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Methoden zur Anwendbarkeit des Benign by Design Konzepts für die biologische Abbaubarkeit in der Umwelt am Beispiel der ionischen Flüssigkeiten

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Zusammenfassung

Um schädliche Wirkungen auf Menschen und Umwelt zu vermeiden, sollten Chemikalien und Pharmazeutika entsprechend des Konzepts *Benign by Design* (BbD) so designt sein, dass sie in Abwasserbehandlungsprozessen und der Umwelt vollständig mineralisieren (10. Prinzip der Grünen Chemie, *design for degradation*). Die ionischen Flüssigkeiten (engl. ionic liquids, ILs) werden aufgrund ihres niedrigen Dampfdrucks als „grüne“ Alternative zu flüchtigen organischen Verbindungen gesehen. Allerdings sind die ILs nicht mehr „grün“ unter Berücksichtigung, dass a) ihre Synthese mit einem hohen Energieverbrauch, dem Einsatz schädlicher Lösungsmittel und nicht erneuerbarer Ressourcen verbunden ist, b) viele ILs persistent in der Umwelt und (öko-)toxisch sind und c) Anwendungen und Produkte zu einem Eintrag der ILs in die Umwelt führen. Folglich bedarf es für die ILs nachhaltigere Ansätze für das Design. Für die Umsetzung von BbD müssen die computergestützten Methoden auf verschiedene Substanzklassen angepasst werden. Daher sollte in dieser Arbeit die Anpassung und Weiterentwicklung der Methoden mit dem Ziel einer Anwendbarkeit des BbD-Konzepts auf ILs näher untersucht werden, um einen Beitrag zur Entwicklung von in der Umwelt mineralisierenden ILs zu leisten.

Um dies zu erreichen wurden a) mithilfe einer Literaturrecherche bereits etablierte Vorgehensweisen für BbD identifiziert und ein Arbeitsablauf für die systematische Kombination von Laborexperimenten und computergestützten Methoden entwickelt, b) entsprechend des *targeted Re-Designs*, als eine der Vorgehensweisen in BbD, neue Strukturfragmente in vorhandene ILs eingebaut und die Anwendbarkeit der Vorgehensweise untersucht, c) mithilfe einer systematischen Literaturrecherche Bioabbaudaten von ILs aufbereitet, in einem Datensatz zusammengestellt und mittels einer Clusteranalyse Struktur-Bioabbaubarkeitsbeziehungen (engl. structure-biodegradability relationships, SBRs) identifiziert und d) Modelle zur quantitativen SBR (QSBR) für die Anwendung in dem BbD-Arbeitsablauf entwickelt. Die Ergebnisse wurden in internationalen peer-reviewed Fachzeitschriften veröffentlicht.

Die Untersuchung vorhandener Studien hinsichtlich der Vorgehensweisen zur Umsetzung von BbD und der Kombination von Laborexperimenten und computergestützten Methoden lieferte vier Vorgehensweisen, das *targeted* und *non-targeted Re-Design* sowie *targeted* und *non-targeted de novo Design* (**Publikation 1**). Für BbD wurde ein Arbeitsablauf entwickelt, welcher eine systematische Kombination aus Laborexperimenten und computergestützten

Methoden zur Bewertung verschiedener Eigenschaften einer Substanz nutzt. Für die Bioabbaubarkeit verwendet der Arbeitsablauf (Q)SBR-Modelle, um Substanzen mit den geforderten Eigenschaften zu identifizieren und zu priorisieren. Die Möglichkeiten und Herausforderungen in der Umsetzung des Arbeitsablaufs für BbD wurden diskutiert (**Publikation 1**). Im Rahmen des *targeted Re-Designs* wurden vier Gemini-L-Phenylalanin-ILs (Gemini-Phe-Ester-ILs) entwickelt, welche sich in vier Strukturfragmenten unterschieden, der Ester- und Amidbindung sowie der *n*-Butyl- und *n*-Hexylkette (**Publikation 5**). Anhand der Ergebnisse aus den Bioabbautests nach OECD 301D und 301F und der Analytik über hochauflösende Massenspektrometrie wurden SBRs ermittelt, die für das Design von mineralisierenden ILs genutzt werden können. Die Gemini-Phe-Ester-ILs sind geeignet, um mögliche toxische Wirkungen von langen Alkylketten in ILs zu reduzieren und gleichzeitig vollständige Mineralisierung zu erreichen. Anhand der Ergebnisse konnte die erfolgreiche Umsetzung des *targeted Re-Designs* für ILs aufgezeigt werden (**Publikation 5**).

Die Aufbereitung der Bioabbaudaten der ILs, welche mittels der systematischen Literaturrecherche erhalten wurden, verdeutlichte, dass viele ILs in den standardisierten Tests zur leichten biologischen Abbaubarkeit kaum oder nicht bioabbaubar sind (**Publikation 2**). Weiterhin wurde dargelegt, dass valide und zuverlässige Bioabbaudaten benötigt werden, um diese für die Analyse von SBRs und der Entwicklung von QSBR Modellen zu nutzen (**Publikation 2**). Die Auswertung der Literaturdaten zur leichten biologischen Abbaubarkeit mittels Clusteranalyse erwies sich als geeignet, um die ILs nach ihrer strukturellen Ähnlichkeit zu gruppieren. Für das Kation, dessen Seitenketten und das Anion wurden die SBRs bestimmt und Datenlücken identifiziert. Die Ergebnisse wurden im Hinblick auf Empfehlungen für das Design bioabbaubarer ILs und zu testende Kombinationen von Kationen und Anionen diskutiert (**Publikation 3**). Für die Anwendbarkeit des Arbeitsablaufs auf ILs wurden fünf fragmentbasierte QSBR-Modelle für die ILs auf Basis von OECD 301D-Daten (**Publikation 4**). Die Modellvalidierung zeigte, dass die Performance der Modelle ausreichend ist, um Vorhersagen für gängige ILs wie Imidazolium, Pyridinium, quartäre Ammoniumverbindungen und Cholinium zu treffen. Es konnte dargelegt werden, wie die Modelle für den Einsatz in der Testbatterie im Arbeitsablauf für BbD eingesetzt werden können (**Publikation 4**).

Die im Rahmen dieser Arbeit erfolgte Anpassung und Weiterentwicklung neuer und spezifischer Vorgehensweisen und Methoden von der Datenrecherche, Datenaufbereitung, Datensatzerstellung, Datenanalyse bis zur Modellentwicklung ermöglicht die Anwendbarkeit von BbD auf ILs (**Publikation 1**–**5**). In dieser Arbeit konnten das Potential von

computergestützten Methoden für BbD demonstriert und für ILs anwendbar gemacht werden. Des Weiteren verdeutlichen die Ergebnisse, dass bereits etablierte Vorgehensweisen und Methoden individuell für verschiedene Substanzklassen angepasst werden müssen, um eine Anwendbarkeit des BbD-Konzepts zu ermöglichen. Voraussetzung dafür sind Kenntnisse zu SBRs und QSBR-Modelle mit einem Anwendungsbereich für die jeweilige Substanzklasse. Mit den weiterentwickelten Vorgehensweisen und Methoden für BbD in dieser Arbeit wird ein Beitrag zur Umsetzung der *Chemikalienstrategie für Nachhaltigkeit* der Europäischen Kommission geleistet, da die Bioabbaubarkeit ein wichtiges Kriterium von sicheren und nachhaltigeren Chemikalien ist und bereits im Designprozess berücksichtigt werden muss.

Abstract

In order to avoid adverse side effects on humans and the environment, the design of chemicals and pharmaceuticals should follow the concept *Benign by Design* (BbD) to develop chemicals and pharmaceuticals that are completely mineralising in wastewater treatment processes and the environment (10^{th} principle of Green Chemistry: design for degradation). Due to their low vapor pressure, ionic liquids (ILs) are being discussed as a "green" alternative to volatile organic compounds. However, the ILs appear no longer "green" considering that a) the synthesis needs a lot of energy, uses harmful solvents and non-renewable resources, b) many ILs are persistent in the environment and (eco)toxic and c) applications and products will release ILs into the environment. Consequently, more sustainable design approaches are needed for ILs. For the implementation of BbD, computer-based methods have to be adapted to the different substance classes. Therefore, in this study, the adaptation and development of methods was investigated with the aim of making the BbD concept applicable to the ILs and to contribute to the development of mineralising ILs.

For this purpose, a) a literature search was used to identify approaches in BbD and a workflow for the systematic combination of laboratory experiments and computer-based methods was developed, b) the applicability of the *targeted re-design* as an approach in BbD to the ILs was investigated, c) available biodegradation data was gathered in a systematic literature search, compiled in a data set and evaluated with regard to structure-biodegradability relationships (SBRs) by means of a cluster analysis and d) quantitative SBR (QSBR) models for the application in the BbD workflow were developed. The results were published in international peer-reviewed journals.

The examination of available studies regarding the applied approaches and the combination of laboratory experiments and computer-based methods resulted in four approaches, the *targeted* and *non-targeted re-design* and *targeted* and *non-targeted de novo design* (**publication 1**). In this work, a workflow for BbD using a systematic combination of laboratory experiments and computational methods was developed to evaluate different properties of a given substance. For biodegradability, the workflow uses (Q)SBR models to identify and prioritise substances that fulfil the required properties. The opportunities and challenges in the implementation of the workflow for BbD were discussed (**publication 1**). The *targeted re-design* was applied to the ILs in order to develop four gemini-L-phenylalanine-ILs (gemini-Phe-ester-ILs) (**publication 5**). They differed in four structural fragments, the ester and amide bond as well

as the *n*-butyl and *n*-hexyl chain. Based on the results of the biodegradation tests according to OECD 301D and 301F and the analysis by high-resolution mass spectrometry, SBRs were identified that can be used for the design of mineralising ILs. The gemini-Phe-ester-ILs are suitable for reducing potential toxic effects of long alkyl chains in ILs while maintaining ready biodegradability. The results demonstrate the successful implementation of the *targeted re-design* for ILs (**publication 5**).

The available biodegradability data of ILs, obtained by means of the systematic literature search, showed that many ILs are partially or not at all biodegradable in standardised tests for ready biodegradability (**publication 2**). Furthermore, this work highlights the need for valid and more reliable biodegradation data to use them in the analysis of SBRs and the development of QSBR models (**publication 2**). The evaluation of the ready biodegradability data using the cluster analysis proved to be suitable for grouping ILs according to their structural similarity (**publication 3**). SBRs were identified for the cation, the side chains attached to the cation and the anion. Data gaps became visible. The results were discussed with regard to recommendations for the design of readily biodegradable ILs and combinations of cations and anions that need to be tested (**publication 3**). For the applicability of the workflow for BbD to ILs, five fragment-based QSBR models were developed for the ILs based on OECD 301D data (**publication 4**). The validation results showed an adequate performance of the models to make predictions for common ILs such as imidazolium, pyridinium, quaternary ammonium compounds and cholinium. A test battery was presented, which shows how the QSBR models could be applied in the workflow for BbD (**publication 4**).

In this study new and specific methods from data research, data assessment, data set creation, data analysis to model development were adapted and developed to enable the applicability of BbD to ILs (**publication 1**–**5**). This study demonstrated the possibilities of *in silico* methods for BbD and made them applicable to ILs. Furthermore, the results underline that the developed approaches and methods need to be adapted individually to different substance classes to enable the applicability of the BbD concept. SBRs and QSBR models with an applicability domain adapted to the respective substance class are required. The developed approaches and methods for BbD contribute to the implementation of the *EU Chemicals Strategy for Sustainability*, as biodegradability is one important criterion in the design of safe and more sustainable substances.

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Inhaltsverzeichnis

Tabellenverzeichnis

Abbildungsverzeichnis

Abkürzungsverzeichnis

AD	Anwendungsbereich (engl. applicability domain)
AOP	Erweiterte Oxidation (engl. advanced oxidation process)
AP	Arbeitspaket
API	Arzneiwirkstoff (engl. active pharmaceutical ingredient)
AUC	Area under the curve
BbD	Benign by Design
$CholPheC_6$	Monokationische IL mit Cholinium-Kation, an der eine L-Phenylalanin-Seitenkette gebunden ist, an die wiederum über eine Esterbindung eine *n*-Hexylkette gebunden ist
C_n	linearer Alkylrest
DABCO	1,4-Diazabicyclo[2.2.2]octanium
ECFP	Extended-Connectivity Fingerprints
ECHA	Europäische Chemikalienagentur (engl. European Chemicals Agency)
HPLC-HRMS	Hochleistungsflüssigkeitschromatographie gekoppelt mit hochauflösender Massenspektrometrie (engl. high performance liquid chromatography coupled to high resolution mass spectrometre)
ILs	Ionische Flüssigkeiten (engl. ionic liquids)
ImAc	1-(Carboxymethyl)-3-methyl-1H-imidazol-3-ium
$ImPheC_6$	Monokationische IL mit 3-Methylimidazolium-Kation, an der eine L-Phenylalanin-Seitenkette gebunden ist, an die wiederum über eine Esterbindung eine *n*-Hexylkette gebunden ist
INSC	Institut für Nachhaltige Chemie an der Leuphana Universität Lüneburg (engl. Institute of Sustainable Chemistry)
ISO	International Organization for Standardization
LR	Logistische Regression (engl. logistic regression)
OECD	Organisation für wirtschaftliche Zusammenarbeit und Entwicklung (engl. Organisation for Economic Co-operation and Development)
OLS	Regressionsverfahren der kleinsten Quadrate (engl. ordinary least squares)
Phe	L-Phenylalanin
$PyPheC_n$	Monokationische IL mit Pyridinium-Kation, an der eine L-Phenylalanin-Seitenkette gebunden ist, an die wiederum über eine Esterbindung eine *n*-Butylkette oder eine *n*-Hexylkette gebunden ist

$[PyPhe]_2C_nEster$	Dikationische IL mit zwei Pyridinium-Kationen, an die eine L-Phenylalanin-Seitenkette gebunden ist. Die zwei Phenylalanin-Seitenketten sind über eine Esterbindung an einer *n*-Butyl- oder einer *n*-Hexylkette verbunden
QACs	Quartäre Ammoniumverbindungen (engl. quaternary ammonium compounds)
(Q)SAR	(Quantitative) Struktur-Wirkungsbeziehung (engl. (quantitative) structure-activity relationship)
(Q)SBR	(Quantitative) Struktur-Bioabbaubarkeitsbeziehung (engl. (quantitative) structure-biodegradability relationship)
(Q)SPR	(Quantitative) Struktur-Eigenschaftsbeziehung (engl. (quantitative) structure-property relationship)
REACH	Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe (engl. Registration, Evaluation, Authorisation and Restriction of Chemicals)
Set_IL	OECD 301D-Datensatz von ionischen Flüssigkeiten
Set_ILNI	OECD 301D-Datensatz von ionischen Flüssigkeiten, organischen Anionen in Kombination mit anorganischen Kationen und nicht-ionische Substanzen
SSbD	Inhärent sicher und nachhaltig (engl. safe-and-sustainable-by-design)
TNR	Spezifität (Richtig-negativ-Rate, engl. true negative rate)
TP	Transformationsprodukt
TPR	Sensitivität (Richtig-positiv-Rate, engl. true positive rate)

1 Einleitung

1.1 Benign by Design als Strategie zur Verminderung des Vorkommens von Chemikalien in der Umwelt

Weltweit sind etwa 350.000 Chemikalien und Mischungen aus Chemikalien registriert, die zum Teil aufgrund ihrer Eigenschaften, wie Persistenz, Bioakkumulation und (Öko-)Toxizität, schädliche Auswirkungen auf Mensch und Umwelt haben können (Schwarzenbach et al., 2006; Wang et al., 2020). Nicht alle dieser Chemikalien können im Kreislauf geführt werden. Durch ihre Anwendung beispielsweise als Pestizid, Körperpflegeprodukt oder Pharmazeutika gelangen einige Chemikalien nach ihrer Anwendung direkt oder in Folge eines unvollständigen Abbaus und Rückhalts in Kläranlagen in die Umwelt. Darüber hinaus werden 80 % des weltweiten Abwassers ohne Behandlung in die Umwelt eingetragen (United Nations World Water Assessment Programme, 2017).

Durch Hydrolyse, Photoabbau und Bioabbau können Chemikalien in Kläranlagen und der Umwelt unvollständig zu Transformationsprodukten (TPs) abgebaut werden oder vollständig zu Kohlenstoffdioxid, Wasser und ggf. weiteren anorganischen Verbindungen mineralisiert werden (Sijm et al., 2007). Einige Chemikalien sind aber auch aufgrund von hohen Halbwertszeiten gegenüber (a)biotischen Abbauprozessen persistent in Kläranlagen und in der Umwelt (Scheringer et al., 2012). Die anthropogenen Chemikalien sind meist nicht bioabbaubar, da Mikroorganismen und Enzyme nicht an diese Chemikalien angepasst sind (Rieger et al., 2002). Folglich können diverse Chemikalien, wie z.B. Biozide, Herbizide, Arzneiwirkstoffe (engl. active pharmaceutical ingredient, API), Flammschutzmittel, UV-Blocker, Weichmacher, Imprägniermittel und Desinfektionsmittel, in Oberflächengewässern nachgewiesen werden (Bester et al., 2008).

