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Membrane partitioning of ionic liquid cations, anions and ion pairs – Estimating the bioconcentration potential of organic ions☆

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ABSTRACT

Recent efforts have been directed towards better understanding the persistency and toxicity of ionic liquids (ILs) in the context of the "benign-by-design" approach, but the assessment of their bioaccumulation potential remains neglected. This paper reports the experimental membrane partitioning of IL cations (imidazolium, pyridinium, pyrrolidinium, phosphonium), anions ($[C(CN)_3]^-$, $[B(CN)_4]^-$, $[FSO_2)_2N]^-$, $[(C_2F_5)_3PF_3]^-$, $[(CF_3SO_2)_2N]^-$) and their combinations as a measure for estimating the bioconcentration factor (BCF). Both cations and anions can have a strong affinity for phosphatidylcholine bilayers, which is mainly driven by the hydrophobicity of the ions. This affinity is often reflected in the ecotoxicological impact. Our data revealed that the bioconcentration potential of IL cations and anions is much higher than expected from octanol-water-partitioning based estimations that have recently been presented. For some ILs, the membrane-water partition coefficient reached levels corresponding to BCFs that might become relevant in terms of the "B" (bioaccumulation potential) classification under REACH. However, this preliminary estimation need to be confirmed by *in vivo* bioconcentration studies.

1. Introduction

According to the formal definition, ionic liquids (ILs) are salts with melting points below 100 °C that are formed solely from cations and anions. In recent years, ILs have attracted much interest, which has led to an enormous number of publications (>70,000), including >12,000 patents.[1] The expanding number of publications and increasing interest in ILs is mainly due to the unique physicochemical properties of certain ILs, for instance, their excellent electrochemical and thermal stability, low vapour pressure, wide range of viscosity and favourable solvation properties, which make them interesting for different fields of research and applications. Several applications and potential implementations of ILs exist in, e.g., aluminium plating, gas compression, lithium-ion batteries and dye-sensitized solar cells or as plant protection agents and active pharmaceutical ingredients (Lewandowski et al., 2014; Plechkova and Seddon, 2008; Stoimenovski et al., 2010). Increased utilisation of ILs comes with the increased probability that these compounds will be continuously released into the environment, for example, via consumer products, as process effluents or in larger amounts as accidental spills. Accordingly, ILs are gaining environmental relevance and have recently been described as "contaminants on the horizon" (Richardson and Kimura, 2016). In the context of environmental protection, the so-called PBT (persistency, bioaccumulation, toxicity) assessment is of major concern – especially from an environmental legislation viewpoint. Although recent efforts have been directed towards better understanding and predicting IL persistency (P) (Cho et al., 2016; Docherty et al., 2007; Harjani et al., 2009; Jordan and Gathergood, 2015; Stolte et al., 2012, 2011a) and toxicity (T) (Amde et al., 2015; Cho et al., 2013; Latała et al., 2009; Łuczak et al., 2010; Petkovic et al., 2011; Samorì et al., 2007; Ventura et al., 2012), the assessment of the bioaccumulation potential (B) of ILs remains

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neglected. It is especially important to fill this gap because the majority of investigated (particularly imidazolium-based) ILs are assumed to be rather recalcitrant towards biotic and abiotic degradation (Neumann et al., 2014, 2010; Steudte et al., 2012a), which increases their risk of being environmentally persistent. Organisms can accumulate chemical substances either directly from the surrounding environment, which is referred to as bioconcentration, or from their diet, which is called biomagnification. The sum of both of these processes then constitutes the bioaccumulation of a substance within an organism. Depending on the underlying uptake mechanisms, the extent to which a contaminant will concentrate in an organism can be expressed as the bioaccumulation factor (BAF) or bioconcentration factor (BCF). According to Gobas et al. BAF is defined as "ratio of the steady state chemical concentrations in an aquatic water-respiring organism and the water determined from field data in which sampled organisms are exposed to a chemical in the water and in their diet". Whereas BCF is described as "ratio of the steady state chemical concentrations in an aquatic water-respiring organism and the water determined in a controlled laboratory experiment in which the test organisms are exposed to a chemical in the water (but not in the diet)" (Gobas et al., 2009). In accordance with the European regulation concerning the registration, evaluation, authorization and restriction of chemicals (REACH), BCF assessment of substances manufactured or imported at rates above 100 tons/year is mandatory. Experimental measurements of BCFs are time-consuming, expensive, and due to ethical concerns regarding animal welfare not feasible for large sets of chemicals. Hence, such assessments are normally not conducted within the proactive hazard assessment of novel chemicals, such as ILs, which have the potential but are not being applied on a larger scale yet. Due to these circumstances, attention is drawn to estimating these BCFs by quantitative structure–activity relationships (QSARs), which make use of the fact that the bioaccumulation of substances is determined mainly by partitioning between aqueous and lipid phases. The octanol-water partition coefficient (log K_{ow}) is useful in estimating the BCFs of non-ionic organics of intermediate lipophilicity, assuming an absence of rapid metabolism and excretion (Pavan et al., 2008). The water-saturated octanol phase mimics to some extent the average molecular interaction potential of storage lipids and biological membranes and thus can be used as a measure of the partitioning into biological membranes (Klamt et al., 2008). The models based on log K_{ow} fail for charged species (Escher et al., 2000a; Klamt et al., 2008; Smejtek and Wang, 1993) (underestimation of partitioning into organisms) because they do not consider the ion–macromolecule interactions of charged compounds with the heterogeneous membrane system that contains, e.g., polar and charged phospholipids. Grisoni et al. presented recently that more complex models i.e. EPI Suite BCFBAF, VEGA CAE- SAR, VEGA Meylan, VEGA Read-across and VEGA *consensus* seem to be relevant in bioconcentration predictions of organic chemicals if they are adequately selected and properly applied (Grisoni et al., 2015). However several drawbacks were highlighted and the authors pointed out that in order to reduce the possibility of false positives or false negatives, there is the need to better predict metabolism or specific interactions with tissues. Also Endo et al. revealed that distinguishing between storage and membrane lipids is necessary to account for the bioaccumulation of H-bond donor compounds (Endo et al., 2011).

Nonetheless, recently, log K_{ow}-based thresholds have been applied to estimate the bioaccumulation potential of ILs - indicating a generally low potential of accumulation for most of the considered structures (Rybinska et al., 2016; Zakari et al., 2013).

Since lipid membrane-water partitioning coefficients (log K_{MW}) are generally better correlated with bioconcentration than the log K_{ow} is (Fujikawa et al., 2009), the main aim of this study was to use the log K_{MW} to obtain a more realistic estimation of the bioconcentration potential of IL cations and anions. In this context, the interactions of organic ions, and ion pairs in particular, with biological membrane lipids and their consequences in terms of bioconcentration are discussed. This approach might allow for screening the bioconcentration potential of not only novel IL structures but also other charged compounds in a proactive manner and is of high environmental relevance because approximately 70,000 compounds that are already preregistered under REACH are ionogenic or charged chemicals (Franco et al., 2010). To date, no approach to estimate the bioaccumulation potential of hydrophobic ions is in regulation (Treu et al., 2015).

