoxicity assay using the WST-1 reagent was described by Ranke et al.[45] Briefly, promyelotic rat cells from the IPC-81 cell line are incubated for 4h in 96-well plates with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) reagent. Each plate contained 8 wells for blank and 16 wells for controls (no toxicant). Each compound carried out with triplicates and diluted 1:1 for 9 parallel dilution series. 1% dimethylsufoxide (DMSO), proven to have no effect on cells, was added to improve the solubility of these compounds. After preparation of dilution, each well contained 50µL of substances solution and 50 µL of cell concentration of $15*10^5$ cell mL^{-1} (in RPMI with 16%HS). After keeping in incubator for 48h (37 °C, 5 % CO_2), 10 µL of WST-1 (diluted 1:4 with phosphate buffer) were added and the samples were kept in incubator for another 4 hours before scan at 450 nm in a microplate reader (MRX Dynatech). Positive controls with carbendazim were checked in regular intervals.

Reproduction inhibition assay with limnic green algae

For this assay, the unicellular limnic green algae *Scenedesmus vacuolatus* (strain 211-15, SAG (Culture Collection of Algae), University Goettingen, Germany) were used, and toxicity tests were done using a synchronized culture. The stock culture was grown under photoautotrophical conditions at 28 °C (±0.5 °C) in an inorganic sterilized medium (pH 6.4) with saturating white light (intensity of 22−33 kilolux) (Lumilux Daylight L 36 W-11 and Lumilux Interna L 36 W-41, Osram, Berlin, Germany). Cells were aerated with 1.5 vol % CO_2 and synchronized by using a 14 h light and 10 h darkness cycle. The stock culture was diluted every day to a cell density of 5×10^5 cells mL^{-1}. The toxicity tests started with autospores (young algal cells at the initial growth cycle). Algae were exposed the test substances for one growth cycle (24 h). The endpoint of this assay is inhibition of algal reproduction measured as inhibition of population growth. All cell numbers (stock culture and test) were determined with the Coulter Counter Z2 (Beckmann, Nuernberg, Germany). The tests were performed in sterilized glass tubes (20 mL Pyrex tubes sealed with caps containing a gastight Teflon membrane), algae were stirred over period of 24 h, and test conditions were the same as for the stock culture, except for the CO_2 [20] source. Here, 150 µL of $NaHCO_3$ solution was added to each test tube. The methods for stock culturing and testing are described by Matzke et al.[20] All substances were tested twice. First, a range finding was undertaken (four concentrations, two replicates), and in a second test, the results were verified with eight concentrations per substance in two replicates. The growth inhibition was calculated using the cell counts of the treated samples in relation to the untreated controls (pure medium). For each assay at least six controls were used.

Acute effects on Daphnia magma

The 48 h acute immobilisation test with the crustacean *D. magna* was performed using the commercially available Daphtoxkit (MicroBioTest Incorporation, Gent, Belgium) referred to in OECD guideline 202. The tests with neonates less than 90 h old, obtained by the hatching of ephippia, were performed at 20 °C in the dark. 5 pre-fed animals were incubated with the toxicants in a volume of 10 mL of mineral medium. For each test 5 different concentrations of the ionic liquids in 5 parallels and 5 controls were investigated. All the experiments were performed twice. The numbers of immobilised organisms were checked after 24 and 48 h. The sensitivity of the organisms to $K_2Cr_2O_7$ was checked routinely once a new batch of organisms was obtained.

Ready Biodegradability

The manometric respirometry test was performed according to OECD guideline 301 F. The biological oxygen demand of the substance was determined for 28 days using a BOD System (OxiTop, thermostatically controlled from WTW GmbH, Weilheim, Germany). Acquired from the wastewater treatment plant in Achim (Germany), the inoculum was filtered and aerated prior to use. A mineral medium containing final concentrations of 85 mg L^{-1} KH_2PO_4, 217.5 mg L^{-1} K_2HPO_4, 221.3 mg L^{-1} Na_2HPO_4*$2H_2O$, 17 mg L^{-1} NH_4Cl, 36.4 mg L^{-1} $CaCl_2$*$2H_2O$, 22.5 mg L^{-1} $MgSO_4$*$7H_2O$, and 0.25 mg L^{-1} $FeCl_3$ (pH 7.2) was added to the filtrate. 1.16 mg L^{-1} allylthiourea was also added in order to inhibit nitrification. The samples, containing inoculated media and the test substance, were prepared, along with both blank samples (inoculated media without test substance) and controls (inoculated media with benzoic acid). The concentrations of test compounds and benzoic acid were calculated to equal BOD of 200 mg O_2 L^{-1}, except of [MeHDecIm]Cl where due to its toxicity the BOD of 40 mg O_2 L^{-1}

was chosen. This corresponded to 186 mg L^{-1} of [MeBn^{F}Im]Br, 104 mg L^{-1} of [BnBnIm]Br, 83 mg L^{-1} of [NBu_4]Br, 15 mgL^{-1} of [MeHDecIm]Cl, and 102 mg L^{-1} of benzoic acid. In this experiment, a bacteria density of 10^4 cells L^{-1} was applied (determined by Paddle-Tester; Hach Europe, Düsseldorf). The flasks containing vessels with sodium hydroxide (CO_2 absorbent) were closed with gastight stoppers and stored in the dark at 20 °C. The oxygen consumption was determined manometrically. The microbial inhibition was determined by following the BOD of benzoic acid (102 mg L^{-1}) in presence of the test substance (in concentrations corresponding to BOD of 200 mg O_2 L^{-1}, except of [MeHDecIm]Cl where due to its toxicity the BOD of 40 mg O_2 L^{-1} was chosen [1]) for 5 days.

Biodegradation of the test substance was calculated by the oxygen uptake 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.

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