Die Persistenz ist eine wichtige Eigenschaft in der Gefährdungs- und Risikobewertung, da persistente Chemikalien die Umwelt in hohen Konzentrationen kontaminieren und sich global verteilen können (Cousins et al., 2019; Joerss et al., 2020). Darüber hinaus müssen auch Umweltrisikobewertungen für TPs durchgeführt werden. Einerseits können diese toxischer und persistenter sein als die Ausgangssubstanz (Cui et al., 2011; Garavagno et al., 2024; Hensen et al., 2020; Menz et al., 2017; Tian et al., 2021). Andererseits sind die Wirkungen auf Mensch und Umwelt aufgrund fehlender Daten weitgehend unbekannt (Escher & Fenner, 2011; Hensen et al., 2020; Menz et al., 2017; Zahn et al., 2024). Die Dringlichkeit der Vermeidung des Eintrags von persistenten und (öko-)toxischen Chemikalien und TPs in die Umwelt wird durch

die Überschreitung der planetarischen Grenze für neuartige Stoffe betont (Persson et al., 2022). Allerdings ist die erweiterte Abwasserbehandlung zur Verminderung des Eintrags von Chemikalien in die Umwelt, wie die erweiterte Oxidation (engl. advanced oxidation process, AOP), mit vielen Problemen verbunden und als *end-of-pipe* Maßnahme nicht nachhaltig (Kümmerer, 2007; Kümmerer et al., 2019). Es können unbekannte TPs entstehen (Funke et al., 2016; Konstantinou & Albanis, 2003). Außerdem sind die Anwendbarkeit und Effektivität der AOPs gegenüber der Vielfalt an Chemikalien und Pharmazeutika limitiert (Kümmerer et al., 2019; Magdeburg et al., 2014; Margot et al., 2013). Daher müssen Maßnahmen an der Quelle des Problems, den Chemikalien selbst, vorgenommen werden. In der *Chemikalienstrategie für Nachhaltigkeit* wurden notwendige Maßnahmen aufgestellt, um die Entwicklung von inhärent sicheren und nachhaltigen (engl. safe-and-sustainable-by-design, SSbD) Chemikalien zu fördern (Europäische Kommission, 2020). Einen Beitrag dazu liefert das Konzept *Benign by Design* (BbD) über die Umsetzung des 10. Prinzips der Grünen Chemie, *design for degradation* (Anastas, 1994; Anastas & Eghbali, 2010; Kümmerer, 2007). Dementsprechend müssen leicht biologisch abbaubare Chemikalien entwickelt werden, die nach ihrem bestimmungsgemäßen Gebrauch bestenfalls in der Kläranlage oder in der Umwelt schnell und vollständig mineralisiert werden (Kümmerer, 2007).

1.2 Beitrag von *in silico* Methoden für die Entwicklung von in der Umwelt mineralisierenden Substanzen

Für die Umsetzung von BbD sind Strukturmerkmale zu identifizieren, die einen leichten Abbau begünstigen oder vermindern. Gleichzeitig müssen die Substanzen während ihrer Lagerung und Anwendung ausreichend stabil sein, um ihre Funktion zu gewährleisten (Kümmerer, 2007). Dabei können bereits kleine Änderungen in der chemischen Struktur einen Einfluss auf die Eigenschaften oder die Aktivität nehmen. Diese Struktur-Bioabbaubarkeitsbeziehungen (engl. structure-biodegradability relationships, SBRs) können für das Design von Substanzen genutzt werden (Kümmerer, 2007). Als Vorgehensweisen für BbD wurden das *de novo* Design und Re-Design beschrieben (Leder et al., 2015). Beim *de novo* Design werden Substanzen unter Berücksichtigung der vollständigen Mineralisierung in der Umwelt von Grund auf neu entwickelt. Beispielsweise kann die Mineralisierung in der Umwelt in das Design von APIs eingebunden werden (Puhlmann et al., 2021). Beim Re-Design werden vorhandene Substanzen durch den Einbau neuer Strukturfragmente verändert, um die Mineralisierung in der Umwelt

zu erwirken. Die für die Funktion unerlässliche Grundstruktur bleibt dabei erhalten (Leder et al., 2015; Leder et al., 2021; Rastogi et al., 2015a; Suk et al., 2023).

Um BbD anzuwenden ist folglich Expertenwissen zu SBRs notwendig. Dieses Wissen kann durch das Testen der Bioabbaubarkeit von einer Serie struktureller Analoge als auch durch die computergestützte Auswertung eines Datensatzes aufgebaut werden (Grabitz et al., 2021; Grabitz et al., 2020; Peltason & Bajorath, 2009; Stumpfe & Bajorath, 2012; Wawer et al., 2010). Weiterhin können Modelle für die Bewertung von vorhandenen und neu entwickelten Chemikalien als auch von TPs angewendet werden, um die Wissenslücken zu Eigenschaften und Aktivitäten zu schließen (Hensen et al., 2020; Leder et al., 2015; Rücker & Kümmerer, 2012). Es wird dabei zwischen Modellen zur (quantitativen) Struktur-Wirkungsbeziehung (engl. (quantitative) structure-activity relationship, (Q)SAR), (quantitativen) Struktur-Eigenschaftsbeziehung (engl. (quantitative) structure-property relationship, (Q)SPR) und (quantitativen) SBR (engl. (quantitative) SBR, (Q)SBR) unterschieden. Im Folgenden werden zur Vereinfachung die Begriffe (Q)SAR und (Q)SBR verwendet, wenn allgemein Modelle zu Aktivitäten und Eigenschaften gemeint sind bzw. zur Bioabbaubarkeit im Speziellen.

Die (Q)SAR-Modelle haben im Vergleich zu Laborexperimenten den Vorteil, dass sie a) nur die Strukturformel der Substanz benötigen, b) zeitsparend sind, c) Tierversuche minimieren können und d) keine Laborabfälle und Chemikalienabfälle produzieren (Gramatica et al., 2018). Ein weiterer Vorteil ist, dass die Europäische Chemikalienagentur (engl. European Chemicals Agency, ECHA) die Vorhersagen für die Registrierung, Bewertung, Zulassung und Beschränkung chemischer Stoffe (engl. Registration, Evaluation, Authorisation and Restriction of Chemicals, REACH) anerkennt, wenn die Modelle und Vorhersagen bestimmte Voraussetzungen erfüllen, u.a. die Prinzipien für die Validierung von (Q)SAR-Modellen der Organisation für wirtschaftliche Zusammenarbeit und Entwicklung (engl. Organisation for Economic Co-operation and Development, OECD) (ECHA, 2016; OECD, 2006). Die OECD-Prinzipien umfassen a) einen definierten Endpunkt, b) einen eindeutigen Algorithmus, c) einen definierten Anwendungsbereich (engl. applicability domain, AD), d) geeignete Messgrößen für die Anpassungsgüte (engl. goodness-of-fit), Robustheit (engl. robustness) und Vorhersagbarkeit (engl. predictivity) und e) eine mechanistische Interpretation, wenn möglich (OECD, 2006).

Es gibt verschiedene Methoden in der Modellierung, die alle ihre Vorteile und Nachteile haben. Dadurch gibt es keine Methode, die für alle QSAR-Probleme geeignet ist (Yee & Wei, 2012). Einerseits sind lineare und logistische Regressionsmodelle einfacher zu modellieren und zu

interpretieren hinsichtlich SBRs als Neuronale Netzwerke (Gramatica, 2020; Guha, 2008; Yee & Wei, 2012). Andererseits können sich Neuronale Netzwerke der Zielfunktion, welche den realen Zusammenhang zwischen Deskriptoren und Eigenschaft einer Chemikalie mathematisch abbildet, genauer annähern, wodurch eine höhere Genauigkeit in der Vorhersage erreicht wird (Gramatica, 2020; Guha, 2008; Yee & Wei, 2012).

Die Ergebnisse aus den Modellvorhersagen werden dann im *de novo* Design und Re-Design verwendet, um Substanzen mit besserer Bioabbaubarkeit zu identifizieren. (Q)SAR- und (Q)SBR-Modelle wurden vereinzelt erfolgreich für BbD eingesetzt (Rastogi et al., 2014, 2015a, 2015b). Allerdings hat die Anwendung von *in silico* Methoden gezeigt, dass für eine entsprechende Nutzung in BbD eine individuelle Anpassung der Modelle auf die zu untersuchende Substanzklasse erfolgen muss (Lorenz, 2023; Rastogi et al., 2014, 2015a, 2015b).

1.3 Benign by Design am Beispiel der ionischen Flüssigkeiten

Ionische Flüssigkeiten (engl. ionic liquids, ILs) sind Salze, die aufgrund der asymmetrischen und großen Molekülstruktur des Anions und Kations einen Schmelzpunkt unter 100°C aufweisen (Greer et al., 2020). Die häufig genutzten Kationen und Anionen sind in Abbildung 1 dargestellt. ILs können sowohl aus einem einzigen, bestimmten Kation und Anion bestehen oder aus verschiedenen Kationen und Anionen in einer Mischung. In dieser Arbeit bezieht sich der Begriff „ILs“ ausschließlich auf ILs, die ein einziges bestimmtes Kation und Anion enthalten. Durch die vielfältigen Kombinationen aus Kation, dessen Seitenkette und Anion sind nahezu unendlich viele ILs möglich. Folglich ergibt sich ein großes Potenzial, ILs entsprechend den geforderten Eigenschaften und Funktionen zu designen (Plechkova & Seddon, 2008). ILs werden daher auch *designer solvents* genannt (Plechkova & Seddon, 2008).

ILs zeichnen sich durch ihre Vielfalt an Anwendungsbereichen aus z.B. in den Bereichen Elektrochemie, chemische Prozesse (z.B. Synthese, Katalyse, Extraktion), Additive (z.B. Dispersionsmittel, Reinigungszusätze, Antistatika, Schmiermittel), Gasabscheidung und -speicherung, Pharmazie und analytische Chemie (z.B. als Säulenmaterial in der Gaschromatographie) (Greer et al., 2020; Gutowski, 2018; Plechkova & Seddon, 2008). ILs wurden u.a. als Lösungsmittel für Zellulose, Elektrolyt in Lithium-Batterien, Lösungsmittel für die Herstellung von Perowskit-Photovoltaikmodulen, API, Biozid und Herbizid eingesetzt (Egorova et al., 2017; Vereshchagin et al., 2021; Wang et al., 2023; Watanabe et al., 2017; Wilms et al., 2020; Zhang et al., 2017).

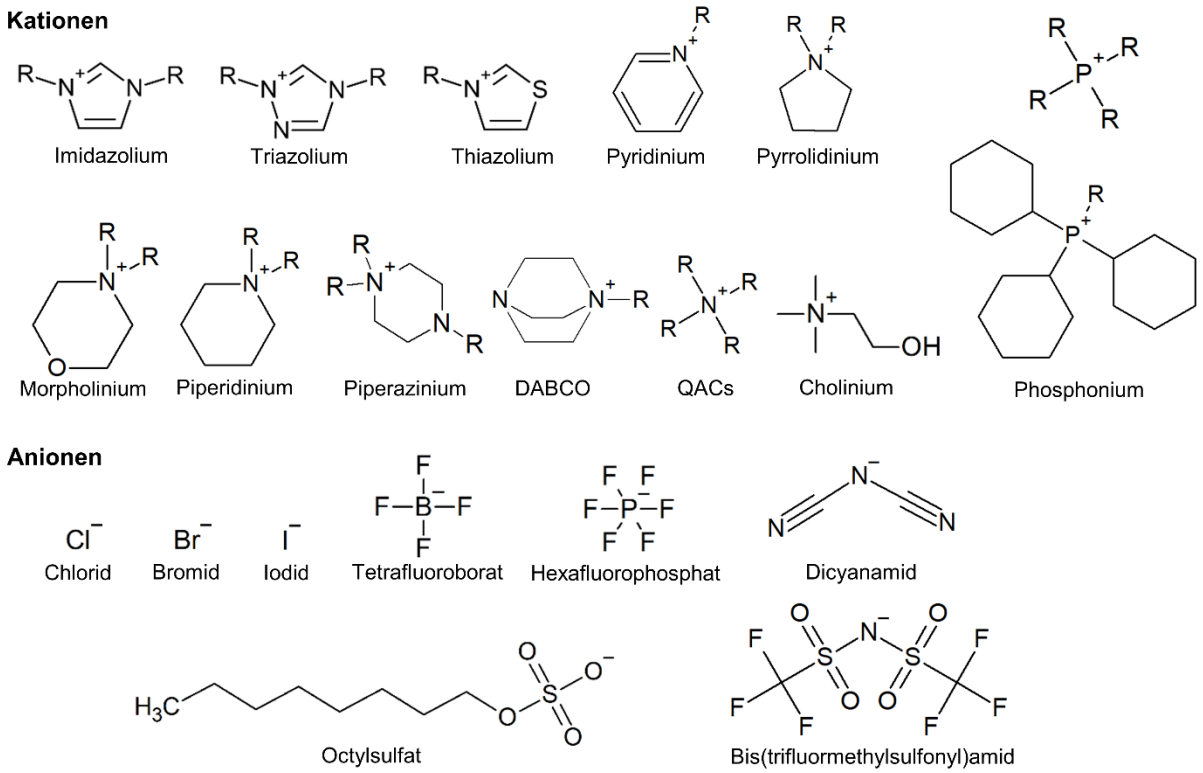

Abbildung 1: Chemische Strukturen der gängigen Kationen und Anionen der ILs. 1,4-Diazabicyclo[2.2.2]octanium (DABCO), quartäre Alkylammoniumverbindungen (engl. quaternary alkylammonium compounds, QACs).

Die Anwendungen als API, Biozid oder Herbizid machen einen Eintrag der ILs in die Umwelt unvermeidlich. Aber auch bei anderen Anwendungen ist ein Eintrag der ILs am Ende des Lebenszyklus der genannten Produkte oder durch unbeabsichtigte Freisetzungen aus der Industrie oder Produkten nicht auszuschließen. Einige quartäre Alkylammoniumverbindungen (engl. quaternary alkylammonium compounds, QACs), Imidazolium-, Pyridinium-, Pyrrolidinium-, Phosphoniumkationen und Tetrafluroborat-, Hexafluorophosphat-, Bis(trifluormethylsulfonyl)amidanionen konnten bereits in Oberflächengewässern, Sedimenten und Kläranlagenabläufen nachgewiesen werden (Brand et al., 2018; Neuwald et al., 2021; Pati & Arnold, 2020). Viele QACs wie Benzylalkyldimethylammonium- oder Alkyltrimethylammoniumverbindungen werden in der Literatur nicht als IL bezeichnet, können aber grundsätzlich auch der Gruppe der ILs zugeordnet werden. Diese werden u.a. kommerziell als Desinfektionsmittel verwendet und konnten in Kläranlagenabläufen und Oberflächengewässern nachgewiesen werden, wo sie toxisch auf Wasserorganismen wirken (Zhang et al., 2015).

Aufgrund ihres niedrigen Dampfdrucks werden ILs als „grüne“ Alternative zu flüchtigen organischen Verbindungen beschrieben (Earle & Seddon, 2000). Allerdings wurde in diesem

Zusammenhang lediglich eine Eigenschaft betrachtet. Bereits die Synthese und Aufreinigung der ILs sind mit einem hohen Energieverbrauch, einem schlechten E-Faktor, einer schlechten Atomökonomie, dem Einsatz schädlicher Lösungsmittel und nicht erneuerbarer Ressourcen verbunden (Deetlefs & Seddon, 2010; Righi et al., 2011). Viele ILs sind zudem persistent in der Umwelt und (öko-)toxisch (Costa et al., 2017; Jordan & Gathergood, 2015; Pham et al., 2010). Weiterhin wurde gezeigt, dass einige ILs zytotoxisch sind und das Enzym Acetylcholinesterase im Nervensystem des Menschen hemmen können, welches zu Störungen in der Neurotransmission führt (Arning et al., 2008; Stock et al., 2004; Stolte et al., 2007). Außerdem wurde gezeigt, dass toxische Zersetzungs- und Abbauprodukte, z.B. in der Aufreinigung von ILs mit einem Hexafluorophosphatanion und in AOPs entstehen können (Calza et al., 2018; Pieczyńska et al., 2015; Siciliano et al., 2019; Swatloski et al., 2003). Um schädliche Wirkungen auf Mensch und Umwelt zu vermeiden, sollten ILs nicht (öko)toxisch, nicht bioakkumulativ, in der Umwelt leicht biologisch abbaubar sein und gleichzeitig ihre Funktion erfüllen. Folglich ist mittels BbD ein Beitrag zur Entwicklung von in der Umwelt mineralisierenden ILs zu leisten.

Bislang wurden im Bereich BbD individuelle Serien von ILs designt, die Strukturfragmente enthalten, die die Bioabbaubarkeit begünstigen können, z.B. Ester, Octylsulfat als Anion und natürliche Verbindungen auf Basis von Aminosäuren (Haiß et al., 2016; Harjani et al., 2009; Jordan et al., 2016; Markiewicz et al., 2016; Prydderch et al., 2017; Suk et al., 2020; Suk & Kümmerer, 2023). Die Serien wurden hinsichtlich ihrer Bioabbaubarkeit untersucht und SBRs identifiziert. Auf Basis der experimentellen Daten und SBRs wurden allgemeinere Faustregeln für die Bioabbaubarkeit abgeleitet (Boethling et al., 2007; Coleman & Gathergood, 2010; Costa et al., 2017; Jordan & Gathergood, 2015). Hingegen wurde die Kombination von Laborexperimenten und computergestützten Methoden bisher noch nicht für das systematische Design von mineralisierenden ILs eingesetzt. Ebenso wenig wurden Datensätze zur Bioabbaubarkeit erstellt und mit computergestützten Methoden systematisch auf SBRs analysiert.