2. Materials and methods

2.1. Chemicals

Benzocaine (4-aminobenzoaic acid ethyl ester, CAS: 94-09-7) and potassium hydrogen sulphate ($KHSO_4$) were purchased from Sigma Aldrich (Darmstadt, Germany). Acetonitrile (HPLC grade), dimethyl sulfoxide (DMSO), sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), 1-ethyl-3-methylimidazolium chloride (IM12 Cl), 1-butyl-3-methylimidazolium chloride (IM14 Cl), 1-octylpyridinium chloride (Py8 Cl), 1-octyl-3-methylpyridinium chloride (Py8-3Me Cl), 1-octylpyrrolidinium chloride (Pyr18 Cl), potassium tricyanomethanide ($KC(CN)_3$), lithium bis(trifluoromethylsulfonyl)imide ($Li(CF_3SO_2)_2N$), potassium tetracyanoborate ($KB(CN)_4$), potassium tris(perfluoroalkyl) trifluorophosphate ($K(C_2F_5)_3PF_3$) were purchased from Merck KGaA (Darmstadt, Germany). Potassium bis(-fluorosulfonyl)imide ($K(FSO_2)_2N$) was provided by IoLitec Ionic Liquids Technologies GmbH (Heilbronn, Germany). 1,3-diheptylimidazolium bis(trifluoromethylsulfonyl)imide (IM77 $(CF_3SO_2)_2N$) and triethyloctylphosphonium bis(trifluoromethylsulfonyl)imide (P2228 $(CF_3SO_2)_2N$) were obtained from Klüber Lubrication München KG. 1-(7-carboxyheptyl)-3-methylimidazolium bromide (IM17COOH Br) and 1-(8-hydroxyoctyl)-3-methylimidazolium bromide (IM18OH Br) were synthesized at the UFT according to (Jastorff et al., 2005). 1-dodecyl-3-methylimidazolium hydrogen sulphate (IM1-12 HSO_4) was synthesized according to (Nowicki et al., 2016). 1,1'-(1,12-dodecanediyl)bis[3-methylimidazolium] dibromide (IM1-12-IM1 Br_2) was synthesized according to (Steudte et al., 2014).

2.2. Lipid membrane-water partitioning experiment

Solid-supported lipid membranes (SSLMs), which are commercially available under the trademark Transil™ (Loidl-Stahlhofen et al., 2001a), were used to measure the log K_{MW}. Although the SSLMs approach only partially mimics the bioconcentration phenomena in living cell, it has been considered recently as highly physiologically relevant due to the fluidity of a true bilayer system (Loidl-Stahlhofen et al., 2001b). Moreover other advantages of SSLMs are: the stability over a longer period of time in comparison to small unilamellar liposomes, short-time experimental procedure and that separation from the aqueous phase is very easy (Escher et al., 2000a; Loidl-Stahlhofen et al., 2001b). The main drawback of this approach is the limitation concerning determination of membrane affinity of highly polar compounds. It allows screening membrane affinities >1.5 (Loidl-Stahlhofen et al., 2001a).

SSLM silica beads (diameter, 10 μm) coated with egg yolk phosphatidylcholine (unilamellar liposomal membrane that was noncovalently bound to the bead) were purchased from Sovicell

GmbH (Germany). The exact lipid volume of the SSLM was given by the supplier in a material certificate as follows: dry weight, 240 mg/mL; lipid content, 12.0 ± 0.6 μL/mL suspension; and lipid concentration, 15.7 ± 0.8 μmol/mL. The details of this experimental approach are described elsewhere (Loidl-Stahlhofen et al., 2001a). Stock solutions (0.2–1 mM) of the ILs containing 10% DMSO were prepared in phosphate-buffered saline (PBS buffer containing 10 mM PO_4^{3-}, 137 mM NaCl, and 2.7 mM KCl; pH 7.4; ionic strength, 162.7 mM). The experiment was carried out in Eppendorf test tubes in a total volume of 150–1500 μL containing 50–100 μM of each respective IL (or a mixture of a constant concentration of IM1-12 HSO_4 at 50 μM and the respective concentration of the organic anion as an inorganic salt; the following salts were used at concentration range of 10–1500 μM in 1% DMSO and PBS buffer: $KB(CN)_4$, $KC(CN)_3$, $K(FSO_2)_2N$, and $KHSO_4$). At 1%, DMSO has been proven to not influence the membrane coating of the silica beads (according to the manufacturer). The volume of added Transil™ beads depends on the membrane affinity of the IL cation/anion and the total volume. Since compound binding that is too high might overload the membrane coating and result in irreproducible membrane-water partition coefficients, the ratio between the Transil™ beads and liquid phase was established in preliminary experiments to allow a binding of approximately 50% of test compound (detailed information is presented in Table S1). Hence, this ratio of bound and unbound compound gave the best detection in the instrumental analysis (section 2.3). After mixing the respective volumes of stock solution and buffer, the SSLM beads were added. These prepared mixtures were thoroughly shaken (vortex, 10 s) and incubated for 30 min at 37 °C and 1400 rpm (Thermomixer compact, Eppendorf AG, Hamburg, Germany). Afterwards, the samples were centrifuged (bench centrifuge, Eppendorf AG, Hamburg, Germany) at 10,000 rpm for 10 min. The IL cation or anion concentration in the supernatant was determined via HPLC or ion chromatography (Section 2.3). For all substances, controls without beads (PBS buffer was used instead) were measured as a reference (n_{total}). Calculation of log K_{MW} is derived in detail elsewhere (Loidl-Stahlhofen et al., 2001a) and can be simplified to:

$$\log K_{MW} = \log\left(\frac{V_{total}}{V_{lipid}} \cdot \frac{(n_{total} - n_{supernatant})}{n_{supernatant}}\right) \quad (1)$$

V_{total} represents the total volume of the sample, and V_{lipid} is known from the certificate of analysis from the supplier (in this case, 12.0 ± 0.6 $\mu L/mL_{suspension}$), whereas $n_{supernatant}$ refers to the amount of IL in the water phase (Loidl-Stahlhofen et al., 2001a).

The validity of the obtained log K_{MW} was confirmed by determining a reference compound (benzocaine) for this assay, for which our log K_{MW} (2.00 ± 0.05) results agree with the literature data (Stolte et al., 2007).

2.3. Instrumental analysis

The HPLC system used to determine the cation or anion concentrations remaining in the water phase after lipid-water partitioning was a VWR Hitachi system containing an L-2130 HTA-pump, L-2130 degasser, L-2200 autosampler, L-2300 column oven, L-2450 diode array detector and EZChrom Elite software. The column (Multospher® 100 RP18-5 μm, 4.6 × 125 mm) and guard column were purchased from CS-Chromatographie (Germany). The mobile phase was composed of acetonitrile (HPLC grade) and aqueous 20 mM KH_2PO_4/3.9 mM H_3PO_4 buffer (pH = 3). All cations were analysed using isocratic elution mode.