Mögliche Gründe dafür könnten sein, dass computergestützte Methoden für ILs noch nicht ausreichend erforscht wurden und diese im Gegensatz zu computergestützten Methoden für nicht-ionische Substanzen auch nicht zugänglich sind (Koutsoukos et al., 2021). Zurzeit sind viele (Q)SBR-Modelle nicht auf ILs anwendbar, da diese Substanzen nicht in den Trainingsdaten der Modelle enthalten sind und daher außerhalb der AD liegen. Die einzigen bekannten QSBR-Modelle für ILs sind in der *AquaBoxIL* hinterlegt, die die Vorhersagen aus

den QSBR-Modellen zusammen mit Vorhersagen zu physiko-chemischen Eigenschaften als Eingangswerte für ein Modell zur Verteilung einer IL in der Umwelt zwischen Wasser, Sediment und organischem Material verwendet (Barycki et al., 2018). Ein weiterer Grund könnte sein, dass zwar experimentelle Bioabbaudaten in der Literatur vorhanden sind, aber keine Datenbanken zur Bioabbaubarkeit von ILs zugänglich sind, die für die Erstellung von Datensätzen für (Q)SBR-Modelle genutzt werden können. Während die *UFT Merck Ionic Liquids Biological Effects Database* nicht mehr online zugänglich ist (UFT Merck Ionic Liquids Biological Effects Database), sind für physiko-chemische und toxikologische Eigenschaften von ILs mit *ILThermo* und *ILTox* Datenbanken vorhanden (Dong et al., 2007; Yan et al., 2023).

Daher besteht die Forschungslücke darin, dass computergestützte Methoden für die Anwendbarkeit von BbD auf ILs bislang nicht weiterentwickelt wurden.

2 Aufbau und Ziele der Arbeit

Das Ziel dieser Arbeit war die Anpassung und Weiterentwicklung von Methoden, um das BbD-Konzept auf die ILs zu anwendbar zu machen und einen Beitrag zur Entwicklung von mineralisierenden ILs zu leisten. Folglich sollten Wissenslücken in SBRs und der Anwendbarkeit von experimentellen als auch computergestützten Methoden aus dem Bereich BbD auf ILs geschlossen werden. Die Erkenntnisse sollten einen Beitrag zur Umsetzung von bestehenden Strategien und Gesetzgebungen zur Bewertung und Regulatorik von Substanzen liefern. Weiterhin sollten die Erkenntnisse in die weitere Forschung und praktische Anwendung der Strategien im Bereich des Designs von mineralisierenden ILs einfließen.

Anhand des übergeordneten Ziels ergaben sich vier Arbeitspakete (APs) und Teilziele (Abbildung 2). In AP 1 lag der Fokus darauf zu verstehen, welche Vorgehensweisen für BbD in der Literatur beschrieben sind und welche Methoden zum Einsatz kommen. Das *targeted Re-Design*, eines der identifizierten Vorgehensweisen in BbD, wurde in AP 2 auf die ILs angewendet, um die Anwendbarkeit zu prüfen und SBRs zu ermitteln. AP 3 zielte darauf ab die vorhandenen Bioabbaudaten der ILs besser zu verstehen. Dafür wurden die Bioabbaudaten aus der Literatur aufbereitet und in einem Datensatz zusammengestellt, der hinsichtlich SBRs analysiert wurde. Das Teilziel in AP 4 war die Entwicklung von QSBR-Modellen für BbD und einer Testbatterie. Folgende Fragestellungen sollten in den vier APs beantwortet werden:

i. **AP 1**: Welches Vorgehen gibt es im Design von in der Umwelt mineralisierenden Chemikalien und Pharmazeutika? Wie können computergestützte Methoden (*in silico*) systematisch bei dem Design von nachhaltigeren Chemikalien eingesetzt werden? Wie würde das in einem Arbeitsablauf umgesetzt werden können?
ii. **AP 2**: Kann das *targeted Re-Design* auf die ILs angewendet werden? Kann das Einfügen einer L-Phenylalanin-Gruppe (Phe) in Gemini-ILs als gezielte strukturelle Veränderung zu einer zunehmenden Bioabbaubarkeit führen?
iii. **AP 3**: Wie ist die Datenverfügbarkeit zur Bioabbaubarkeit von ILs? Wie ist die Qualität der Daten? Welche SBRs können identifiziert werden und wie stimmen diese mit den bestehenden Faustregeln aus der Literatur überein?
iv. **AP 4**: Kann die Bioabbaubarkeit von ILs modelliert werden? Wie können die QSBR-Modelle in BbD eingesetzt werden?

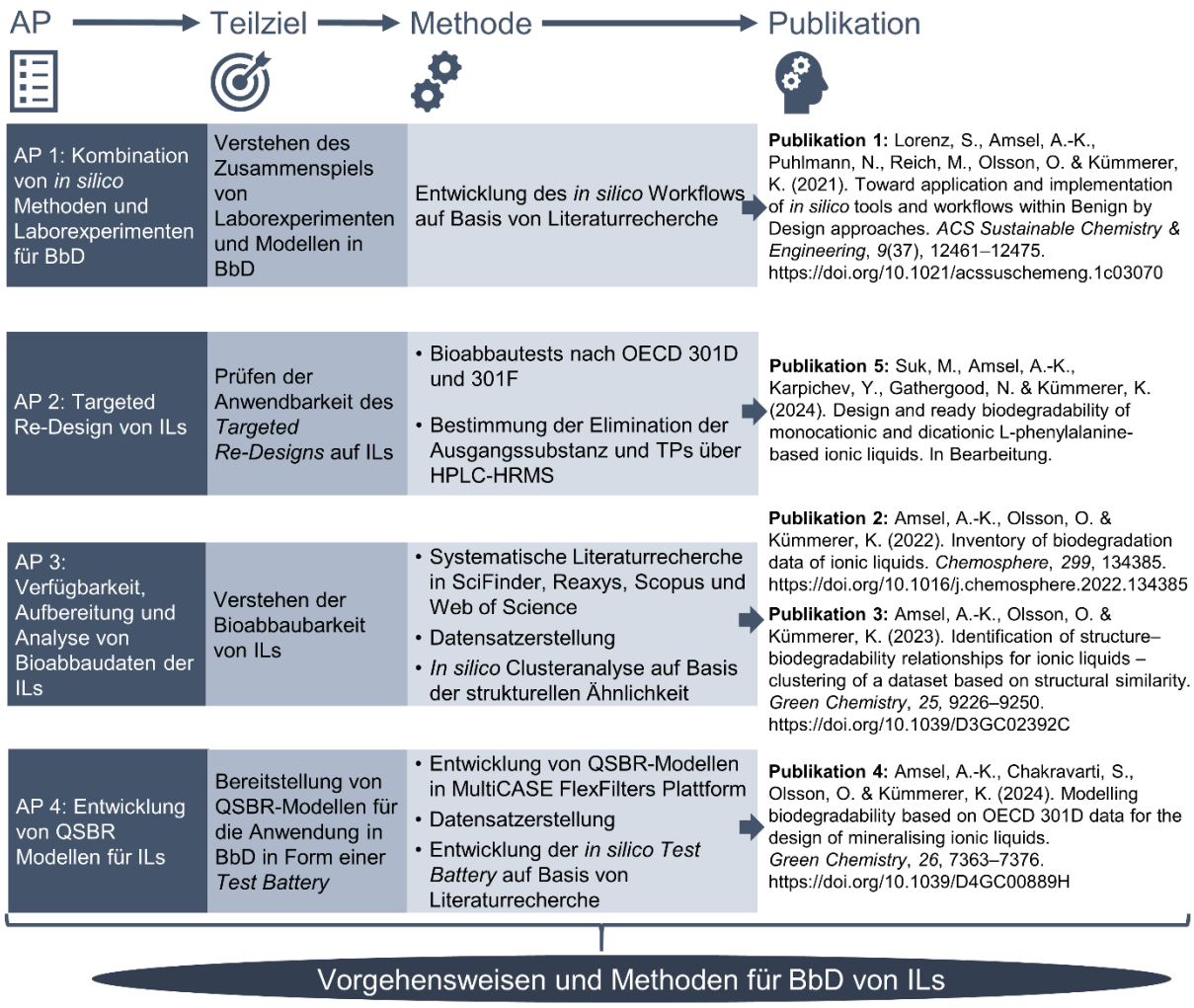

Abbildung 2: Arbeitspakete und Teilziele der Arbeit, methodisches Vorgehen und die daraus resultierenden Publikationen.

Bestehende Strategien wurden analysiert, angepasst und weiterentwickelt. Darauf aufbauend wurden deren Potenzial und Übertragbarkeit auf die Substanzklasse der ILs untersucht. Jedes dieser APs wurde wissenschaftlich bearbeitet und die Forschungsergebnisse in Artikeln in internationalen peer-reviewed Fachzeitschriften veröffentlicht (Abbildung 2). Die Ergebnisse der APs werden in den Kapiteln 3.1–3.5 dargelegt und ihr Beitrag zu Strategien und Gesetzgebungen zur Bewertung und Regulatorik von Substanzen und BbD in Kapitel 4 eingeordnet.

3 Ergebnisse und Diskussion

3.1 Kombination von *in silico* Methoden und Laborexperimenten in Benign by Design

3.1.1 Forschungslücke

Wie in Kapitel 1.1 gezeigt wurde, ist die Umsetzung von BbD erforderlich, um durch die Entwicklung von in der Umwelt vollständig mineralisierenden Chemikalien und Pharmazeutika einen Beitrag zur Umsetzung der *Chemikalienstrategie für Nachhaltigkeit* und dem darin vorgestellten SSbD-Konzept zu leisten. Für die erfolgreiche Umsetzung von BbD ist es notwendig die Arbeitsabläufe zu verstehen, geeignete Endpunkte und Modelle auszuwählen und die Vorhersagen zu interpretieren. Das *de novo* Design und Re-Design für BbD wurden beschrieben und sowohl Laborexperimente als auch *in silico* Methoden in BbD eingesetzt (Kapitel 1.2). Allerdings gab es bislang keine systematische Aufstellung der Arbeitsabläufe für die Kombination von Modellen und Laborexperimenten für die Stoffbewertung im Kontext von BbD. Ziel dieser Arbeit war es daher die Vorgehensweisen von BbD und den Beitrag von computergestützten Methoden besser zu verstehen. Um schnellere und bessere Entscheidungen im Design von Chemikalien und Pharmazeutika zu treffen, wurde ein Arbeitsablauf entwickelt, der zeigt, wie Modelle zur Vorhersage von Stoffeigenschaften für BbD eingesetzt werden können.

3.1.2 Methode

Für die Entwicklung des Arbeitsablaufs wurden Studien zur praktischen Umsetzung von BbD und Anwendung von Modellen zur Vorhersage von Stoffeigenschaften genutzt, um auf Grundlage dessen einen Arbeitsablauf für die Anwendung von Laborexperimenten und Modellen zu entwickeln (**Publikation 1**).

3.1.3 Ergebnisse und Diskussion

Das allgemeine Vorgehen von BbD kann in fünf Schritte unterteilt werden (**Publikation 1**, Abbildung 1). Im ersten Schritt ist die Funktion der Substanz zu definieren, welche während der Lagerung und zum Zeitpunkt der Anwendung gewährleistet sein sollte. Der zweite Schritt umfasst die Auswahl des konkreten Vorgehens aus vier verschiedenen Vorgehensweisen für das Design neuer Chemikalien und Pharmazeutika (**Publikation 1**, Tabelle 1). Dies führt zu einem Pool verschiedener Moleküle im dritten Schritt. In diesem Pool müssen die Moleküle

identifiziert werden, die die gewünschten Eigenschaften erfüllen (z.B. Funktion wird erfüllt, leichte Bioabbaubarkeit, keine Ökotoxizität und Toxizität, nicht bioakkumulierend). Dafür werden im vierten und fünften Schritt Modelle und Laborexperimente eingesetzt.

Folgende vier Vorgehensweisen für das Design neuer Moleküle wurden anhand der Literatur abgeleitet (**Publikation 1**, Tabelle 1):

i. *non-targeted de novo Design*
ii. *targeted de novo Design*
iii. *non-targeted Re-Design*
iv. *targeted Re-Design*

Die Vorgehensweisen wurden in **Publikation 1** genauer beschrieben und unterscheiden sich in den Methoden, *in silico* oder Laborexperimente, die für die Entwicklung der Moleküle angewendet werden. Der Unterschied zwischen dem d*e novo* Design und Re-Design wurde bereits in Kapitel 1.2 beschrieben. Bei dem *targeted* Ansatz werden im Gegensatz zum *non-targeted* Ansatz gezielt Strukturfragmente in ein bestehendes Molekül eingebaut (Re-Design) oder zu einer neuen Substanz kombiniert (*de novo* Design). Die Strukturfragmente werden auf Basis von Expertenwissen ausgesucht und sollen die gewünschten Eigenschaften verbessern. Bei dem *non-targeted* Ansatz werden Strukturfragmente nicht zielgerichtet zu neuen Molekülen kombiniert oder in bestehende Moleküle eingebaut. So zeichnet sich das *non-targeted Re-Design* dadurch aus, dass Derivate des bestehenden Moleküls durch z.B. Photolyse im Labor erzeugt werden. Die Derivate unterscheiden sich oft nur in kleinen Strukturfragmenten von der Ausgangsverbindung (**Publikation 1**).

Die Moleküle aus allen vier Vorgehensweisen werden dann im Arbeitsablauf näher auf ihre Eigenschaften untersucht und bewertet, ob sie die gewünschten Eigenschaften erfüllen und ihre Funktion beibehalten haben (**Publikation 1**, Abbildung 2). Vor der Anwendung des Arbeitsablaufs sind folgende fünf Schritte durchzugehen, die in **Publikation 1** beschrieben wurden:

i. Darstellung der Molekülstruktur in einem für Computer lesbaren Format
ii. Festlegung der relevanten Endpunkte für die Bewertung
iii. Auswahl geeigneter Modelle, in denen die Substanzen innerhalb der AD liegen
iv. Festlegung der Methoden zur Evaluation der Vorhersagen
v. Festlegung der Dokumentation der Vorhersagen und Datenspeicherung

Ebenfalls wurden Zielwerte diskutiert, die bei der Bewertung hilfreich sein können, ob eine Substanz die gewünschten Eigenschaften aufweist und als „benign“ gelten kann. Ein Zielwert ist die vollständige Mineralisierung in der aquatischen Umwelt in Tests nach OECD 301, wobei die Substanz während der Lagerung und Anwendung stabil sein sollte (**Publikation 1**).

Der Arbeitsablauf soll die Moleküle aus dem Pool auf die vielversprechendsten Kandidaten begrenzen und ihre Anzahl verkleinern (**Publikation 1,** Abbildung 2). Dafür wird für die Moleküle der zuvor festgelegte Endpunkt mit verschiedenen geeigneten Modellen, wie (Q)SAR und Read-Across, vorhergesagt. Für das *non-targeted (Re-)Design* wirkt der Arbeitsablauf wie ein Filter, der Moleküle von der weiteren Bewertung ausschließt, wenn sie die geforderte Eigenschaft nicht erfüllen. Im Gegensatz dazu wird im *targeted (Re-)Design* der Arbeitsablauf wie eine Schleife verwendet. Sollte eine Eigenschaft nicht erfüllt werden, können die Moleküle in ihren Strukturen so geändert werden, dass sie die Eigenschaft in der nächsten Bewertungsrunde möglicherweise erfüllen. Die Anpassung der Molekülstruktur und die Bewertung der Eigenschaften können so lange wiederholt werden, bis ein Molekül mit den geforderten Eigenschaften gefunden wurde. Die so verkleinerte Anzahl an Molekülen kann dann synthetisiert und die Eigenschaften im Labor experimentell überprüft werden. Selbst wenn Moleküle verworfen werden, werden viele Daten generiert, die SBRs, SARs und SPRs liefern und damit Hinweise für das Design geben können. Entsprechend sollten diese dokumentiert werden und in zukünftigen Designprozessen bedacht werden.

Weiterhin wurden die Herausforderungen in der Umsetzung des Arbeitsablaufs diskutiert (**Publikation 1**). Um den Arbeitsablauf erfolgreich einsetzen zu können, ist während des Designs immer zu berücksichtigen, dass das Molekül auch synthetisiert werden kann. Auch ist es für manche Substanzklassen, z.B. ILs und siliziumorganische Substanzen, eine Herausforderung geeignete Modelle zu finden, da manche Klassen in den Trainingsdaten nicht hinterlegt sind und somit außerhalb der AD liegen. Zudem sind nicht alle Modellvorhersagen gleich gut interpretierbar, da dies von den verwendeten Algorithmen und Deskriptoren in den Modellen abhängig ist. Außerdem ist Expertenwissen zu SBRs, SARs und SPRs für das Design der Molekülstrukturen erforderlich.

3.1.4 Schlussfolgerung

Erstmals wurde ein Arbeitsablauf für BbD entwickelt, der das systematische Zusammenspiel von *in silico* und experimentellen Methoden für eine erste Priorisierung der Substanzen im Hinblick auf Persistenz, (Öko)Toxizität und Bioakkumulation im Designprozess aufzeigt. Mit dem Arbeitsablauf wird ein Beitrag zum Design von sicheren und nachhaltigeren Chemikalien geleistet, wodurch die Umsetzung der *Chemikalienstrategie für Nachhaltigkeit* unterstützt wird. Aus den Ergebnissen in AP 1 folgt, dass eine höhere Datenqualität, -anzahl und -verfügbarkeit für die Erweiterung der Trainingsdaten erforderlich ist, um die Anwendbarkeit für Substanzklassen, wie ILs, zu erreichen. Ebenso ist die leichte Interpretierbarkeit der Vorhersagen hinsichtlich SARs, SBRs und SPRs in der Modellentwicklung zu berücksichtigen, um auf Basis der Vorhersagen Hinweise auf mögliche Änderungen im Design von Molekülen zu ermöglichen.

3.2 Targeted Re-Design von ionischen Flüssigkeiten

3.2.1 Forschungslücke

Ein Ansatz zur Entwicklung bioabbaubarer ILs besteht darin, natürlich vorkommende Strukturfragmente, z. B. von Aminosäuren, in bestehende ILs einzubauen (Haiß et al., 2016; Kapitanov et al., 2019; Suk et al., 2020; Suk & Kümmerer, 2023). Eine Serie von monokationischen ILs enthielt Phe. Die ILs unterschieden sich in der Kationengruppe (Pyridinium, Cholinium, Imidazolium) und dem linearen Alkylrest (C_n). Es konnte gezeigt werden, dass mit zunehmender Kettenlänge bis C_8 die Bioabbaubarkeit in Tests nach OECD 301D gestiegen ist und ab C_{10} abgenommen hat (Haiß et al., 2016; Kapitanov et al., 2019; Suk et al., 2020). Gleichzeitig stieg die Ökotoxizität von ILs mit zunehmender Kettenlänge unabhängig von den kationischen Strukturen (Kapitanov et al., 2019; Kusumahastuti et al., 2021; Kusumahastuti et al., 2019; Suk et al., 2020). Eine Verringerung der Ökotoxizität wurde durch dikationische Imidazolium-ILs im Vergleich zu den entsprechenden monokationischen ILs erreicht (Steudte et al., 2014). Allerdings war keine der untersuchten dikationischen ILs bioabbaubar (Steudte et al., 2014). Um die Bioabbaubarkeit zu fördern, werden Ester- und Amidbindungen vorgeschlagen (Boethling et al., 2007). Allerdings gab es bislang keine Untersuchungen zur Bioabbaubarkeit von dikationischen Phe-basierten ILs (Phe-ILs), die beide Designstrategien kombinieren, den Einbau natürlich vorkommender Strukturfragmente und die dikationische Struktur, um bioabbaubare ILs bei gleichzeitiger geringerer Ökotoxizität zu entwickeln. Daher wurden in dieser Arbeit entsprechend des *targeted Re-Designs* gezielte

Veränderungen über den Einbau einer Phe-Gruppe sowie Ester- und Amidbindung in dikationische ILs vorgenommen, um zu untersuchen, ob dieses die Bioabbaubarkeit begünstigt.

3.2.2 Methoden

Eine Serie von dikationischen Phe-ILs wurde entwickelt und entsprechend der Methoden OECD 301D und 301F auf die leichte Bioabbaubarkeit getestet (OECD, 1992) (**Publikation 5,** Kapitel 2.1 und 2.2). Zusätzlich wurden drei monokationische ILs entsprechend OECD 301F getestet und die Ergebnisse mit denen aus OECD 301D-Tests aus der Studie von Suk et al. (2020) verglichen. Für alle Proben aus den Bioabbautests von Tag 0 und 28 wurde die Elimination der Ausgangssubstanz mittels Hochleistungsflüssigkeitschromatographie gekoppelt mit hochauflösender Massenspektrometrie (HPLC-HRMS) gemessen und TPs identifiziert (**Publikation 5,** Kapitel 2.3).

Für die monokationischen ILs wurden die Kopfgruppen Pyridinium (Py), 3-Methylimidazolium (Im), Cholinium (Chol) verwendet, die an das Phe über eine Amidbindung gebunden wurden (Abbildung 3). An das Phe wurde eine *n*-Hexylkette über eine Esterbindung gebunden. Für die vier dikationischen ILs wurden Gemini-Analoge von PyPheC_n (n = 4 und 6) verwendet (Abbildung 3). Die zwei PyPhe Fragmente wurden über eine *n*-Butyl- und eine *n*-Hexylkette verbunden. Die lineare Alkylkette bindet dabei an den Phe-Rest über eine Ester- oder Amidbindung (Gemini-Phe-Ester-ILs und Gemini-Phe-Amid-ILs). Das Kation 1-(Carboxymethyl)-3-methyl-1H-imidazol-3-ium (ImAc) wurde ebenfalls getestet. Alle ILs waren Bromid-Salze. Eine Substanz wird als leicht biologisch abbaubar eingestuft, wenn sie innerhalb von 10 Tagen ab einem Abbauwert von 10 % zu ≥ 60 % abgebaut wird (OECD, 1992).

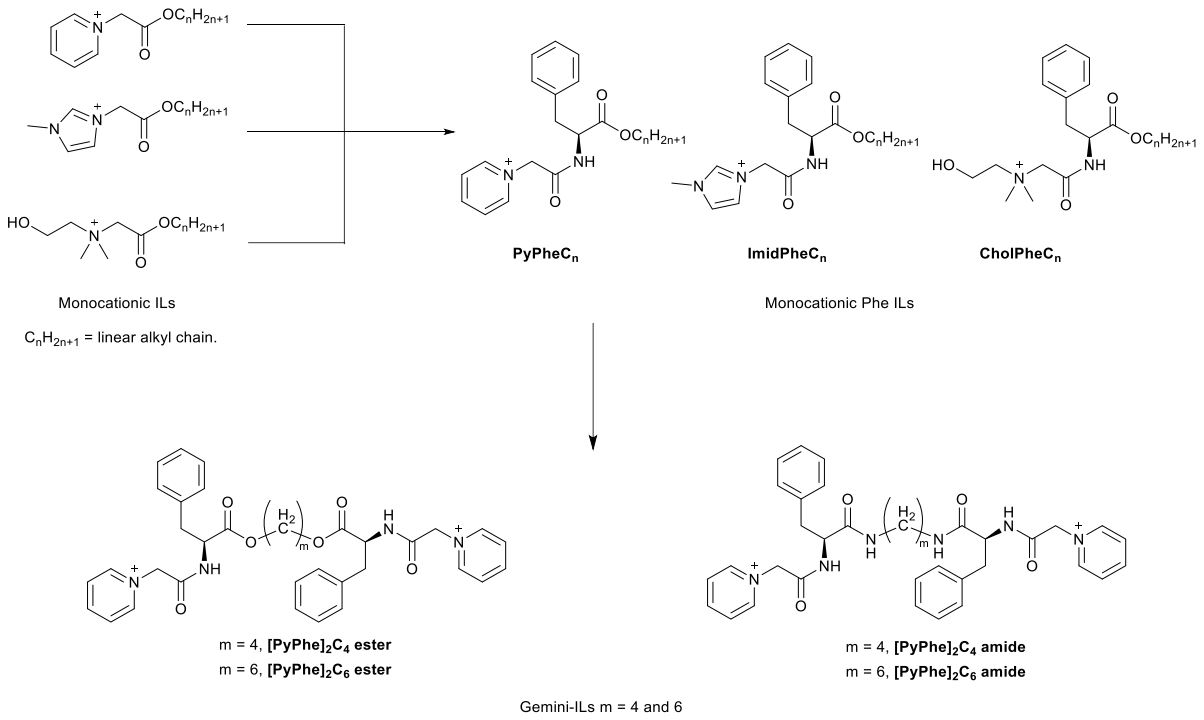

*Abbildung 3: Strukturen der monokationischen Phe-ILs und Gemini-Phe-ILs durch den Einbau einer Phe-Gruppe und Ester- und Amidbindungen in kommerziell verwendeten monokationischen ILs. Alle ILs sind Bromid-Salze. (Quelle: **Publikation 5**)*

3.2.3 Ergebnisse und Diskussion

Keine der acht untersuchten ILs war in den Tests nach OECD 301D leicht biologisch abbaubar. Hingegen waren in den Tests nach OECD 301F PyPheC$_6$, [PyPhe]$_2$C$_4$Ester und [PyPhe]$_2$C$_6$Ester leicht biologisch abbaubar (**Publikation 5**, Tabelle 1). In beiden Tests konnte in der Toxizitätskontrolle keine Inhibition des Inokulums durch die ILs festgestellt werden. Entsprechend waren alle ILs nicht toxisch für die Mikroorganismen.

Bei den monokationischen ILs erhöhte sich die Bioabbaubarkeit in den Tests nach OECD 301F in der Reihenfolge ImPheC$_6$ < CholPheC$_6$ < PyPheC$_6$. Damit änderte sich die Reihenfolge im Vergleich zu den Tests nach OECD 301D, welche CholPheC$_6$ < PyPheC$_6$ < ImPheC$_6$ war (Suk et al., 2020). Die Änderung der Reihenfolge ergibt sich aus zwei Gründen. Zum einen führte die höhere Konzentration und Vielfalt der Mikroorganismen im Belebtschlamm, welcher als Inokulum für die Tests nach OECD 301F verwendet wurde, zu einer vollständigen Mineralisierung von PyPheC$_6$ und CholPheC$_6$, wie die Ergebnisse aus der Analytik mittels HPLC-HRMS zeigten. Zum anderen ist ImAc, das TP von ImPheC$_6$, persistent in Tests nach OECD 301D und OECD 301F (**Publikation 5**, Tabelle 1), wodurch ImPheC$_6$ nicht vollständig mineralisiert werden konnte.

$CholPheC_6$ wurde in den vorherigen Tests nach OECD 301D in einem einphasigen Prozess zu CholPhe abgebaut (Suk et al., 2020). Die Ergebnisse aus den Tests nach OECD 301F zeigen einen in zwei Phasen aufgeteilten Abbauweg (**Publikation 5**, Abbildung 2b). In der ersten Abbauphase wurde $CholPheC_6$ zu 34 ± 4.0 % $ThOD_{NH3}$ nach 18 Tagen zu CholPhe infolge der Mineralisierung der *n*-Hexylkette abgebaut. Nach einem entscheidenden Anpassungsschritt der Mikroorganismen in den ersten 18 Tagen konnte eine weitere Abbauphase beobachtet werden, die zur Mineralisierung von CholPhe führte (**Publikation 5**, Abbildung 3). Dies wurde von den Ergebnissen aus der Analytik mittels HPLC-HRMS bestätigt. In den Proben nach 28 Tagen von $ImPheC_6$ konnte hingegen das persistente ImAc nachgewiesen werden. Die geringere Bioabbaubarkeit von $CholPheC_6$ in Tests nach OECD 301D im Vergleich zu $PyPheC_6$ und $ImPheC_6$ steht im Widerspruch zu den SBRs, die eine Verbesserung der Bioabbaubarkeit durch das Cholinium-Kation und Aminosäure-Reste zeigten (Costa et al., 2017; Jordan & Gathergood, 2015). Eine Kombination beider Fragmente ist also nachteilig.

Bei den Gemini-Phe-ILs zeigen die Ergebnisse der Bioabbautests nach OECD 301D und 301F, dass die Gemini-Phe-Amid-ILs geringer abbaubar waren als Gemini-Phe-Ester-ILs. Folglich wurde die Bioabbaubarkeit durch die Ester- und Amidbindung beeinflusst. Für die Gemini-Phe-Ester-ILs wurde keine Auswirkung der linearen Alkylkette (*n*-Butyl- oder *n*-Hexylkette) auf die Bioabbaubarkeit festgestellt, da beide ILs in den Tests nach OECD 301F leicht biologisch abbaubar eingestuft wurden und in den Tests nach OECD 301D abbaubar waren. Hingegen konnte in den Tests nach OECD 301F für die Gemini-Phe-Amid-ILs mit einer *n*-Hexylkette eine Zunahme der Abbaubarkeit im Vergleich zur *n*-Butylkette beobachtet werden. In den Tests nach OECD 301D konnte der Einfluss der linearen Alkylkette nicht festgestellt werden. Beide Gemini-Phe-Amid-ILs waren nicht bioabbaubar. Der Vergleich der Bioabbaubarkeitsergebnisse aus **Publikation 5** und Suk et al. (2020) von $PyPheC_n$ (n = 4 und 6) und den Gemini-Phe-Ester-ILs zeigte keinen Einfluss der Gemini-Struktur auf das Bioabbauverhalten, da alle ILs nahezu zu gleichen Anteilen abgebaut wurden.

Die Analyse der OECD 301D Proben nach 28 Tagen mittels HPLC-HRMS zeigte, dass die Gemini-Phe-Ester-ILs nicht mineralisiert wurden. Der Ester wurde gespalten und es entstanden zwei TPs (**Publikation 5**, Abbildung 6). In den OECD 301F-Proben konnte die Analyse über HPLC-HRMS die vollständige Mineralisierung bestätigen. Der Abbauweg der Gemini-Phe-Ester-ILs umfasst zwei Abbauschritte. Im ersten Schritt findet eine Esterhydrolyse statt. Im zweiten Schritt wird die Amid-Bindung hydrolysiert (**Publikation 5**, Abbildung 6). Die Ergebnisse der HPLC-HRMS-Analyse zeigen, dass in den Sterilkontrollen eine

vollständige primäre Elimination über die abiotische Esterhydrolyse zu zwei TPs stattgefunden hat (**Publikation 5**, Abbildung 6).

Die HPLC-HRMS-Analyse der OECD 301D Proben nach 28 Tagen bestätigte für die Gemini-Phe-Amid-ILs, dass keine primäre Elimination stattgefunden hat. In den OECD 301F Proben nach 28 Tagen wurden keine TPs identifiziert. In den Sterilkontrollen wurde keine primäre Elimination der Gemini-Phe-Amid-ILs beobachtet. Dementsprechend sind diese ILs gegenüber der abiotischen Hydrolyse stabil im Gegensatz zu den Gemini-Phe-Ester-ILs. Aufgrund des schlechteren biotischen und abiotischen Abbaus der Gemini-Phe-Amid-ILs im Vergleich zu den Gemini-Phe-Ester-ILs lässt sich sagen, dass Amidbindungen nicht immer gut abbaubar sind, wie auch schon von Boethling et al. (2007) beschrieben wurde.

3.2.4 Schlussfolgerung

Die Untersuchung der Bioabbaubarkeit von sieben Phe-ILs und ImAc nach OECD 301D und OECD 301F zeigte, dass die Anwendung beider Testmethoden hilfreich ist, um den Abbauweg einer IL zu verstehen, wie im Beispiel von CholPheC$_6$ gezeigt wurde. Mit der Entwicklung der leicht biologisch abbaubaren ILs, PyPheC$_6$, [PyPhe]$_2$C$_4$Ester und [PyPhe]$_2$C$_6$Ester in AP 2, wurde das *targeted Re-Design* erfolgreich umgesetzt. Mit dem Beispiel der Phe-ILs wird bewiesen, dass BbD auf ILs angewendet werden kann. SBRs wurden von den Ergebnissen abgeleitet, die für das Design von mineralisierenden ILs hilfreich sind. Insbesondere die Verwendung von Gemini-Phe-Ester-ILs hat sich als geeignet erwiesen, mögliche toxische Wirkungen von langen linearen Alkylketten in ILs zu reduzieren und gleichzeitig die leichte biologische Abbaubarkeit zu erhalten. Da QSBR-Modelle für die ILs nicht zugänglich waren, konnte der Arbeitsablauf aus **Publikation 1** nicht für die Bewertung der Bioabbaubarkeit der verschiedenen ILs angewendet werden. Statt über Modelle wurde die Bioabbaubarkeit experimentell bestimmt.

3.3 Verfügbarkeit und Aufbereitung von Literaturdaten zur Bioabbaubarkeit von ionischen Flüssigkeiten

3.3.1 Forschungslücke

Um die Anforderung von BbD zu erfüllen, dass Kation und Anion einer IL in Kläranlagen und in der Umwelt bioabbaubar sein müssen, ist es zunächst notwendig, einen aktuellen Überblick über den Stand des Wissens zur Bioabbaubarkeit der ILs zu erhalten. In einigen Studien wurde ein Überblick zu angewendeten Methoden und experimentellen Bioabbaudaten gegeben, um biologisch gut abbaubare ILs und SBRs zu identifizieren (Coleman & Gathergood, 2010; Costa et al., 2017; Jordan & Gathergood, 2015; Stolte et al., 2015; Stolte et al., 2011). Die Studien, die experimentelle Bioabbaudaten berichten, wurden bisher jedoch nicht systematisch analysiert. Dementsprechend war das Ziel dieser Arbeit, durch eine systematische Literaturrecherche einen Überblick über die angewendeten Methoden, die Einhaltung der entsprechenden Richtlinien, die Erfüllung der Validitätskriterien und die Bioabbaubarkeit der getesteten ILs zu erhalten.

3.3.2 Methoden

Eine systematische Literaturrecherche wurde durchgeführt, um Studien mit experimentellen Daten zur Bioabbaubarkeit von ILs zu sammeln und hinsichtlich der angewandten Methoden und der Bioabbaubarkeit von ILs auszuwerten (**Publikation 2**, Kapitel 2.1). Für die Untersuchung der Studien wurden die Strukturformeln der getesteten ILs, der Bioabbauwert und die verwendete Methode erfasst (**Publikation 2**, Kapitel 2.2). Die angewendeten Methoden (z. B. nach OECD und *International Organization for Standardization*, (ISO)) wurden auf Übereinstimmung mit der Standardmethode und Validität geprüft (**Publikation 2**, Kapitel 2.2). Die Validitätskriterien sind in Tabelle A.1 im Anhang der **Publikation 2** zusammengefasst.