The proportions of acetonitrile and buffer for the cations and reference compound (benzocaine) were as follows: benzocaine: 40:60 (v/v); Py8: 40:60 (v/v); Py8-3Me: 40:60 (v/v); IM1-12-IM1: 35:65 (v/v); IM1-12: 50:50 (v/v). For IM77, the proportion of acetonitrile and buffer (40 mM KH_2PO_4/2 mM H_3PO_4) was 45:55 (v/v). The analytical wavelength was 212 nm for the imidazolium ILs, whereas the pyridinium ILs and benzocaine were analysed at 254 nm and 289 nm, respectively.

The anions were analysed using a Metrohm 881 Compact IC system (Metrohm, Herisau, Switzerland) equipped with an online eluent degasser, 20 μL injection loop and conductometric detector (maintained at 30 °C). A self-regenerating suppressor module (MSM) and CO_2-suppressor (MCS) (both Metrohm, Herisau, Switzerland) were used. All chromatographic data were recorded by Metrohm software (MagICNet version 2.4 compact). Chromatographic separations of anions were performed on 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 Metrosep RP guard (all purchased from Metrohm, Herisau, Switzerland). The packing material consisted of polyvinyl alcohol and quaternary ammonium groups. The flow rate was 0.7 mL/min. The eluent was composed of acetonitrile and an aqueous buffer solution containing 3.2 mM Na_2CO_3 and 1.0 mM $NaHCO_3$. The proportions of acetonitrile and buffer for the anions were as follows: $[B(CN)_4]^-$: 33:67 (v/v); $[(C_2F_5)_3PF_3]^-$: 38:62 (v/v); $[(CF_3SO_2)_2N]^-$: 30:70 (v/v); $[C(CN)_3]^-$: 31:69 (v/v); and $[(FSO_2)_2N]^-$: 33:67 (v/v). For cation separation (Pyr 18 and P2228), a silica-based (modified with carboxylic groups) Metrosep C4 ion exchange column (dimensions—50 × 4.0 mm ID and 5 μm mean particle size) coupled with a Metrosep C4 guard and Metrosep RP guard was used (all purchased from Metrohm, Herisau, Switzerland). A flow rate of 0.9 mL/min was applied. The mobile phase for cation analysis consisted of acetonitrile and 2 mM aqueous nitric acid (35:65, v/v). For IM12 and IM14 the method presented elsewhere (Stolte et al., 2011b) was applied.

All methods were validated by analysing at least 7 different concentrations (dissolved in PBS buffer) ranging between 5.0 and 200.0 μM for the investigated anions and 1–100 μM for the cations. Validation parameters such as the linearity; correlation coefficient (R^2); intra-day precision, expressed by coefficient of variation (CV); accuracy; limit of detection (LOD); and limit of quantification (LOQ) were established (Table S2). The linearity was determined by analysing a minimum of seven different concentrations of each compound with six replicates (for each method, $R^2 \geq 0.9973$). The linear ranges of the obtained calibration curves covered the tested concentrations, assuming up to 80% partitioning (Table 2S). Method accuracy was determined by assessing the agreement between the measured and nominal concentration of the analytes and ranged from 82.3% to 120.1%. The intra-day precision of the methods was determined by calculating the CV of replicated measurements (n = 6) and did not exceed 5% in each case. Therefore, the analytical method used for the lipid partitioning assessment attained the validation requirements for quantitative analyses.

2.4. Statistical analysis

The statistical analysis for linear regression and validation study was performed using SPSS 12.0.1 for Windows.

3. Results and discussion

In general, phospholipid membranes are composed of phosphatidylcholine, which interacts with both cations and anions because the zwitterionic head group (net charge of zero) supports electrostatic interactions. Moreover, the fatty acid tails in the inner region of the bilayer offer hydrophobic interactions. In the following subsection, the interaction between the different

structural elements of ILs and the membrane bilayer will be discussed.

3.1. In vitro lipid membrane-water partitioning

3.1.1. Influence of the side chain

The log K_{MW} of imidazolium compounds with different side-chain length ranged between ≤1.5 and 4.48 and generally increased with an increasing number of -CH_2 units (Table 1). In non-logarithmic scale, the concentration of IM1-16 was 30,000 times higher in (or adsorbed onto) the membrane than in the water phase. In contrast, compounds with side-chain lengths ≤6 as well as hydroxylated (IM18OH) and carboxylated compounds (IM17COOH) were less than 30 times enriched in the zwitterionic phosphatidylcholine membrane. The di-heptyl-substituted cation had a similar partitioning coefficient as that of IM1-14 (log K_{MW} of approximately 4), whereas for the dicationic moiety with a dodecyl spacer between the two imidazolium head groups, the log K_{MW} was comparable to that of IM18 (log K_{MW} of approximately 2). The high membrane affinity of cations with long alkyl side chains corresponds well with the so-called "side-chain effect" (decreasing EC_{50} with increasing hydrophobicity) that has been widely reported for ILs and in several different biological tests (Markiewicz et al., 2013; Ranke et al., 2007; Ventura et al., 2012).

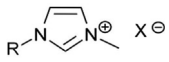
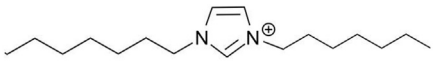
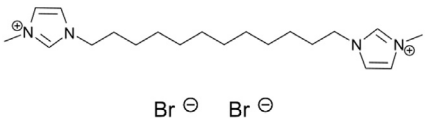
The increased affinity of compounds with longer alkyl chains was experimentally determined by using immobilized artificial membranes (IAMs) and HPLC-derived lipophilicity parameters (Stepnowski and Storoniak, 2005).

In a recent study, Jing et al. investigated the impact of IM14, IM16, IM18, and IM1-12 (chlorides) on the morphology of L-α-phosphatidylcholine model lipid bilayers (Jing et al., 2016). The IL cations intercalated into the bilayer in a manner that was dependent on their hydrophobicity and caused swelling of the lipid bilayers. At concentrations above the critical micelle concentration (CMC), the ILs caused disintegration of the lipid bilayer (Jing et al., 2016). Molecular dynamics (MD) simulations revealed that IL cations at millimolar concentrations spontaneously insert into the lipid bilayer regardless of the alkyl chain length (Bingham and Ballone, 2012; Hartmann et al., 2015; Lim et al., 2015). For membranes composed of 1-palmiltoyl-2-oleoylphosphatidylcholine (POPC), the insertion of IL cations was calculated to be thermodynamically favourable, with a Gibbs free energy change of 27 kJ mol^{-1} for IM14 and 37 kJ mol^{-1} for IM1-12 (Yoo et al., 2014).