Die ILs wurden auf Grundlage der Kernstruktur des Kations in Gruppen eingeteilt, z.B. in Imidazolium, Pyridinium, QAC. Die Gruppierung wurde für ILs, die aus einem bestimmten Kation und Anion bestehen, und Mischungen aus ILs, die Kationen aus einer Gruppe und verschiedene Anionen enthalten, getrennt vorgenommen. Die Bioabbaudaten zur leichten biologischen Abbaubarkeit wurden näher untersucht (**Publikation 2**, Kapitel 2.3). Dafür wurden die Studien ausgewählt, die die leichte biologische Abbaubarkeit testeten, z.B. nach OECD 301, ISO 7827, ISO 9408 und ISO 14593. Die Studien waren geeignet, wenn a) die

Tests über 28 Tage durchgeführt wurden, b) die von der Richtlinie erlaubte Quelle für das Inokulum verwendet wurde und c) die Mineralisation gemessen wurde. Die Daten zur leichten Bioabbaubarkeit wurden anschließend klassifiziert (rot: 0–19 %, orangefarben: 20–59 %, grün: ≥ 60 % Bioabbaubarkeit).

3.3.3 Ergebnisse und Diskussion

Über die systematische Literaturrecherche wurden 109 Studien erhalten, die experimentelle Daten zu 716 ILs, davon 29 Mischungen aus ILs, ermittelt über eine Standardmethode, berichten. Die Auswertung der Methoden ergab, dass die meisten Studien die Bioabbaubarkeit nach OECD 301D und 301F getestet haben (**Publikation 2**, Abbildung 2). Die Auswertung der Methodenbeschreibungen und die Beurteilung der Übereinstimmung mit den Richtlinien zur Methode ergab, dass nicht alle Studien alle Testparameter berichteten, um einen vollständigen Vergleich mit den Vorgaben in den Richtlinien vorzunehmen. Außerdem wurden in keiner Studie alle Validitätskriterien, wie sie in den Richtlinien definiert sind (**Publikation 2,** Tabelle A.1), dargelegt. Folglich ist die Zuverlässigkeit und Qualität der vorhandenen Daten zur Bioabbaubarkeit eingeschränkt. Um einen Vergleich der Bioabbaudaten zu ermöglichen und die Bioabbaubarkeit besser zu verstehen, ist es erforderlich, dass die Daten gemäß den Kriterien (**Publikation 2**, Tabelle A.1) valide sind. Darüber hinaus bieten valide Daten die Möglichkeit in der Bewertung von SBRs, der Entwicklung von QSBR-Modellen und der Registrierung unter REACH eingesetzt zu werden.

In den 716 ILs aus 109 Studien wurden insgesamt 16 verschiedene Kationengruppen identifiziert (**Publikation 2**, Tabelle 1). Die ILs unterschieden sich in der Kationengruppe, den Seitenketten am Kation und dem Anion. Die Kationengruppen, die am meisten getestet wurden, sind Imidazolium (245 ILs), QACs (134 ILs) und Pyridinium (109 ILs). Weniger als 9 ILs wurden für die Kationengruppen Piperidinium, Prolinium, Quinolinium, Piperazinium, Thiazolium, Triazolium, Guandinium und Sulfonium getestet (**Publikation 2**, Abbildung 3A). Der Vergleich von 716 ILs, für die Bioabbaudaten vorliegen, mit 2482 ILs, für die physiko-chemische Eigenschaften untersucht wurden (Dong et al., 2007), zeigt eine große Datenlücke zur Bioabbaubarkeit.

Die vorab definierten Kriterien für die Eignung zur weiteren Auswertung der leichten biologischen Abbaubarkeit wurden von 67 der 109 Studien erfüllt. Diese untersuchten die leichte biologische Abbaubarkeit nach OECD 301, ISO 7827, ISO 9408 und ISO 14593. Die Auswertung ergab, dass zu 508 ILs Daten vorliegen. Davon wurden 120 ILs zu ≥ 60 %

abgebaut. Allerdings wurden nur 34 ILs als leicht biologisch abbaubar eingestuft. Ihre Bioabbaubarkeit war ≥ 60 % innerhalb von 10 Tagen ab einem Abbau von 10 %, wie von der OECD (1992) definiert. Die meisten ILs wurden demnach zu weniger als 60 % biologisch abgebaut (**Publikation 2**, Abbildung 4A). Keine IL der Kationengruppen Phosphonium, Prolinium, Piperazinium, 1,4-Diazabicyclo[2.2.2]octanium (DABCO, in **Publikation 2** als Octanium abgekürzt) und Thiazolium war zu ≥ 60 % bioabbaubar. Bei den Cholinium-ILs wurden mehr als die Hälfte der ILs zu ≥ 60 % abgebaut (**Publikation 2**, Abbildung 4A). Die meisten ILs wurden entsprechend OED 301D getestet. Von den 29 Mischungen aus ILs gehörten die meisten zu den QACs (24 ILs). Zehn der Mischungen aus ILs waren zu ≥ 60 % biologisch abbaubar, von denen acht leicht biologisch abbaubar eingestuft wurden (**Publikation 2**, Abbildung 4B). Die meisten Mischungen aus ILs wurden entsprechend OED 301F getestet.

3.3.4 Schlussfolgerung

Anhand der systematischen Literaturrecherche in AP 3 konnte ein umfassender Überblick über angewandte Standardmethoden und die verschiedenen Kationengruppen erhalten werden. Die Auswertung der Methodenbeschreibungen und die Bewertung der Übereinstimmung mit den Standardmethoden ergab, dass die Methode vollständig und alle wichtigen Testparameter beschrieben werden sollten, um die Daten verschiedener Studien besser vergleichen und unterschiedliche Bioabbaubarkeit besser verstehen zu können. Die Ergebnisse zeigen, dass mehr und qualitativ bessere Tests nach OECD 301 erforderlich sind. Dabei sollten insbesondere alle Validitätskriterien, die in den Richtlinien hinterlegt sind, angewendet und mit den experimentellen Daten veröffentlicht werden. Für zukünftige Tests ist die Methode OECD 301D vorzuziehen, da diese die strengste der OECD 301-Methoden hinsichtlich der Testbedingungen ist und am häufigsten für die ILs angewendet wurde. So könnte eine Datenbasis geschaffen werden, die Daten aus einer Standardmethode von vielen verschiedenen ILs enthält. Folglich könnte eine zuverlässige Datenbasis geschaffen werden, die einen Vergleich zwischen den nach einer Standardmethode erzeugten Daten ermöglicht. Darüber hinaus sind zuverlässige Daten für zukünftige Anwendungen wie die Bewertung von SBRs, die Entwicklung von (Q)SBR-Modellen und der Registrierung unter REACH, erforderlich. Weitere Tests sind erforderlich, insbesondere für die Kationengruppen Morpholinium, DABCO, Piperidinium, Prolinium, Piperazinium, Quinolinium, Triazolium, Guandinium, Sulfonium und Thiazolium, da nur wenige Daten zu diesen vorlagen.

3.4 Analyse von Bioabbaudaten zur Identifizierung von Struktur-Bioabbaubarkeitsbeziehungen für das Design von ionischen Flüssigkeiten

3.4.1 Forschungslücke

Der Einsatz von Expertenwissen zu SARs, SBRs, und SPRs für das strukturelle Design von Substanzen wurde bereits in **Publikation 1** diskutiert. Dieses Wissen ist für die strukturelle Gestaltung der Moleküle im *targeted (Re-)Design* und für die Umsetzung des Arbeitsablaufs erforderlich (**Publikation 1**). Um die Entwicklung von bioabbaubaren ILs zu unterstützen, sind die SBRs zu verstehen. In der Literatur sind Faustregeln für das Design von bioabbaubaren Substanzen vorhanden (Boethling et al., 2007). Speziell für die ILs wurden in mehreren Studien SBRs auf Grundlage der verfügbaren Literaturdaten identifiziert und Faustregeln für die Bioabbaubarkeit von ILs aufgestellt (Beil et al., 2021; Coleman & Gathergood, 2010; Costa et al., 2017; Jordan & Gathergood, 2015; Stolte et al., 2015). Eine systematische computeruntergestützte Identifizierung von SBRs in einem Datensatz fand bislang nicht statt. In **Publikation 2** wurden die Daten zu leichten Bioabbaubarkeit, z. B. nach den OECD 301-Methoden, von 508 ILs aus 12 Kationengruppen zusammengestellt. Allerdings wurde dieser Datensatz noch nicht hinsichtlich SBRs ausgewertet. Um die Unterschiede in den Strukturen zu überblicken, sind die ILs entsprechend ihrer Ähnlichkeit zu gruppieren. Jedoch ist die manuelle Gruppierung von ILs schwierig, da sie sich in vielen Strukturfragmenten unterscheiden. Daher wurde *in silico* Clustering auf die ILs angewendet, um sie nach ihrer strukturellen Ähnlichkeit zu gruppieren. Dies bildete die Grundlage für den Vergleich von Strukturen und Bioabbaubarkeit. Ziel dieser Arbeit war zu verstehen, welche SBRs den ILs zugrunde liegen und wie diese mit den bekannten Faustregeln aus der Literatur übereinstimmen, um dieses Wissen in der Gestaltung bioabbaubarer ILs anwenden zu können.

3.4.2 Methoden

Von den gesammelten Studien aus **Publikation 2** waren 68 Studien und Bioabbaudaten zu 508 ILs für die Erstellung des Datensatzes geeignet, da sie a) experimentelle Bioabbaudaten vorlegten, b) die leichte biologische Abbaubarkeit untersuchten, c) über 28 Tage die Bioabbaubarkeit gemessen haben, d) die erlaubte Quelle für das Inokulum verwendeten und e) die Mineralisierung gemessen haben. Daten zu 508 ILs lagen vor, die nach der Kernstruktur des Kations sortiert waren (181 Imidazolium-ILs, 60 QACs, 96 Pyridinium-ILs, 80 Cholinium-ILs, 36 Phosphonium-ILs, 21 Pyrrolidinium-ILs, 12 Morpholinium-ILs, 8 DABCO-ILs,

7 Piperidinium-ILs, 3 Prolinium-ILs, 2 Piperazinium-ILs und 2 Thiazolium-ILs) (**Publikation 3**, Tabelle 2). Die ILs der Kationen Imidazolium, QAC, Pyridinium, Cholinium, Phosphonium und Pyrrolidinium, zu denen experimentellen Daten zur leichten biologischen Abbaubarkeit aus **Publikation 2** vorlagen, wurden entsprechend der strukturellen Ähnlichkeit nach dem Tanimoto-Koeffizienten unter Verwendung des Softwarepakets *Canvas* von Schrödinger (Version 4.3.013) mittels einer hierarchischen Clusteranalyse in Cluster geteilt. Das genaue Vorgehen ist in **Publikation 3** beschrieben. Anschließend wurden die Bioabbauwerte den ILs zugeordnet und klassifiziert (rot: 0–19 %, orangefarben: 20–59 %, grün: ≥ 60 % Bioabbaubarkeit), um zu visualisieren, wie viele ILs welcher Klasse in einem Cluster zugeordnet wurden. SBRs wurden identifiziert, indem einzelne Cluster betrachtet und mehrere Cluster einer Kationengruppe verglichen wurden. Da für Morpholinium, DABCO-, Piperidinium-, Prolinium-, Piperazinium- und Thiazolium-ILs jeweils weniger als 12 ILs vorlagen, wurden die ILs visuell auf Ähnlichkeiten in der Struktur untersucht, z. B. auf funktionelle Gruppen und Alkylketten, wie in **Publikation 3** erläutert wird. Die Ergebnisse wurden mit den Faustregeln aus der Literatur verglichen (**Publikation 3**, Tabelle 1).

3.4.3 Ergebnisse und Diskussion

Die Anzahl an gebildeten Clustern für die Kationengruppen Imidazolium, Pyridinium, QAC, Cholinium, Phosphonium und Pyrrolidinium und die Klassifizierung der Bioabbaubarkeit der ILs pro Cluster werden in Abbildung 4 dargestellt. Die rote und orangefarbene Klassifizierung der Bioabbaubarkeit ist insbesondere bei Imidazolium und Phosphonium vorherrschend und zeigt, dass die meisten ILs nicht oder nur begrenzt bioabbaubar sind. Hingegen ist die orangefarbene und grüne Klassifizierung der Bioabbaubarkeit bei den Cholinium-ILs vorherrschend. Somit sind die Cholinium-ILs am besten bioabbaubar.

In **Publikation 3** werden die SBRs für das Kation, dessen Seitenketten und das Anion in den Abschnitten zu der jeweiligen Kationengruppen beschrieben und tabellarisch zusammengefasst (**Publikation 3**, Tabelle 4). Für alle 12 Kationengruppen wurde der Einfluss von Kation (monokationisch und dikationisch), der Seitenketten (z.B. Aminosäurereste, Alkylketten, Ester, Ether, Hydroxygruppe) und Anion (z.B. Aminosäuren, Alkylsulfonate, Alkylsulfate, Carboxylsäuren) ermittelt. Für Imidazolium- und Pyridinium-ILs konnten mehr SBRs identifiziert werden im Vergleich zu den anderen zehn Kationengruppen. Aufgrund der begrenzten Datenverfügbarkeit und fehlender Referenzsubstanzen, die Rückschlüsse auf den Abbau nur des Kations oder des Anions zulassen, konnten einige SBRs z.B. für Alkylketten,

Ester- und Ethergruppen nicht abgeleitet werden, wie in Tabelle 4 in der **Publikation 3** dargestellt ist. Insbesondere für Morpholinium-, DABCO-, Piperidinium-, Prolinium-, Piperazinium- und Thiazolium-ILs konnten die SBRs aufgrund begrenzter Daten nicht eindeutig identifiziert werden. Auf Basis von Tabelle 4 in **Publikation 3** wurden Empfehlungen für das Design bioabbaubarer ILs und zu testende Kombinationen von Kationen und Anionen abgeleitet, um Lücken in den SBRs zu schließen. Die Kationen auf Basis von Cholinium und seinen Derivaten Acetylcholin, Betain und Carnitin in Kombination mit einem gut biologisch abbaubaren Anion zeigten sich geeignet, um bioabbaubare ILs zu entwickeln. Imidazolium- und Phosphonium-ILs sollten vermieden werden.

Die Clusteranalyse erwies sich als geeignet, die Identifizierung von SBRs zu erleichtern (**Publikation 3**). Allerdings bestand eine Herausforderung darin, dass sich die ILs oft in vielen Strukturfragmenten unterschieden und Referenzsubstanzen fehlten, wodurch es schwierig war das Strukturfragment zu identifizieren, das die bessere Bioabbaubarkeit beim Vergleich zweier ILs begünstigte. Die Auswahl der Studien waren auf ILs begrenzt. Studien, die QACs, kationische Tenside, wie Benzalkoniumchlorid, oder anionische Tenside nicht als IL beschrieben, wurden aufgrund der Suchbegriffe in der systematische Literaturrecherche nicht berücksichtigt. Diese Substanzen können weitere Einblicke in SBRs geben. Dafür ist ein globaler Datensatz von ionischen Substanzen in einer Datenbank erforderlich.

Da es keine Datenbank mit hochwertigen Daten zur Bioabbaubarkeit von ILs gab, wurden die Daten aus der Literaturrecherche aus **Publikation 2** verwendet. Damit einhergehend ergeben sich für diese Studie Einschränkungen (**Publikation 3**). Da die Validitätskriterien nicht oder unvollständig in den Studien berichtet wurden, konnte nicht festgestellt werden, ob alle Bioabbaudaten valide sind (**Publikation 2**, Kapitel 3.2). Um trotzdem eine Auswertung der vorhandenen Bioabbaudaten vorzunehmen, wurde dies als Limitierung in **Publikation 2** (Kapitel 3.6) diskutiert. Auch ist zu berücksichtigen, dass die Vergleichbarkeit der aus der Literatur gewonnenen Daten ebenfalls begrenzt ist. Für die Bestimmung der leichten biologischen Abbaubarkeit nach OECD 301 und ISO kann Inokulum aus unterschiedlichen Quellen verwendet werden, was zur unterschiedlichen Zusammensetzung der Mikroorganismen führt (z. B. unterschiedliche Arten und Dichte) (Rücker & Kümmerer, 2012). Außerdem kann die Bioabbaubarkeit einer bestimmten IL innerhalb eines Jahres und an verschiedenen Standorten variieren, da sich die Zusammensetzung der Mikroorganismen aufgrund wechselnder Umweltbedingungen (z. B. Temperatur, pH-Wert, konkurrierende Arten, Nährstoffe) ändert (Rücker & Kümmerer, 2012). Für die Entwicklung biologisch leicht

abbaubarer ILs ist es notwendig, diese Faktoren zu berücksichtigen. Im besten Fall ist eine IL unter verschiedenen Umweltbedingungen abbaubar.