Hartmann et al. investigated the effect of a series of 1-alkyl-3-methylimidazolium chlorides on the plasma membrane of the filamentous fungus *Aspergillus nidulans*. Compounds with long alkyl substituents (≥6) were able to permeate the membrane, causing cell death (Hartmann et al., 2015). Supported by results from MD simulations, these authors suggested that the charged imidazolium moiety associates with the negatively charged phosphocholine or phosphoserine head groups of the membrane bilayer, whereas the (long) alky chains interact with the hydrophobic regions of the phospholipids (Hartmann et al., 2015).

Accordingly, the lower log K_{MW} values of IM18OH and IM17COOH (in comparison to IM18) were expected due to the increased polarity of the side chain and reduced interactions with the hydrophobic fatty acid tails of the phospholipids. In the case of IM1-12-IM1, the long hydrophobic dodecyl chain is surrounded by two cationic groups, which apparently reduces the possibility of the alky chain intercalating into the membrane, resulting in a much lower log K_{MW} compared to the terminal dodecyl chain of IM1-12. For IM77, the two heptyl chains appeared to intercalate equally as

Table 1
Influence of the side chain on lipid-water partitioning.

Structural formula	Acronym	R=	Membrane-water partitioning (log K_{MW})
	IM12 Cl	C2	≤1.5
	IM14 Cl	C4	≤1.5
	IM16 Cl	C6	≤1.5[a]
	IM17COOH Br	C7COOH	≤1.5
	IM18OH Br	C8OH	≤1.5
	IM18 Cl	C8	2.06 ± 0.15[a]
	IM1-10 Cl	C10	3.15 ± 0.19[a]
	IM1-12 HSO_4	C12	3.76 ± 0.04
	IM1-14 Cl	C14	4.09 ± 0.17[a]
	IM1-16 Cl	C16	4.48 ± 0.03[a]
	IM77$(CF_3SO_2)_2N$	–	4.03 ± 0.02
	IM1-12-IM1 Br_2	–	2.29 ± 0.03

Definitions to the acronyms provided in Table 1: IM12 Cl: 1-ethyl-3-methylimidazolium chloride, IM14 Cl: 1-butyl-3-methylimidazolium chloride, IM16 Cl: 1-hexyl-3-methylimidazolium chloride, IM17COOH Br: 1-(7-carboxyheptyl)-3- methyl-imidazolium bromide, IM18 Cl: 1-octyl-3-methylimidazolium chloride, IM18OH Br: 1-(8-hydroxyoctyl)-3-methyl-imidazolium bromide, IM1-10 Cl: 1- decyl-3-methylimidazolium chloride, IM1-12 HSO_4: 1-dodecyl-3-methylimidazolium hydrogen sulphate, IM1-14 Cl: 1-tetradecyl-3-methylimidazolium chloride, IM1-16 Cl: 1-hexadecyl-3-methylimidazolium chloride, IM77 $(CF_3SO_2)_2N$: 1,3-diheptylimidazolium bis(trifluoromethylsulfonyl)imide, IM1-12-IM1 Br_2: 1,1'-(1,12-dodecanediyl)bis[3-methylimidazolium] dibromide.

[a] (Stolte et al., 2007).

well as the tetradecyl side chain of IM1-14.

Plotting the log K_{MW} against the number of carbon atoms in the chain for the 1-alkyl-3-methylimidazolium chlorides (Fig. 1) provided a concave curve, suggesting a reduced increase in the log K_{MW} with elongation of the side chain. These observations correspond well with the findings from the (eco)toxicological data. At a certain side-chain length, the toxic effects reached a maximum towards, e.g., IPC 81 cells (Cho et al., 2013), the green algae *Scenedesmus vacuolatus* (Stolte et al., 2007), the greater wax moth (*Galleria mellonella*) (Megaw et al., 2015) and several different fungal and algae strains (Łuczak et al., 2010; Markiewicz et al., 2011; Pernak et al., 2003; Stolte et al., 2007). The deviation from linearity, i.e., lower toxicity than expected based on the chain length, is known as the "cut-off effect". The reason for this effect might be the reduced (bio)availability when the CMC is exceeded or the low solubility of highly hydrophobic compounds (in the latter case, the nominal concentrations can deviate significantly from the real test concentrations) (Birnie et al., 2000; Cho et al., 2013). Moreover, Devínsky et al. suggested that longer alkyl chains better mimic the lipid bilayer and thus cause less disruption of the membrane (Devínsky et al., 1990). In our membrane partitioning experiments, the complete dissolution of the test compounds was analytically confirmed, the applied test concentrations were set well below the CMC and an improved intercalation of long chains would rather increase the log K_{MW} values.

On the other hand, unbound phospholipids in the aqueous phase (released e. g. due to mechanic damage of the beads or due to the disruption of the bilayer caused by ILs) would theoretically offer a competitive phase for sorption processes. In such case a decreased log K_{MW} (more IL in the aqueous phase) would be expected for compounds with increased phospholipid affinity which would also correspond to results shown in Fig. 1. Nevertheless, according to the supplier it is very unlikely as long as mechanical damage of the beads does not occur. Moreover it has been shown in the literature that residual silica charge at the surface of the beads does not falsify membrane affinity data of charged molecules (Loidl-Stahlhofen et al., 2001a) and results obtained when using solid supported lipid membranes (SSLM, e.g. TRANSIL beads) correlate well with those obtained by using lipid bilayer vesicles (Escher et al., 2000a; Loidl-Stahlhofen et al., 2001a). Therefore, we assume that mainly kinetic aspects are responsible for the lesser increase in the log K_{MW} values with elongation of the side chain. The size as well as the increased flexibility of longer alkyl side chains (steric hindrance) might reduce the diffusibility into the lipid membranes and result in a lower-than-expected increase in the log K_{MW} values.

A diminished ability to intercalate into liposomes was observed for a series of (p-methylbenzyl)alkylamines with increasing chain length, and steric effects were used to explain this observation (Escher and Sigg, 2004; Fruttero et al., 1998).

3.1.2. Influence of the cationic head group

To ascertain the influence of the head group on membrane partitioning, a set of different cations substituted with octyl side chains were tested (Table 2). The log K_{MW} values of the investigated compounds were very similar and ranged between 2.06 and 2.34, indicating a low contribution of the type of cationic head group to the partitioning process. The minor influence of the head group to the hydrophobicity of an IL cation was demonstrated using an HPLC-derived lipophilicity parameter (log k_o) (Ranke et al., 2007; Stolte et al., 2007). However, Malferrari et al. used chromatophore vesicles isolated from a photosynthetic bacterium, *Rhodobacter sphaeroides*, as a model to study how different ILs affect the membrane permeability to ions (Malferrari et al., 2015). Pyr14 was much more effective than IM14 at increasing the membrane permeability *inter alia* due to the higher propensity of Pyr14 to bind to the chromatophore membrane that also contained integral membrane proteins. This observation could not be confirmed in our partitioning experiments using a very simple lipid bilayer that did not consider (specific) interactions with integral proteins. Since the uptake and efflux of charged substances into cells is strongly mediated by organic anion transporters (OATs) and organic cation transporters (OCTs) (e.g. proteins and polypeptides) (Roth et al., 2012) the further studies involving these constituents are immensely important.