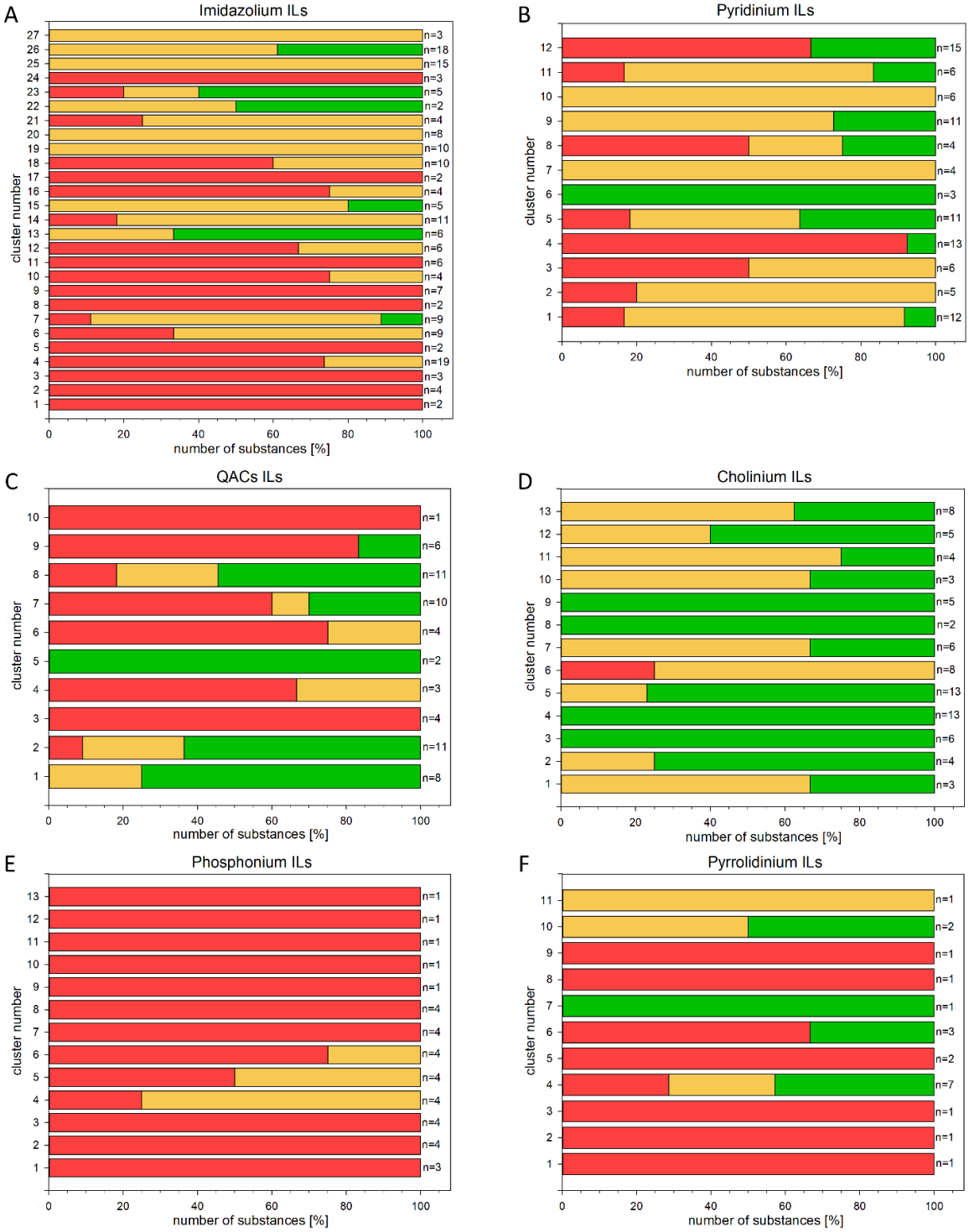

Abbildung 4: Bioabbaubarkeit von ILs in den Kationengruppen (A) Imidazolium, (B) Pyridinium, (C) QACs, (D) Cholinium, (E) Phosphonium und (F) Pyrrolidinium pro Cluster. Rot bedeutet 0–19 %, orangefarben bedeutet 20–59 % und grün bedeutet ≥60 % Bioabbaubarkeit. n = Anzahl der Substanzen pro Cluster. (Quelle: ***Publikation 3****)*

3.4.4 Schlussfolgerung

Mit AP 3 und der zugehörigen **Publikation 3** wurde erstmals eine *in silico* Clusteranalyse auf einen Datensatz von ILs angewendet, um SBRs zu identifizieren. Das methodische Vorgehen der Clusteranalyse war geeignet, um die ILs entsprechend ihrer strukturellen Ähnlichkeit zu gruppieren. Die Clusteranalyse erleichterte die Auswertung von Bioabbaudaten zu 508 ILs, indem sie die ILs hinsichtlich ihrer strukturellen Ähnlichkeit Clustern zuordnete. Die Unterschiede in den Strukturen der ILs wurden erkennbar. Somit konnten beim Vergleich der Bioabbaubarkeit der ILs innerhalb eines Clusters als auch zwischen Clustern leichter Rückschlüsse auf SBRs gezogen werden. Das Vorgehen der Clusteranalyse könnte auch für Datensätze anderer Substanzklassen verwendet werden, um SBRs zu identifizieren. Die Zusammenfassung der SBRs für das Kation, dessen Seitenketten und das Anion kann als Grundlage für das Design von mineralisierenden ILs dienen. Außerdem sind die Wissenslücken in den SBRs (**Publikation 3**, Tabelle 4) zu schließen, um die Anwendbarkeit von SBRs bei der Entwicklung von mineralisierenden ILs zu verbessern.

3.5 Entwicklung von QSBR-Modellen für ionische Flüssigkeiten

3.5.1 Forschungslücke

Das Zusammenspiel von experimentellen und computergestützten Methoden für die Umsetzung von BbD wurde in **Publikation 1** aufgezeigt. (Q)SBR-Modelle können im Design von Substanzen unterstützen, indem sie zeit- und ressourcensparend die Bioabbaubarkeit bewerten können. Für die Anwendbarkeit des Arbeitsablauf aus **Publikation 1** zur Bewertung und Priorisierung von Substanzen im Hinblick auf die Bioabbaubarkeit von ILs sind QSBR-Modelle notwendig. Bislang sind die meisten vorhandenen Modelle nur für nicht-ionische Substanzen entwickelt worden (Boethling et al., 2003; Dimitrov et al., 2011; Jaworska et al., 2002; Klopman & Tu, 1997; Lombardo et al., 2014). Diese nutzen hauptsächlich den MITI-Datensatz (OECD 301C). Insbesondere die Modellierung der Beziehungen zwischen Strukturfragmenten und Bioabbaubarkeit, auch *alerts* genannt, hat sich aufgrund ihrer leichten Interpretierbarkeit nützlich in dem Design von Substanzen gezeigt (Rastogi et al., 2014, 2015b). Mit Hilfe der *alerts* können gezielt strukturelle Anpassungen vorgenommen werden, um eine vollständig mineralisierende Chemikalie zu entwickeln. Die vorhandenen Modelle können nicht für die ILs verwendet werden, da diese Substanzklasse nicht in den Trainingsdaten der Modelle hinterlegt ist und sich grundlegend von nicht-ionischen Substanzen unterscheidet. ILs bestehen aus einem Kation und Anion. Sollten das Kation und das Anion

organisch sein, können sie in unterschiedlichem Maß zur Bioabbaubarkeit einer IL beitragen. Ein Ion kann sehr gut abbaubar sein, während das andere gar nicht oder nur zum Teil abgebaut wird. Anorganische Ionen enthalten keinen Kohlenstoff, der von den Mikroorganismen verstoffwechselt werden könnte. Somit tragen diese nicht zur Abbaurate einer IL bei. Aufgrund dessen können die ILs nicht wie nicht-ionische Substanzen behandelt werden und vorhandene Modellierungsverfahren müssen an ILs angepasst werden. Die einzigen bekannten Modelle zur Bioabbaubarkeit von ILs sind in der *AquaBoxIL* hinterlegt. In der *AquaBoxIL* werden die Vorhersagen aus den QSBR-Modellen zusammen mit Vorhersagen zu physiko-chemischen Eigenschaften als Eingangswerte für ein Modell zur Verteilung einer IL in der Umwelt zwischen Wasser, Sediment und organischem Material verwendet (Barycki et al., 2018). Die QSBR-Modelle basieren auf OECD 310-Daten von 77 ILs (CO_2-Headspace-Test). Allerdings sind dies keine fragmentbasierten Modelle, die durch *alerts* anzeigen, welche strukturellen Änderungen vorgenommen werden müssen, um bioabbaubare ILs zu entwickeln. Daher war das Ziel dieser Arbeit fragmentbasierte QSBR-Modelle auf Basis von OECD 301D-Daten zu entwickeln.

3.5.2 Methoden

Es wurden fünf fragmentbasierte QSBR-Modelle für ILs in Zusammenarbeit mit MultiCASE, Inc. in der *FlexFilters*-Plattform entwickelt. Die OECD 301D-Daten stammen aus der Literatur (**Publikation 2**) und in-house OECD 301D-Tests am *Institut für Nachhaltige Chemie* (engl. Institute of Sustainable Chemistry, INSC) an der *Leuphana Universität Lüneburg* (Arbeitsgruppe von Prof. Kümmerer) (**Publikation 4**, Kapitel 2.1). Das Vorgehen der Modellierung und die Erstellung des Datensatzes werden in den Kapiteln 2.1–2.4 von **Publikation 4** beschrieben. Die wichtigsten Punkte sollen hier kurz zusammengefasst werden. Hervorzuheben ist, dass zwei Datensätze erstellt wurden (**Publikation 4**, Kapitel 2.2). Diese unterscheiden sich darin, dass ein Datensatz ausschließlich OECD 301D-Daten von ILs enthielt (set_IL), während der andere Datensatz zusätzlich auch Daten von organischen Anionen in Kombination mit anorganischen Kationen und nicht-ionischen Substanzen enthielt (set_ILNI). Außerdem enthielt set_IL Stereoisomere für die ILs, die am INSC getestet wurden. Diese wurden in set_ILNI zu einer IL zusammengefasst. Set_IL und set_ILNI wurden in einen Trainingsdatensatz mit 233 bzw. 321 Substanzen und einen Testdatensatz mit 26 bzw. 36 Substanzen unterteilt. Für beide Trainingsdatensätze wurde eine Clusteranalyse durchgeführt, um die strukturellen Unterschiede der Substanzen und die AD darzustellen (**Publikation 4**, Kapitel 2.4).

Das Regressionsverfahren der kleinsten Quadrate (engl. ordinary least squares, OLS) wurde verwendet, um Modelle zu entwickeln, die einen kontinuierlichen Bioabbauwert vorhersagen. Logistische Regression (engl. logistic regression, LR) wurde für die Entwicklung von Modellen verwendet, die eine Klassifizierung in bioabbaubar/nicht bioabbaubar vornehmen. Als Deskriptoren wurden einerseits Strukturfragmente auf Basis von *Extended-Connectivity Fingerprints* (ECFP) und andererseits Elemente des von Chakravarti (2018) entwickelten Fingerabdrucks verwendet (siehe **Publikation 4**, Kapitel 1 im Anhang). Die Kombination von OLS oder LR mit Deskriptoren auf Basis von Strukturfragmenten wurde gewählt, da sie ein gut untersuchtes und leicht zu interpretierendes Modellierungsverfahren ist (**Publikation 4**, Kapitel 2.3). Die für die Bioabbaubarkeit relevanten Fragmente, die als Deskriptoren dienten, wurden anhand der L1-Regularisierung ausgewählt, um eine Überanpassung (engl. overfitting) zu vermeiden und Regressionskoeffizienten für die Fragmente zu erhalten, die angeben, ob die Fragmente die biologische Abbaubarkeit fördern bzw. verringern (Friedman et al., 2010; Tibshirani et al., 2012) (**Publikation 4**, Kapitel 1 im Anhang). Die Strukturfragmente auf Basis der ECFP werden in den Modellen als *alert* angegeben. Die fünf Modelle unterscheiden sich im Trainingsdatensatz, den Deskriptoren und dem Regressionsverfahren (Tabelle 1). Es wurde eine interne und eine externe Validierung durchgeführt, wie von der OECD vorgegeben, und den OECD-Prinzipien für (Q)SAR-Modelle gefolgt (OECD, 2014) (**Publikation 4**, Kapitel 2.4). Eine Testbatterie wurde für die gezielte Verwendung der QSBR-Modelle in dem Arbeitsablauf aus **Publikation 1** entwickelt (**Publikation 4**, Kapitel 2.5).

*Tabelle 1: Neu entwickelte QSBR-Modelle auf Basis von OECD 301D-Daten für ILs. Logistische Regression (LR), Regressionsverfahren der kleinsten Quadrate (OLS). (Quelle: übersetzt aus **Publikation 4**)*

	Modell 1 IL_FP_cont	**Modell 2 IL_Al_cont**	**Modell 3 IL_Al_class**	**Modell 4 ILNI_Al_cont**	**Modell 5 ILNI_Al_class**
Trainingsdaten Anzahl an Substanzen	233 ILs			321 ILs und nicht-ionische Substanzen	
Testdaten Anzahl an Substanzen	26 ILs			36 ILs und nicht-ionische Substanzen	
Anzahl an Deskriptoren/ alerts	61	70	29	130	60
Modellierung	Fingerabdruck, OLS	alerts, OLS	alerts, LR,	alerts, OLS	alerts, LR,
Vorhersage	kontinuierliche Abbaurate in % des ThOD	kontinuierliche Abbaurate in % des ThOD	Klassifizierung in bioabbaubar oder nicht	kontinuierliche Abbaurate in % des ThOD	Klassifizierung in bioabbaubar oder nicht

3.5.3 Ergebnisse und Diskussion

Es wurden fünf QSBR-Modelle entwickelt, die Strukturfragmente der Substanzen in den Trainingsdaten als Deskriptoren nutzen, um die Bioabbaubarkeit vorherzusagen (**Publikation 4**). Im Gegensatz zu vorhandenen Modellen in der *AquaBoxIL* (Barycki et al., 2018), wurden für diese Modelle Strukturfragmente und deren Beziehung zur Bioabbaubarkeit (ausgedrückt als Regressionskoeffizient) genutzt. Positive Regressionskoeffizienten zeigen eine Begünstigung der Bioabbaubarkeit, negative Koeffizienten eine Verminderung an. Dies ermöglicht eine leichte Interpretierbarkeit der *alerts* und unterstützt die Entscheidungsfindung bei der Entwicklung leicht biologisch abbaubarer ILs.

Die Zusammenstellung der OECD 301D-Daten aus Literaturdaten und internen Daten des INSC für set_IL und set_ILNI ist in **Publikation 4** im Abschnitt 3.1 beschrieben. Hervorzuheben ist, dass mehr als die Hälfte der Daten in set_IL und set_ILNI im INSC gemessen wurde. Dementsprechend ist die Qualität der Daten hoch, da sie im selben Labor unter Verwendung desselben OECD 301D-Testprotokolls, Einhaltung der Validitätskriterien und derselben Quelle des Inokulums erhoben wurden. Im Gegensatz dazu sind die Literaturdaten von weniger guter Qualität, da unterschiedliche Quellen, Konzentrationen des Inokulums und Diversität der Mikroorganismen verwendet wurden. Außerdem wurden nicht immer alle Validitätskriterien angegeben, wie auch schon in **Publikation 2** bei der Auswertung der Literaturdaten diskutiert wurde.

Die Klassifizierung der Substanzen in den Trainingsdatensätzen nach der Kopfgruppe der Kationen zeigt, dass die Modelle Imidazolium-, Pyridinium-, Cholinium-ILs und QACs abdecken (**Publikation 4**, Abbildungen 1 und 2). Die interne und externe Validierung für die Modelle IL_FP_cont, IL_Al_cont, ILNI_Al_cont ergab ein R^2 von 0,620 bis 0,854. Für die Modelle IL_Al_class, ILNI_Al_class lag die Genauigkeit, Sensitivität (Richtig-positiv-Rate, engl. true positive rate, TPR) und Spezifität (Richtig-negativ-Rate, engl. true negative rate, TNR) bei 62–100 % (Tabelle 2). Da die *area under the curve* (AUC) für IL_FP_cont, IL_Al_cont, ILNI_Al_cont über 0,5 liegt, sind die Modelle in der Lage, zwischen biologisch abbaubaren und nicht biologisch abbaubaren ILs zu unterscheiden. Die Modelle sind entsprechend geeignet die Bioabbaubarkeit gängiger ILs wie Imidazolium-, Pyridinium-, Cholinium-ILs und QACs vorherzusagen.

*Tabelle 2: Ergebnisse für die interne und externe Validierung. Fläche unter der Kurve (engl. area under the curve, AUC), Spezifität (Richtig-negativ-Rate, engl. true negative rate, TNR), Sensitivität (Richtig-positiv-Rate, engl. true positive rate, TPR). (Quelle: übersetzt aus **Publikation 4**)*

		Modell 1 IL_FP_cont	**Modell 2 IL_Al_cont**	**Modell 3 IL_Al_class**	**Modell 4 ILNI_Al_cont**	**Modell 5 ILNI_Al_class**
interne Validierung	Genauigkeit	-	-	98 %	-	92 %
	TPR	-	-	91 %	-	80 %
	TNR	-	-	100 %	-	96 %
	AUC	-	-	0,99	-	0,97
	R^2	0,814	0,843	-	0,788	-
externe Validierung	Genauigkeit	-	-	96 %	-	81 %
	TPR	-	-	75 %	-	62 %
	TNR	-	-	100 %	-	91 %
	AUC	-	-	0,82	-	0,90
	R^2	0,854	0,687	-	0,620	-

Da sich die Modelle sowohl im Trainingsdatensatz als auch Deskriptoren und Algorithmen von vorhandenen Modellen unterscheiden, kann kein Vergleich vorgenommen werden. Entsprechend kann keine Aussage darüber gemacht werden, dass die hier vorgestellten Modelle besser oder schlechter Vorhersagen treffen. Grundsätzlich liegen die Ergebnisse der Validierung in Bereichen, die auch von vorhandenen Modellen für nicht-ionische Substanzen erreicht wurden (**Publikation 4,** Kapitel 3.2). Für den Testdatensatz von *AquaBoxIL* wurde in der Validierung eine höhere Genauigkeit (96 %), Sensitivität (94 %), Spezifität (100 %) und R^2 (0,726 für das Modell für persistente ILs und 0,881 für das Modell für biologisch leicht abbaubare ILs) als bei den in dieser Studie vorgestellten Modellen erreicht. Dennoch führte die hier verwendete Kombination von Strukturfragmenten als Deskriptoren mit OLS und LR zu angemessenen Modellen für die Vorhersage der Bioabbaubarkeit von ILs.