3.1.3. Influence of the organic anion

To assess the membrane affinity of selected organic anions, $[B(CN)_4]^-$, $[C(CN)_3]^-$, $[(FSO_2)_2N]^-$, $[(CF_3SO_2)_2N]^-$ and $[(C_2F_5)_3PF_3]^-$, the membrane partitioning of their inorganic salts (mainly K^+) were measured (Table 3).

Generally, the phosphatidylcholine membrane has a zero net charge, but the ester group in phospholipids causes an internal dipole moment that is oriented with its positive pole inside the lipid bilayer, which favours adsorption as well as absorption of negatively charged hydrophobic ions (Flewelling and Hubbell, 1986a, 1986b).

A low log K_{MW} of <1.5 was determined for the $[C(CN)_3]$ anion, whereas $[(C_2F_5)_3PF_3]^-$ showed a high membrane affinity (log K_{MW}

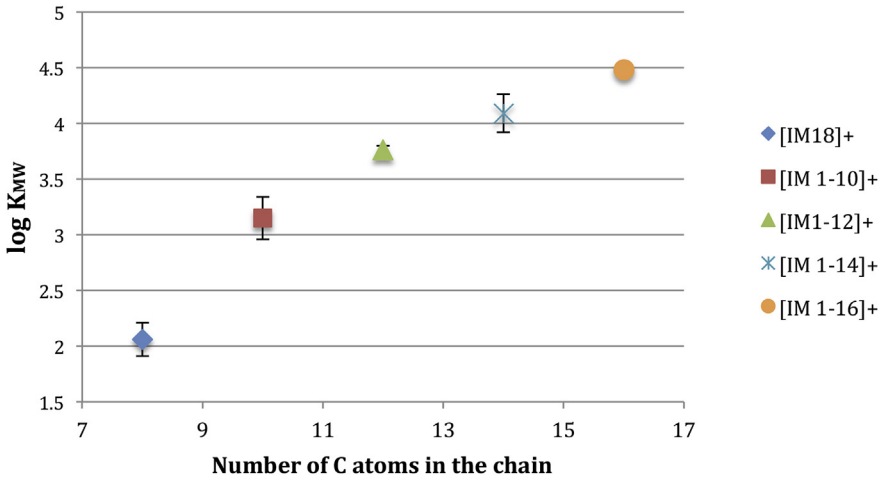
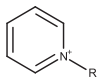
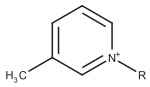
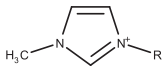
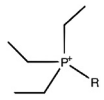


Fig. 1. Log K_{MW} vs. the number of carbon atoms in 1-alkyl-3-methylimidazolium chlorides.

Table 2
IL core influence on lipid-water partitioning.

Structure of the cation	Acronym	R=	Membrane-water partitioning (log K_{MW})
	Py8 Cl	-C8	2.28 ± 0.07
	Py8-3Me Cl	-C8	2.34 ± 0.05
	IM18 Cl	-C8	2.06 ± 0.15[a]
	Pyr18 Cl	-C8	2.18 ± 0.17
	P2228 $(CF_3SO_2)_2N$	-C8	2.24 ± 0.04

Definitions to the acronyms provided in Table 2: Py8 Cl: 1-octylpyridinium chloride, Py8–3Me Cl: 1-octyl-3-methylpyridinium chloride, IM18 Cl: 1-octyl-3-methylimidazolium chloride, Pyr18 Cl: 1-octylpyrrolidinium chloride, P2228 $(CF_3SO_2)_2N$: Triethyloctylphosphonium bis(trifluoromethylsulfonyl)imide.
[a] (Stolte et al., 2007).

3.67) that is comparable with the hydrophobic IL cation IM1-12 (log K_{MW} 3.76). For $[(FSO_2)_2N]^-$, $[(CF_3SO_2)_2N]^-$ and $[B(CN)_4]^-$, similar values in the range of 2.0 and 2.5 were determined that roughly correspond to the membrane affinity of the IM18 cation. In MD simulations with Cl^-, $[BF_4]^-$, $[PF_6]^-$ and $[(CF_3SO_2)_2N]^-$, the latter three had an effect on the structural properties of the bilayer, but only $[(CF_3SO_2)_2N]^-$ inserted into the POPC lipid bilayers (Yoo et al., 2014). The free energy change for the insertion of the $[(CF_3SO_2)_2N]$ anion was calculated to be thermodynamically favourable at -10 kJ mol^{-1} (Yoo et al., 2014). It was assumed that the anion inserts due to the hydrophobic interactions of $[(CF_3SO_2)_2N]^-$ with the non-polar regions of the bilayer as well as due to its weaker tendency to be solvated by water.

The anionic hydrophobicity (Ha) was determined previously by our group to investigate the correlation between the membrane affinity and hydrophobicity of anions (Cho et al., 2014). The obtained data are presented in Table 3. The large log K_{MW} of $[(C_2F_5)_3PF_3]^-$ can be explained by its Ha value; however, a higher membrane affinity was assumed for $B(CN)_4^-$ compared to the fluorinated imide anions.

3.1.4. Membrane partitioning of the IM1-12 cation and different organic anions at equal concentrations

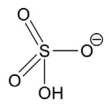
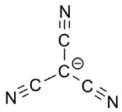
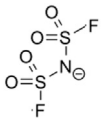
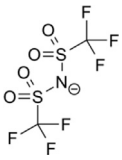
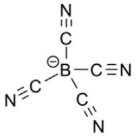
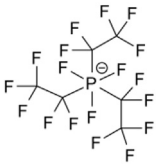
In pharmaceutical research, ion paring is used to increase the transport of ionic drugs across membranes (Neubert, 1989). A charged hydrophilic drug is combined with an oppositely charged hydrophobic counter ion. In theory, the ion-pair molecule that is formed has a reduced or neutralized overall electrostatic charge and, consequently, an increased membrane permeation (Sarveiya et al., 2004). Toxicological studies with *Vibrio fischeri* revealed that combinations of IL cations and anions such IM14 $(CF_3SO_2)_2N$ cause much stronger effects than expected due to the toxicities of the cation (tested as IM14 Cl) and anion (tested as $Li(CF_3SO_2)_2N$) (Stolte et al., 2007). The authors speculated that the (temporarily) formed ion pairs of the ILs in aqueous solutions lead to higher bioavailability and greater membrane disturbance.

Based on this assumption, it would follow that the log K_{MW} of a cation should change in the presence of an anion with which it forms ion pairs. In this context, we measured the membrane-water partitioning of the IM1-12 cation combined with different anions at concentrations of 50 μM each (Table 4).

Very similar log K_{MW} were determined for IM1-12 with different counter anions. The values ranged between 3.74 and 3.80, which are within the accuracy of the measurement and do not indicate that IL cation-anion interactions affect the membrane affinity. For $[(CF_3SO_2)_2N]^-$, a minor increase to 4.0 was observed. MD simulations with [IM14] $[(CF_3SO_2)_2N]$ demonstrated that the anion follows the cation into POPC bilayers, reassociates and stays close to the intermediate region between the hydrophobic chains and polar head groups (Bingham and Ballone, 2012). For the most hydrophobic anion $[(C_2F_5)_3PF_3]^-$, the strongest influence was expected, but the log K_{MW} could not be determined due to the low solubility of IM1-12 $[(C_2F_5)_3PF_3]$ in the buffer (below the LOQ of 5 μM).

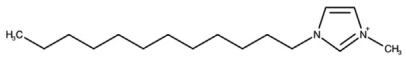
In general, the results for the investigated cation-anion combinations do not suggest a strong influence of ion-pair formation on the log K_{MW} of ILs, but these results might be due to the selected IL combinations as well as the ionic strength of the PBS buffer used. The IM1-12 cation itself has a strong membrane affinity, which might lead to fast sorption to the membrane surface and intercalation of the side chain into the inner region of the membrane without any needed for interaction with a hydrophobic counter ion. On the other hand, for compounds with shorter chains, the affinity for the membrane is less pronounced and might be facilitated by cation-anion interactions. Jing et al. showed that the influence of $[(CF_3SO_2)_2N]^-$ on bilayer disruption is the greatest when combined with IM14 and is subsequently reduced when combined with more hydrophobic cations (Jing et al., 2016). Moreover the ionic strength of the media has an important influence on partitioning and distribution coefficients. For ionogenic compounds, the octanol-water distribution ratios are increased at higher electrolyte concentrations because counter ions are a prerequisite to maintain overall electroneutrality in the octanol phase (Escher et al., 2000b; Jafvert et al., 1990). Generally, lipid-membrane distributions are less affected by ionic strength (Escher, 1996) since the cations and

Table 3
Calculated values of anion hydrophobicity and determined membrane-water partitioning of anions (as inorganic salts); n.d. – not determined.

Structure	Acronym	Hydrophobicity of anion (Ha)	log K_{MW} anion
	$[HSO_4]^-$	0.606, 0.358[c]	n.d.
	$[C(CN)_3]^-$	1.411[a]	<1.5
	$[(FSO_2)_2N]^-$	1.510	2.03 ± 0.08
	$[(CF_3SO_2)_2N]^-$	1.504[a], 1.600[b]	2.50 ± 0.03
	$[B(CN)_4]^-$	1.855[a]	2.33 ± 0.02
	$[(C_2F_5)_3PF_3]^-$	3.170	3.67 ± 0.07

Definitions to the acronyms provided in Table 3: $[HSO_4]^-$: hydrogen sulphate, $[C(CN)_3]^-$: tricyanomethanide, $[(FSO_2)_2N]^-$: bis(fluorosulfonyl)imide, $[(CF_3SO_2)_2N]^-$: bis(trifluoromethylsulfonyl)imide, $[B(CN)_4]^-$: tetracyanoborate, $[(C_2F_5)_3PF_3]^-$: tris(perfluoroalkyl) trifluorophosphate.
[a] The data are from reference (Cho et al., 2014).
[b] The data are from reference (Steudte et al., 2012b).
[c] To calculate the anionic hydrophobicity (Ha) of $[HSO_4]^-$, the log K_{ow} values of ILs with $[HSO_4]^-$ were collected from the literature (Jain and Kumar, 2016; Rybinska et al., 2016).

Table 4
Influence of the anion on the log K_{MW} of the cation.

Ionic liquid		Membrane-water partition (log K_{MW}) of the cation
Cation	Anion	
$[IM1\text{-}12]^+$ 	$[HSO_4]^-$	3.80 ± 0.06
	$[C(CN)_3]^-$	3.74 ± 0.02
	$[(FSO_2)_2N]^-$	3.82 ± 0.02
	$[B(CN)_4]^-$	3.76 ± 0.03
	$[(CF_3SO_2)_2N]^-$	4.00 ± 0.10

Definitions to the acronyms provided in Table 4: IM 1-12 HSO_4: 1-dodecyl-3-methylimidazolium hydrogen sulphate, IM1-12 $C(CN)_3$: 1-dodecyl-3-methylimidazolium tricyanomethanide, IM1-12 $(FSO_2)_2N$: 1-dodecyl-3-methylimidazolium bis(fluorosulfonyl)imide, IM1-12 $(CF_3SO_2)_2N$: 1-dodecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, IM1-12 $B(CN)_4$: 1-dodecyl-3-methylimidazolium tetracyanoborate.

anions do not necessarily partition as ion pairs (Bittermann et al., 2016). Nevertheless, ion-pair formation should perhaps not always be neglected (Austin et al., 1998; Escher et al., 2000b).

Anions such as $[(C_2F_5)_3PF_3]^-$ and $[(CF_3SO_2)_2N]^-$ form ILs with low water solubility, which indicates interactions between the cation and anion. However, the log K_{MW} experiments were performed in PBS buffer with high ionic strength (≈163 mM). The electrolyte provides enough counter ions to accompany the IL's cation and thus does not necessarily form ion pairs with its original anion to ensure electroneutrality. Moreover, the buffer ions available in large excess may also shield the interactions of IL cations and anions that are present in much lower concentrations (50 μM).

3.1.5. Dependence of the membrane partitioning of the IM1-12 cation on the concentration of the organic anion

The methodology used did not allow reduction of the buffer concentration when determining the log K_{MW}. Nevertheless, to facilitate IL cation and anion interactions, we changed the concentration of organic anions but maintained the IM1-12 concentration. For this, 50 μM IM1-12 HSO_4 was incubated with various concentrations (20–1500 μM) of organic counter ions as potassium salts (Fig. 2). For the most hydrophobic anions $[(C_2F_5)_3PF_3]^-$ and $[(CF_3SO_2)_2N]^-$, no such dependency could be determined because these anions precipitated as the IM1-12 IL, even at the lowest investigated concentration (20 μM).

The log K_{MW} of IM1-12 increased by up to 0.5 log units in the presence of increasing amounts of $[B(CN)_4]^-$ and $[(FSO_2)_2N]^-$, whereas $[C(CN)_3]^-$ and $[HSO_4]^-$ did not clearly change the partitioning of IM1-12 (Fig. 2). It can be hypothesized that the ion pairing of $[B(CN)_4]^-$ and $[(FSO_2)_2N]^-$ with IM1-12 increases the log K_{MW} of the cation but that anions adsorb to the bilayer surface and neutralize the charge of choline, thus reducing its repulsive interactions with IM1-12. Moreover, these anions in high concentrations may also change the integrity of the lipid bilayer, which may facilitate the intercalation of IM1-12. All these hypotheses generally appear to be particularly dependent on the hydrophobicity of the anion.