Um die Leistung der Modelle zu verbessern, könnte eine Vergrößerung der Datensätze bei gleichzeitiger Abdeckung einer größeren Vielfalt von Strukturen in Betracht gezogen werden. Die Trainingsdaten und die daraus abgeleiteten Deskriptoren definieren die AD und die zugrundeliegenden SBRs der Modelle. Daher decken die Modelle möglicherweise wichtige SBRs nicht ab, die für die Vorhersage der Bioabbaubarkeit einer neuen Testsubstanz relevant sind. Entsprechend können Modelle keine zuverlässigen Vorhersagen für Testsubstanzen machen, die sich in zu vielen Strukturfragmenten von den Trainingssubstanzen unterscheiden. Nur innerhalb der AD der Modelle sind zuverlässige Vorhersagen möglich und Extrapolationen werden vermieden.

Darüber hinaus wurde untersucht, wie die neuen QSBR-Modelle in einer Testbatterie angewendet werden können, um die Zuverlässigkeit der Bewertung der Bioabbaubarkeit zu erhöhen (**Publikation 4**, Abbildung 5). Die Testbatterie kann in den Arbeitsablauf aus **Publikation 1** eingebaut werden. Als bioabbaubar vorhergesagte ILs sind in Labortests OECD 301D experimentell zu testen, um die Bioabbaubarkeit zu bestätigen oder zu widerlegen. Daher sind QSBR-Modelle hilfreich in der Planung von Experimenten und der Auswahl der vielversprechendsten Substanzen.

3.5.4 Schlussfolgerung

Aus den Ergebnissen der Modellvalidierung folgt, dass es grundsätzlich möglich ist, für die ILs QSBR-Modelle zur Bioabbaubarkeit in Übereinstimmung mit den OECD-Prinzipien für die Validierung von (Q)SARs zu entwickeln. Mit den vorhandenen OECD 301D-Daten konnten zwei Trainingsdatensätze erstellt werden, die für die Modellentwicklung geeignet waren. Auch mit den verwendeten Methoden zur Modellierung konnten QSBR-Modelle entwickelt werden, die die SBRs der ILs in den Trainingsdatensätzen abbilden konnten. Durch die Kombination der neuen QSBR-Modelle in der entwickelten Testbatterie wird der Arbeitsablauf für BbD aus **Publikation 1** auf ILs anwendbar gemacht. Die Anwendbarkeit der *alerts* und Testbatterie für die Entwicklung von mineralisierenden ILs ist in einer Fallstudie zu überprüfen. Weiterhin sind Untersuchungen der Trainingsdaten vorzunehmen, um Lücken in den strukturellen Merkmalen zu schließen und die AD der Modelle zu erweitern. So könnte auch die Performance der Modelle verbessert werden.

4 Erkenntnisse und Implikationen für Wissenschaft und Praxis

In den folgenden Kapitel 4.1–4.4 werden die Beiträge der wissenschaftlichen Ergebnisse aus AP 1–4 zur Anwendbarkeit von BbD auf ILs, Übertragbarkeit der weiterentwickelten Vorgehensweisen und Methoden auf andere Substanzklassen, Stoffbewertung, Regulatorik von Chemikalien unter REACH und Umsetzung des SSbD-Konzepts diskutiert (Abbildung 5).

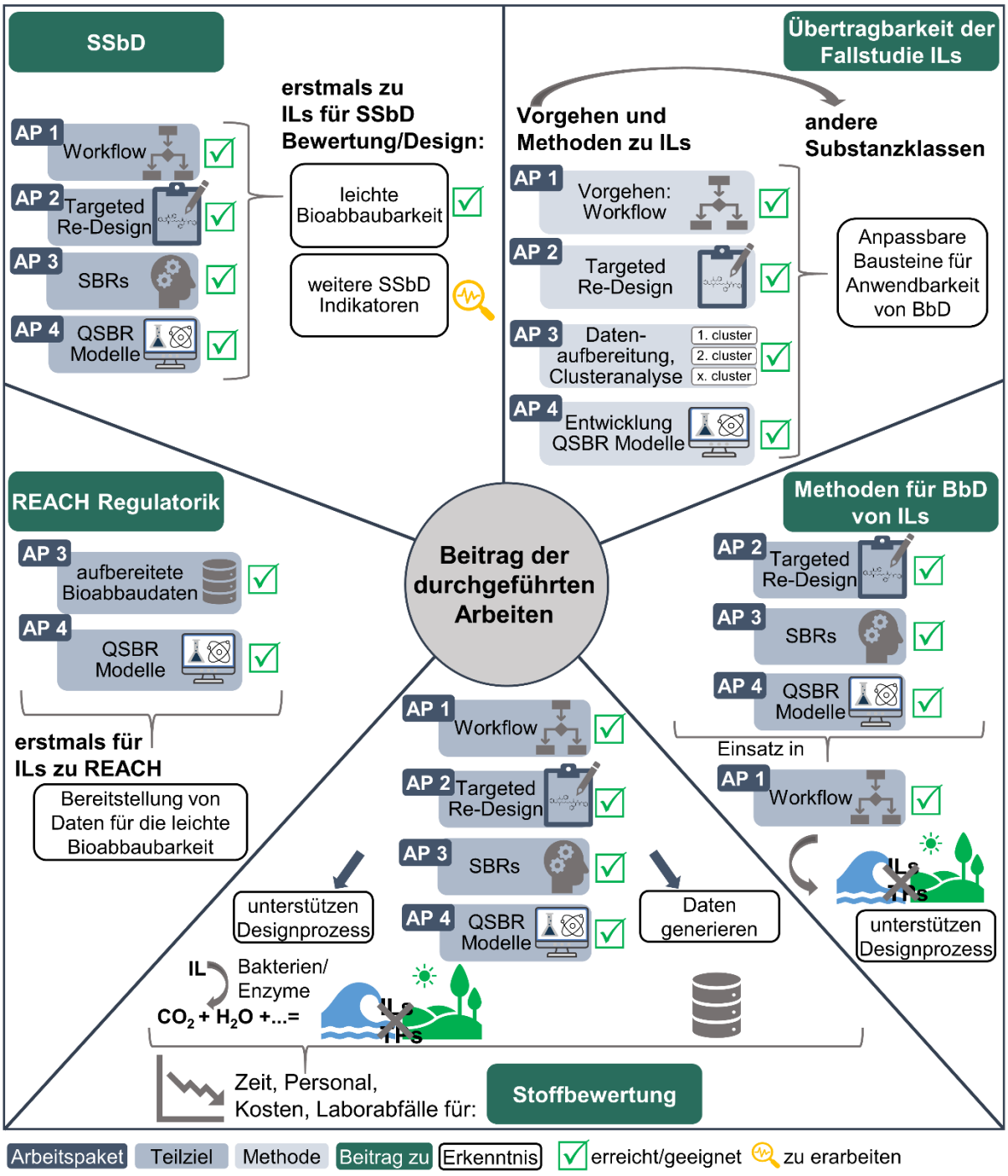

Abbildung 5: Übersicht der verschiedenen Beiträge durch neue Forschungserkenntnisse in dieser Arbeit.

4.1 Kombination von verschiedenen Methoden für die Anwendbarkeit von Benign by Design auf ionische Flüssigkeiten

Die Ergebnisse aus AP 1–4 liefern Vorgehensweisen für BbD und Methoden von der Datenrecherche, Datenaufbereitung, Datensatzerstellung, Datenanalyse bis zur Modellentwicklung, um BbD auf ILs anwendbar zu machen (Abbildung 5). Anhand der Ergebnisse in **Publikation 1** wurde die Anwendbarkeit von Modellen in BbD auf Basis von Beispielen aus der Literatur bestätigt. Die Ergebnisse zum Arbeitsablauf für BbD verdeutlichen erstmals das systematische Zusammenspiel von *in silico* und experimentellen Methoden für die Auswahl von Substanzen im Designprozess.

Es waren mehrere Forschungslücken für die Anwendbarkeit des Arbeitsablaufs aus **Publikation 1** auf die ILs zu schließen, da es bislang weder verfügbare QSBR-Modelle noch Datensätze zur Bioabbaubarkeit für die Modellentwicklung gab. Die Datenrecherche und -aufbereitung von Literaturdaten erfolgte über **Publikation 2**. Clustering erwies sich als geeignet, die gewonnenen Daten auf SBRs zu analysieren (**Publikation 3**). Die SBRs erweitern das vorhandene Expertenwissen und tragen dazu bei im Arbeitsablauf in **Publikation 1** gezielte strukturelle Veränderungen in den ILs vorzunehmen, um die Bioabbaubarkeit zu erhöhen. Aus den Literaturdaten in **Publikation 2** und **3** wurden Bioabbaudaten nach OECD 301D für einen Datensatz zur Entwicklung von QSBR-Modellen gezogen (**Publikation 4**). Die Studie zur Entwicklung der QSBR-Modelle hat gezeigt, dass für die Bioabbaubarkeit von ILs im Bereich des maschinellen Lernens Methoden erfolgreich an die ILs angepasst werden konnten. Die neu entwickelten QSBR-Modelle aus **Publikation 4** schließen die Lücke in den verfügbaren Methoden für BbD von ILs. Über die Testbatterie wird ein Beitrag zur Anwendbarkeit des Arbeitsablaufs in **Publikation 1** auf ILs geliefert. Die Anwendbarkeit des *targeted Re-Designs* auf ILs wurde in **Publikation 5** bestätigt. Der gezielte Einbau von Phe-Substituenten in ILs ermöglichte eine Verbesserung der Bioabbaubarkeit (**Publikation 5**). Allerdings konnte keine Kombination aus QSBR-Modellen und Laborexperimenten angewendet werden wie im Arbeitsablauf in **Publikation 1** aufgezeigt, da geeignete QSBR-Modelle für ILs nicht verfügbar waren.

Für die Anwendung der weiterentwickelten Vorgehensweisen und Methoden in einer Fallstudie zu BbD von ILs sind einige Punkte zu beachten. Durch das Kation, dessen Seitenketten und das Anion ist die Substanzklasse der ILs strukturell sehr vielfältig und kann nach Berechnungen bis zu 10^6 Substanzen umfassen (Plechkova & Seddon, 2008). Folglich

kann es sein, dass die ermittelten SBRs und QSBR-Modelle nicht auf jede IL anwendbar sind. Dies wird durch die Ergebnisse in **Publikation 3** und **Publikation 5** bestätigt. Die Ergebnisse der Clusteranalyse in **Publikation 3** zeigen, dass einige Lücken in SBRs aufgrund fehlender Daten vorliegen, u.a. zu Referenzsubstanzen, um für die Vielfalt an Strukturfragmenten SBRs ableiten zu können. Darüber hinaus zeigte der Abgleich der in der Literatur beschriebenen generalisierten Faustregeln mit den Ergebnissen der Clusteranalyse, dass die Faustregeln nicht immer zutreffen (**Publikation 3**).

Außerdem konnte am Beispiel von CholPheC_6 gezeigt werden, dass vorhandene Faustregeln aus der Literatur nicht immer zutreffen (**Publikation 5**). Die Kombination gut abbaubarer Fragmente entsprechend der Faustregeln führte zu keiner besser abbaubaren IL im Vergleich zu Analogen mit vermeintlich schlechter abbaubaren Kation-Kopfgruppen. Dieser Widerspruch wird auch in anderen Studien bestätigt (Haiß et al., 2016; Suk et al., 2020). Haiß et al. (2016) sieht eine mögliche Erklärung darin, dass die Stereochemie des quartären Amins einen negativen Effekt auf die Bioabbaubarkeit hat, da eine zu CholPheC_6 strukturell ähnliche IL, in der statt einer *n*-Hexylkette eine *n*-Ethylkette vorliegt, in Tests nach OECD 301D weniger abgebaut wurde als das nicht-ionische Derivat mit einem tertiären Amin. Damit unterscheidet sich das Choliniumkation von planaren Imidazolium- und Pyridiniumkationen. Dass SBRs und Faustregeln nicht immer zutreffen, wurde auch für andere Substanzklassen beobachtet, so z.B. für Chinolone und Sulfonamide (Lorenz, 2023; Puhlmann, Olsson & Kümmerer, 2024). Entsprechend ist das Design von bioabbaubaren ILs unter Anwendung von SBRs und Faustregeln herausfordernd. Es verdeutlicht aber auch, dass die SBRs für die Kopfgruppen der Kationen, die Seitenketten des Kations und die Anionen verstanden werden müssen.

Wie für die SBRs ergibt sich die Limitierung in der Anwendbarkeit von QSBR-Modellen infolge der eingeschränkten Datenverfügbarkeit. Wie die Ergebnisse in **Publikation 4** zeigen, sind die QSBR-Modelle nur für ILs geeignet, die innerhalb der AD der Modelle liegen. Folglich sollte immer geschaut werden, wie sich eine neu designte IL von den ILs in dem Datensatz zur Ableitung der SBRs (**Publikation 3**) und denen im Trainingsdatensatz der QSBR-Modelle (**Publikation 4**) unterscheidet, um die Grenzen der Anwendung von SBRs und QSBR-Modellen zu erkennen. Die Limitierung in der Anwendung von SBRs und Faustregeln im Design und QSBR-Modellen verdeutlicht, dass die Überprüfung der Bioabbaubarkeit im Labor für die vermeintlich gut abbaubaren ILs grundsätzlich erforderlich ist.

Um die Implementierung von BbD in der chemischen und pharmazeutischen Industrie zu fördern, sind die vier Vorgehensweisen für BbD und der Arbeitsablauf aus **Publikation 1** in die Vorgehensweisen der Forschungs- und Entwicklungsabteilungen der Industrie zu integrieren, z.B. in den iterativen Innovationsprozess nach dem Stage-Gate-Modell (The European Chemical Industry Council (Cefic), 2024). Für die pharmazeutische Industrie stellte sich die Integration der Optimierung der Bioabbaubarkeit und damit die Anwendung von BbD in der Phase der *Lead*-Optimierung im Wirkstoffentwicklungsprozess als geeignet heraus (Puhlmann et al., 2021; Puhlmann, Vidaurre & Kümmerer, 2024). Da eine große Herausforderung in der Integration von BbD in die Wirkstoffentwicklung die Verfügbarkeit von geeigneten Modellen ist (Puhlmann, Vidaurre & Kümmerer, 2024), wird in dieser Arbeit mit dem entwickelten Arbeitsablauf für BbD und den QSBR-Modellen ein Beitrag zur Anwendbarkeit von BbD in der Phase der *Lead*-Optimierung zur Entwicklung von bioabbaubaren ILs mit der Verwendung als API geleistet.

4.2 Übertragbarkeit der Vorgehensweisen und Methoden auf andere Substanzklassen

Mit den Ergebnissen aus AP 1–4 zu weiterentwickelten Vorgehensweisen und Methoden wurden Möglichkeiten bereitgestellt BbD auf ILs anwendbar zu machen (Kapitel 4.1). Die Übertragbarkeit dieser Methoden auf andere Substanzklassen wird im Folgenden diskutiert. Der Beitrag der angewendeten Datenrecherche in **Publikation 2** liegt darin zu zeigen, wie für Substanzklassen, die recht neu auf den Markt kommen und nicht umfassend in zugänglichen Datenbanken erfasst sind, z.B. in der ECHA-Datenbank, Bioabbaudaten aus der Literatur gewonnen und aufbereitet werden können. Vorteil dieses Vorgehens ist auch, dass Substanzen aus der Forschung oder TPs, die nicht regulatorisch erfasst werden, in die Datensatzerstellung einfließen. Für andere Substanzklassen können sich andere Datenquellen besser eignen. So erwies sich die ECHA-Datenbank als Datenquelle für siliziumorganische Substanzen geeignet, da diese Substanzklasse industriell weit verbreitet eingesetzt wird, aber nicht ausführlich in der Literatur auf die Bioabbaubarkeit bewertet wurde (Grabitz et al., 2021). Entsprechend kann für andere Substanzklassen die Datenrecherche angepasst werden.

Die Datenaufbereitung wurde in **Publikation 2** und **4** für die recherchierten Bioabbaudaten durchgeführt, um die Qualität des darauf aufbauenden Datensatzes zu erhöhen. Mit der Datenaufbereitung wurde ein wichtiger Schritt unternommen, um die Datensätze für die Ermittlung von SBRs und Entwicklung von QSBR-Modellen zu nutzen (Szymańska, 2018;

Tropsha, 2010). Denn die Datenanalyse und Modelle können nur so gut sein wie die Daten selbst (Szymańska, 2018; Tropsha, 2010). Die entwickelten Kriterien für die Datenaufbereitung helfen die in der Literatur beschriebene Methode auf Übereinstimmung mit dem angegeben Standard abzugleichen und richten sich nach den Standardmethoden zur Bestimmung der leichten Bioabbaubarkeit nach OECD (1992). Die Datenaufbereitung ist allerdings davon abhängig, wie ausführlich die recherchierten Studien die Methoden beschreiben und Testparameter benennen. Daher ist für eine Literaturauswertung einer neuen Substanzklasse zu prüfen, ob die Kriterien entsprechend den verfügbaren Informationen in der Literatur übernommen oder erweitert werden können oder gar gekürzt werden müssen.