3.2. Estimation of the bioconcentration potential of the investigated IL cations and anions

According to the European Union's REACH regulation and Annex IX, information on the bioaccumulation potential in aquatic species (preferably fish) is required for substances manufactured or imported in quantities of 100 t/y or more. The bioaccumulation potential is assessed based solely on the BCF, with classification as "bioaccumulative" (BCF > 2,000, B criterion) and "very bioaccumulative" (BCF > 5,000, vB criterion). Annex IX allows for the use of valid QSARs to predict the BCF in the interests of time- and cost-effectiveness and animal welfare. The most common and simplest QSAR models are based on correlations between the BCF and log K_{ow}. A substance is considered to potentially fulfil the B criterion when the log K_{ow} exceeds 4.5 and the vB criterion when log K_{ow} exceeds 5. These models can be used to derive estimates for neutral chemicals but are not applicable to ionic or partly ionized chemicals substances (Pavan et al., 2008). Nonetheless, recently, the log K_{ow} threshold (>5) was applied to estimate the bioaccumulation potential of ILs - indicating a generally low potential to accumulate for most of the considered structures (Rybinska et al., 2016; Zakari et al., 2013). Experimental as well as computed log K_{ow} values of ILs are often negative (IM12 Cl: −3.1 (Rybinska et al., 2016); IM14 Cl: −0.31(Domańska et al., 2003); IM18 Cl: −0.2 (Domańska et al., 2003); IM1-10: −0.15 (Domańska et al., 2003); and IM1-16 Cl: 1.79 (Rybinska et al., 2016)), what might indicate a very low affinity for biological interphases. However our experimental data revealed much greater interaction with lipid bilayers indicating stronger bioaccumulation potential than expected from log K_{ow}. Indeed, membrane-water or liposome-water partition coefficients were shown to be a more suitable descriptor of the bioaccumulation potential than log K_{ow} (Armitage et al., 2013; Bittermann et al., 2016; Escher et al., 2000b; Schmitt, 2008).

To assess the bioconcentration potential of IL cations and anions based on their membrane partitioning, a correlation between the log K_{MW} and log BCF needs to be established. To do so, empirical BCF data for organic chemicals was plotted versus the empirical log K_{MW} values, and a linear correlation was obtained as a result (Fig. 3). This correlation has several major drawbacks (e.g., too few data points and log K_{MW} values that were generated with different methodologies and using different lipids) and thus gives only a very rough and preliminary estimate of the bioconcentration potential as expressed as follows:

$$\log BCF = 0.80 \log K_{MW} - 0.95 \quad (2)$$

Nevertheless, the simple log K_{MW} values correlated well with the experimental BCFs (r^2 = 0.612, RMSE = 0.693 log units, n = 109). In order to validate the linear model Eq. (2), leave-many-out validation (Q^2_{Lmov}) study was performed. For the validation, we randomly split the total dataset into 11 sets i.e., 10 sets composed of 10 and 1 set composed of 9 compounds. For this, we developed models using the dataset excluding each of 11 sets. Then in each case, the estimated coefficients (i.e., intercept and constant) of 11 cases were applied for predicting log BCF values of each of the excluded set. As result, the estimated Q^2_{Lmov} was 0.598. It is higher than 0.5 which is the standard value of an acceptance in cross-validation study.

In accordance with the correlation, log K_{MW} values below 4.0 indicate compounds that are assumed to not fulfil the B criterion. Above a log K_{MW} of 4, there is an increased occurrence of compounds with BCFs of log >3.3 (BCF of 2000 on a non-logarithmic scale). In particular, compounds with long chains such as IM1-16 (log K_{MW} 4.5) show membrane affinities that might be relevant in terms of their bioaccumulation potential. Moreover, according to the Globally Harmonized System of Classification and Labelling of

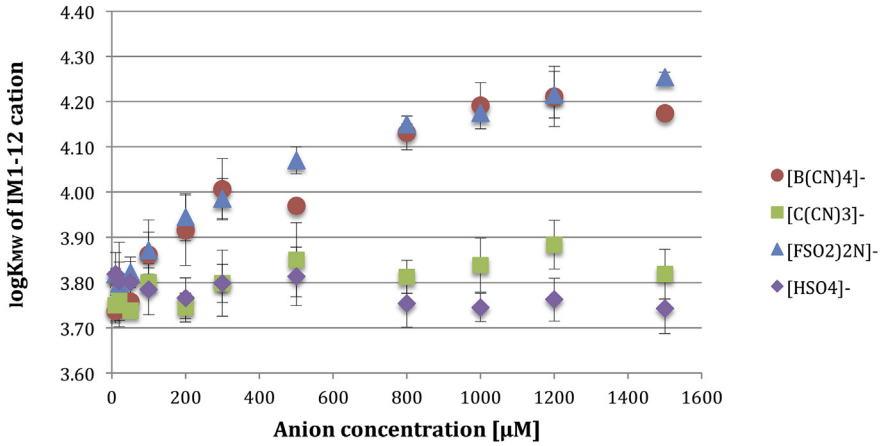


Fig. 2. Anion influence on the membrane affinity of the IM1-12 cation.

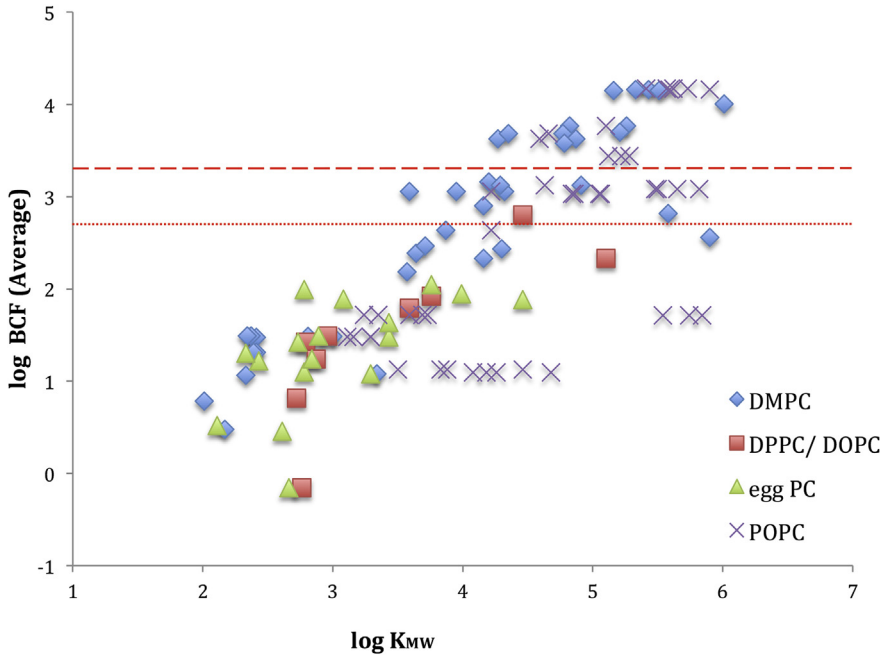


Fig. 3. Correlation between the empirical log K_{MW} values determined by (Endo et al., 2011) and the log BCF values from the EURAS BCF Gold Standard Database. The dashed line indicates the REACH threshold of BCF >2000 (B criterion), the dot line indicates the GHS threshold of BCF > 500 (B criterion). DMPC – dimyristoylphosphatidylcholine; DPPC/DOPC – dipalmitoylphosphatidylcholine/dioleoylphosphatidylcholine; egg-PC – phosphatidylcholine from egg yolk; POPC – palmitoyloleoylphosphatidylcholine.

Chemicals (GHS) a lower BCF threshold is defined for bioaccumulative chemicals (BCF >500 L/kg, log BCF > 2.7) (Matthies et al., 2016) which would include ILs with even lower log K_{MW}.

It needs to be kept in mind that the correlation shown in Fig. 3 is based on BCFs of neutral (non-ionizable) organic compounds which can notably sorb to both phospholipids and storage lipids, whereas ionised compounds predominantly sorb to phospholipids (Endo et al., 2011; Escher et al., 2000b; Schmitt, 2008). Taking into account the different phospholipid and storage lipid contents (typically 1–2% vs. 3–9% based on wet-weight of a whole body fish) (van Wezel et al., 1995) the estimation of the ILs' bioconcentration potential is now overestimated (worst case estimation), because IL sorption to storage lipids is also considered. A more realistic estimation would require experimental BCFs for a large set of permanently charged and ionogenic compounds which is not available so far.

The aforementioned assumptions of the bioconcentration potential of IL cations are based only on their equilibrium partitioning behaviour. If the chemical can undergo biotransformation the measured BCF is expected to be well below the predicted value derived from its partitioning. So far little is known about the biotransformation of imidazolium ILs. Sipes et al. could demonstrate that tissue disposition and metabolisation of the IM14 in male F-344 rats and female B6C3F1 mice are negligible. After oral and intravenous application the cation was extensively eliminated via urine (Sipes et al., 2008). Based on theoretical approach several hydroxylated, carboxylated and dealkylated compounds were postulated for the IM14 cation (Jastorff et al., 2003; Stepnowski and Storoniak, 2005) and IM18 cation (Jastorff et al., 2005). For IM18 several of them could be verified in studies with microorganisms from waste water treatment sludge and it was shown that the degradation starts with the hydroxylation terminal carbon atom of the alkyl chain (Stolte et al., 2008). Generally, longer and unbranched alkyl chains seem to promote the microbial biodegradability of imidazolium cations (Jordan and Gathergood, 2015). Similar trends were observed while investigating the hepatic clearance rates of ionizable organic chemicals with liver S9 fractions isolated from rainbow trout. Here, for instance, N,N-dimethyloctylamine showed lower clearance rates than N,N-dimethyldodecylamine, but for the quaternary derivates (trimethyloctylamine and trimethyldodecylamine) no clearance at all was observed (Chen et al., 2016). The elimination via biotransformation may decrease the BCF of hydrophobic IL cations, but has not been studied yet.

The high affinity of hydrophobic and fluorinated anions such as $[(FSO_2)_2N]^-$, $[(CF_3SO_2)_2N]^-$ and $[(C_2F_5)_3PF_3]^-$ to phospholipids might results in elevated concentrations in tissues with elevated phospholipid contents (e.g., liver, kidney, spleen, brain) as it was hypothesised for perfluoroalkyl substances (PFAS) (Armitage et al., 2012). PFAS are an important class of industrial chemicals that are highly persistent, bioaccumulative (dependent upon their alkyl chain length) and detected in the environment on a global scale (Zhang et al., 2013). Due to hydrophobic (fluorinated tail) and hydrophilic (charged head group) interactions, such anions have a high affinity for plasma proteins such as albumin and thus tend to accumulate in blood (Kelly et al., 2009; Ng and Hungerbühler, 2013). Grisoni et al. proposed scheme to classify compounds between three classes (as mainly stored within lipids, affected by additional interactions with non-lipid tissues, or metabolized/eliminated) in order to avoid under or overestimation in bioconcentration modelling, and highlighted thereby significant contribution of i.a. specific interactions with tissues other than lipids in bioconcentration process (Grisoni et al., 2016.)

Apart of the high affinity to phospholipids also this "non-classical" bioaccumulation can be assumed for several IL anions because they exhibit similar interaction potentials to PFAS. Furthermore, the B potential of IL cations might be even higher in mixtures with highly hydrophobic anions, as was proven in this study. Additionally, a higher lipid membrane affinity of IL cations was found for membranes containing 20% negatively charged phosphatidylserine groups (Stolte et al., 2007). This dynamic can be easily explained by the increased ionic interactions between the IL

cations and negatively charged lipids. Thus, the consequences of various factors for the distribution of a cation must also be considered because, e.g., different tissues have membrane lipids with different compositions (e.g., brain tissue is rich in phosphatidylserine (Kim et al., 2014)).

4. Conclusions

In this study, the interactions between organic cations and anions as well as their combinations with biological membrane lipids were investigated to obtain a preliminary assessment of their bioconcentration potential. The membrane-water partition coefficients showed that both cations and anions can have a strong affinity for phosphatidylcholine bilayers that appeared to be mainly (but not solely) driven by the hydrophobicity of the ions. Our preliminary correlation of membrane-partition coefficients vs. BCF clearly demonstrated that the bioconcentration potential of IL cations and anions is much higher than expected from octanol-water partitioning. The membrane affinity of long-chain compounds (≥C14) reached levels corresponding to BCFs that might be relevant in terms of the "B" classification. Moreover, the partitioning and concomitant BCF of organic ions is expected to be more dependent on environmental parameters than what is known for neutral compounds. Our study indicates that interactions with other ions in particular need to be considered when assessing the bioconcentration potential of hydrophobic cations and anions, including the type of counter ion, the concentration and type of the other hydrophobic ions in the medium (other pollutants) and the ionic strength of the medium (e.g., fresh vs. marine water).

For reliable estimation of the bioaccumulation potential of organic ions, much more experimental *in vitro* and *in vivo* data are needed to achieve a mechanistic understanding of the accumulation processes of ions and ion pairs. Very promising LFER and COSMOmic models have been developed to predict the membrane-water partitioning of ionogenic compounds (Bittermann et al., 2016, 2014) that should be applied to IL ions; these models should expanded to consider the influence of ion pairing and the ionic strength of the surrounding medium.

Such an improved understanding would allow screening of the bioconcentration potential of not only novel IL structures but also other charged compounds in a proactive manner, which would close an urgent knowledge gap in fundamental research and environmental regulation because approximately 70,000 compounds already preregistered under REACH are ionogenic or charged chemicals (Franco et al., 2010). The multitude of possible structural variations in protic and aprotic, cationic or anionic centres; substitution patterns; and substituted groups (aromatic or aliphatic, wide variety of functional groups) make ILs an ideal substance class for systematic investigations in this field.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.04.079.

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