Vorhandene QSBR-Modelle sind aufgrund der Datenverfügbarkeit in ihrer AD bislang sehr eingeschränkt (Singh et al., 2021). Daher ist zu prüfen, ob für eine neue Substanzklasse, die in BbD untersucht werden soll, die vorhandenen Modelle verwendet werden können oder neue Modelle entwickelt werden müssen. Die in **Publikation 4** entwickelten QSBR-Modelle zeigen, dass eine im Vergleich zu Neuronalen Netzen einfache Methode geeignete Modelle liefern kann, die gleichzeitig den Vorteil mit sich bringen, dass sie einfacher zu interpretieren sind (Gramatica, 2020; Guha, 2008; Yee & Wei, 2012). Ebenfalls wurde gezeigt, dass leicht zu interpretierende Modelle in BbD zu bevorzugen sind, um die SBRs in dem Design einsetzen zu können. Ob das hier beschriebene Vorgehen in der Modellentwicklung auch auf andere Substanzklassen übertragen werden kann, ist in einer Modellvalidierung zu überprüfen. Vorab kann keine Aussage getroffen werden, da Charakteristika des Trainingsdatensatzes (Größe, Qualität der Daten, Diversität in Strukturfragmenten, Anzahl aktiver und inaktiver Substanzen in Bezug auf den Endpunkt) einen Einfluss auf die Performance der Modelle haben (Gramatica, 2020; Shoombuatong et al., 2017). Für N-Heterozyklen wurde beispielweise ein anderes Vorgehen in der Modellentwicklung gewählt. Für den Einsatz in BbD wurde ein *field-based* QSBR-Modell auf Basis von 3D-Strukturen entwickelt, welches die Identifizierung von SBRs unterstützte (Suk et al., 2023).

Für die Anwendung des Arbeitsablaufs in **Publikation 1** und die Clusteranalyse in **Publikation 3** ist für andere Substanzklassen zu prüfen, ob geeignete Methoden für die Darstellung der Molekülstruktur in einem für Computer lesbaren Format, wie Strukturschlüssel, und QSBR-Modelle mit einer geeigneten AD vorhanden sind. Allerdings heißt dies nicht, dass automatisch Substanzen mit verbesserter Bioabbaubarkeit entwickelt werden können. Aufgrund der im vorherigen Kapitel beschriebenen Limitierungen in der Anwendung von SBRs und QSBR-Modellen gibt es keine allgemein gültige Herangehensweise

für BbD, wie die Ergebnisse von Lorenz (2023) und Puhlmann, Olsson und Kümmerer (2024) bestätigen. Die Diskussion zeigt, dass die weiterentwickelten Vorgehensweisen und Methoden von der Datenrecherche, Datenaufbereitung, Datensatzerstellung, Datenanalyse bis zur Modellentwicklung wichtige Bausteine für die Anwendbarkeit von BbD sind. Anpassungen der Methoden können vorgenommen werden. Welche genau ist in Abhängigkeit von der Substanzklassen zu prüfen (Abbildung 5).

4.3 Beitrag zur Stoffbewertung und Regulatorik von Chemikalien

Die Ergebnisse aus AP 1–4 leisten auf zwei verschiedenen Ebenen einen Beitrag zur Bewertung der Persistenz in der Gefährdungs- und Risikobewertung von Chemikalien in der Umwelt. Einerseits können die Ergebnisse dazu beitragen, die auf Persistenz zu bewertende IL zu priorisieren. Andererseits werden Daten bereitgestellt, die für die Bewertung genutzt werden können, z.B. im Rahmen von REACH.

Infolge der weiterentwickelten Vorgehensweisen und Methoden für das Design von in der Umwelt vollständig mineralisierenden ILs (**Publikation 1–5)**, werden das Vorkommen in der Umwelt und schädliche Wirkungen von ILs bestenfalls vermieden. Voraussetzung ist, dass die ILs so schnell mineralisieren, dass Mensch und Umwelt der ILs und ihrer Effekte nicht ausgesetzt sind und keine Pseudo-Persistenz eintritt (Mackay et al., 2014). Weiterhin entstehen keine TPs in der Umwelt. Dadurch werden die mit ihnen einhergehenden Wissens- und Datenlücken zu ihrem Verbleib und Effekten in der Umwelt umgangen und ihre ressourcenintensive Gefährdungs- und Risikobewertung (Zeit, Geld, Personal) vermieden (Escher & Fenner, 2011; Puhlmann et al., 2022) (Abbildung 5).

Da die Mineralisierung von vielen Umweltbedingungen abhängig ist, die nicht überall optimal für den Abbau vorherrschen (Schäffer et al., 2022), sind Gefährdungs- und Risikobewertungen von „benign“ ILs auch weiterhin vorzunehmen. Gefahren und Risiken während der Anwendung werden so verstanden oder fehlerhafte Annahmen aufgrund des Designs vermieden. Aufgrund der Vielzahl an Chemikalien, sind aber die Kapazitäten begrenzt, um eine Bewertung vorzunehmen (Wang et al., 2020). Die Ergebnisse können dazu beitragen, dass in der Bewertung der Persistenz eine Priorisierung von ILs stattfinden kann. Diejenigen, die nicht nach BbD designt wurden, sollten vorrangig auf Persistenz untersucht werden. Damit können die von Mackay et al. (2014) vorgeschlagenen Informationen in der Bewertung der Persistenz zur Priorisierung von Substanzen, u.a. Verwendungsmuster, Freisetzung, und Expositionswege, ergänzt werden. Um Ressourcen zu sparen, können die neu entwickelten

QSBR-Modelle aus **Publikation 4** für die Bewertung von ILs genutzt werden. Die Ergebnisse zeigen, dass diese geeignet sind, wenn die zu bewertende IL innerhalb der AD liegt.

Da für REACH experimentelle Daten oder Vorhersagen durch Modelle zur leichten biologischen Abbaubarkeit für Chemikalien vorgelegt werden müssen, die in einer Menge von ≥ 1t pro Jahr hergestellt oder eingeführt werden (ECHA, 2008, 2023), leisten die Ergebnisse aus AP 3 und AP 4 einen Beitrag die geforderten Daten zu liefern. Die recherchierten Studien mit Literaturdaten zur leichten Bioabbaubarkeit aus **Publikation 2** und die Trainingsdaten der QSBR-Modelle (**Publikation 4**) liefern erstmals auf Qualität geprüfte Daten, womit ein wichtiger Schritt für die Bereitstellung von Daten für REACH gemacht wurde (Abbildung 5). Wie in **Publikation 2** beschrieben, sind in den recherchierten Studien die Methoden und die Validitätskriterien nicht immer vollständig beschrieben sowie die Einstufung als leicht biologisch abbaubar gemäß OECD (1992) nicht immer angegeben oder korrekt angewendet. Daher ist aufbauend auf den Ergebnissen dieser Arbeit, der nächste Schritt für die Anwendung in REACH die fehlende Informationen in den zugrundeliegenden Studien zu ergänzen zu u.a.: a) Einstufung als leicht biologisch abbaubar oder nicht gemäß OECD (1992), b) TPs, wenn die IL primär eliminiert wird und nicht vollständig mineralisiert wird, c) Validität, d) Anzahl an Replikaten und Kontrolltests (ECHA, 2023). Darüber hinaus stehen für REACH erstmals auch QSBR-Modelle für ILs (**Publikation 4**) zur Verfügung. Die Modelle ermöglichen, dass Daten zur leichten biologischen Abbaubarkeit in kurzer Zeit für die Registrierung generiert werden können (Abbildung 5), wenn die IL innerhalb der AD liegt. Da die neuen QSBR-Modelle unter Berücksichtigung der fünf OECD-Prinzipien für die Validierung von (Q)SAR entwickelt wurden, ist eine wichtige Voraussetzung für die Nutzung von (Q)SAR in REACH erfüllt (ECHA, 2023).

4.4 Beitrag zur Umsetzung von Chemikalienstrategien auf EU Ebene

Die Ergebnisse aus den vier APs zeigen, wie Methoden für BbD angepasst und weiterentwickelt werden können und wie in BbD vorgegangen werden kann, um einen Beitrag zur Entwicklung von in der Umwelt mineralisierenden ILs zu leisten. Dieses steht in Einklang mit dem Ziel der *Chemikalienstrategie für Nachhaltigkeit* und dem darauf basierenden *SSbD framework* sichere und nachhaltigere Chemikalien zu entwickeln (Caldeira et al., 2022; Europäische Kommission, 2020). Für die Umsetzung von SSbD sind zuverlässige Datenquellen zu identifizieren, Datenqualität zu bestimmen und Methoden zu erarbeiten (Caldeira et al., 2022). Einerseits werden über die Ergebnisse aus AP 1–4 diese

Voraussetzungen für die Bioabbaubarkeit von ILs geliefert. Andererseits wurde am Beispiel der Bioabbaubarkeit von ILs in dieser Arbeit gezeigt, wie ausgehend von der Prüfung der Datenverfügbarkeit, Datenanalyse, Datenaufbereitung bis zur Entwicklung neuer Datensätze und QSBR-Modelle vorgegangen werden kann, um die genannten Voraussetzungen zu erfüllen. Weiterhin steht das Vorgehen im Arbeitsablauf aus AP 1 im Einklang mit dem *SSbD framework*, welches betont, dass *in silico* Methoden, wie (Q)SAR, in der (Re-)Design Phase zur Bewertung von Chemikalien eingesetzt werden sollen, um Entscheidungen im Design zu lenken ohne eine Synthese vorzunehmen (Caldeira et al., 2022). Allerdings wurde kein Vorgehen zur Umsetzung des SSbD-Designprinzips *design for end-of-life,* welches u.a. die Bioabbaubarkeit betrachtet, für die (Re-)Design Phase beschrieben (Caldeira et al., 2022). An dieser Stelle setzt AP 1 an. Die neuen Erkenntnisse aus AP 1 zum systematischen Einsatz von Modellen und Laborexperimenten in dem Arbeitsablauf für die Bewertung von Chemikalien in der (Re-)Design Phase liefern einen Beitrag, wie das Vorgehen zur Umsetzung des SSbD-Designprinzips *design for end-of-life* zum Endpunkt Bioabbaubarkeit aussehen kann. Folglich kann basierend auf den Ergebnissen aus dieser Arbeit zu dem *in silico* Arbeitsablauf (AP 1), den SBRs von ILs (AP 3) und den QSBR-Modellen (AP 4) der Endpunkt „leichte Bioabbaubarkeit“ in Fallstudien für die Entwicklung von sicheren und nachhaltigeren ILs aufgenommen werden (Abbildung 5).

Der Arbeitsablauf aus **Publikation 1** wurde so gestaltet, dass verschiedene Eigenschaften einer Chemikalie, wie z.B. Bioabbaubarkeit, (Öko-)Toxizität und für die Funktion relevante Endpunkte, bewertet werden können. Folglich kann der Arbeitsablauf auf zusätzliche Endpunkte der SSbD-Designprinzipien im *SSbD framework* erweitert werden, wie z.B. aus dem Bereich Toxizität für das SSbD-Designprinzip *minimise the use of hazardous chemicals/materials* (Abbildung 5). Voraussetzung dafür ist, dass Zielwerte für die Endpunkte definiert werden, um zu entscheiden, ab wann eine Chemikalie den SSbD-Designprinzipien entspricht oder nicht. Um für die ILs spezifische Methoden für die SSbD-Bewertung zu entwickeln, kann das hier verwendete Vorgehen zur Prüfung der Datenverfügbarkeit, Datenanalyse, Datenaufbereitung und Entwicklung neuer Datensätze und QSAR-Modelle für weitere Endpunkte angewendet werden.

Da der Einsatz einiger ILs als API diskutiert wird (Egorova et al., 2017), ist auch der S*trategische Ansatz der Europäischen Union für Arzneimittel in der Umwelt* für die ILs relevant (Europäische Kommission, 2019). In der Arzneimittelstrategie wird der Handlungsbedarf zur Verminderung des Eintrags von Arzneimitteln in die Umwelt und ihrer

schädlichen Wirkungen auf Mensch und Umwelt aufgezeigt. Die Ergebnisse dieser Arbeit leisten einen Beitrag zur Umsetzung der Arzneimittelstrategie, indem Methoden weiterentwickelt und angepasst wurden, um das Design von in der Umwelt mineralisierenden ILs im Rahmen von BbD zu unterstützen. Voraussetzung ist, dass ILs so schnell in der Umwelt mineralisieren, dass sie keine schädlichen Wirkungen auf Mensch und Umwelt ausüben können. Folglich wird das Vorkommen von ILs in der Umwelt verringert.

Sowohl die *Chemikalienstrategie für Nachhaltigkeit* als auch der S*trategische Ansatz der Europäischen Union für Arzneimittel in der Umwelt* betrachten den Lebenszyklus einer Chemikalie bzw. APIs (Europäische Kommission, 2019, 2020). Darin eingeschlossen sind die Synthesebedingungen und -wege und die dort verwendeten Ressourcen. Während die Weiterentwicklung der Synthesebedingungen und -wege Richtung mehr Nachhaltigkeit in dieser Arbeit nicht im Fokus standen, wurde in AP 2 mit der Entwicklung von bioabbaubaren dikationischen Phe-ILs beispielhaft gezeigt, wie natürlich vorhandene Strukturfragmente in ILs eingebaut werden können. Die von Suk und Kümmerer (2023) beschriebenen Synthesewege unter Verwendung von erneuerbaren Ressourcen oder Abfällen für grünere und nachhaltigere ILs sind mit den hier weiterentwickelten Methoden für BbD in Fallstudien zu kombinieren, um den gesamten Lebenszyklus im Design von ILs zu betrachten.

5 Fazit

Durch die Anpassung und Weiterentwicklung von *in silico* Methoden wurden Möglichkeiten bereitgestellt, um BbD zukünftig auf ILs anzuwenden. Im Rahmen dieser Arbeit werden neueste Erkenntnisse zu den konzeptionellen Grundlagen von BbD geliefert. Erstmalig wurden die vier Vorgehensweisen in BbD definiert und mit einem neu entwickelten Arbeitsablauf verknüpft, der das systematische Zusammenspiel von computergestützten Methoden und Laborexperimenten konkretisiert (Teilziel 1). Mit Hilfe der neu generierten Daten zum biologischen Abbau der ILs wurden mehrere Wissenslücken geschlossen, um den Arbeitsablauf für diese Substanzklasse anwendbar zu machen. Mit der Entwicklung der Gemini-Phe-ILs und der Daten zu ihrem Bioabbau konnte die Anwendbarkeit des *targeted Re-Designs* durch den Einbau einer Phe-Gruppe erfolgreich gezeigt werden (Teilziel 2). Erstmalig wurde über eine systematische Literaturrecherche die Verfügbarkeit von Bioabbaudaten zu ILs ermittelt und deren Qualität bewertet. In dieser Arbeit wurde in einem neu aufbereiteten Datensatz zur leichten biologischen Abbaubarkeit die zugrundeliegenden SBRs und die Übereinstimmung mit den Faustregeln aus der Literatur gezeigt (Teilziel 3). Anhand des neu erstellten Datensatzes aus OECD 301D-Daten auf Basis der gewonnenen Literaturdaten und Daten des INSC wurden erstmals fragmentbasierte QSBR-Modelle für ILs entwickelt. Im Rahmen einer Testbatterie machen die neuen QSBR-Modelle den Arbeitsablauf auf die ILs anwendbar (Teilziel 4). Gleichzeitig wurde die Anwendbarkeit von *in silico* Methoden auf die ILs erweitert. In einem Fallbeispiel sind die hier erarbeiteten QSBR-Modelle und SBRs in dem Arbeitsablauf anzuwenden und der weitere Anpassungsbedarf festzustellen.

Anhand des Beispiels der ILs wurde in der Arbeit deutlich, dass wichtige Bausteine für die Übertragbarkeit der Methoden in BbD auf andere Substanzklassen geeignete Strukturschlüssel für die Nutzung von *in silico* Methoden, SBRs auf Basis von qualitativ hochwertigen Daten und QSBR-Modelle mit einer für die Substanzklasse angepassten AD sind. In dieser Arbeit wurde gezeigt, wie in der Datenrecherche, Datenaufbereitung, Datenanalyse und Modellentwicklung vorgegangen werden kann und *in silico* Methoden angepasst und weiterentwickelt werden können, um diese Bausteine zu liefern. Die durchgeführten Arbeiten liefern erstmals eine Aufstellung an auf Qualität geprüfte Literaturdaten zur leichten Bioabbaubarkeit, die unter Beachtung der Vorgaben von REACH für die Registrierung von ILs genutzt werden können. Außerdem stehen erstmals für REACH QSBR-Modelle für ILs zur Verfügung. Deren Vorhersagen können in der Registrierung verwendet werden, wenn die IL innerhalb der AD liegt. Die Diskussion liefert neue Erkenntnisse, wie die weiterentwickelten

Vorgehensweisen und Methoden für BbD von ILs die Umsetzung der *Chemikalienstrategie für Nachhaltigkeit* unterstützen. Unter Berücksichtigung des gesamten Lebenszyklus sind die Synthesebedingungen und -wege mit den hier weiterentwickelten Methoden für BbD in Fallstudien zu kombinieren. Um weitere Kriterien für sichere und nachhaltigere Chemikalien in das Design einzubauen und die Anwendung der bioabbaubaren designten Substanzen zu fördern, ist eine interdisziplinäre Zusammenarbeit von Industrie, akademischer Forschung und Behörden erforderlich.

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Anhang

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Publikation 1

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Perspective

Toward Application and Implementation of *in Silico* Tools and Workflows within Benign by Design Approaches

Stefanie Lorenz,[#] Ann-Kathrin Amsel,[#] Neele Puhlmann, Marco Reich, Oliver Olsson, and Klaus Kümmerer*

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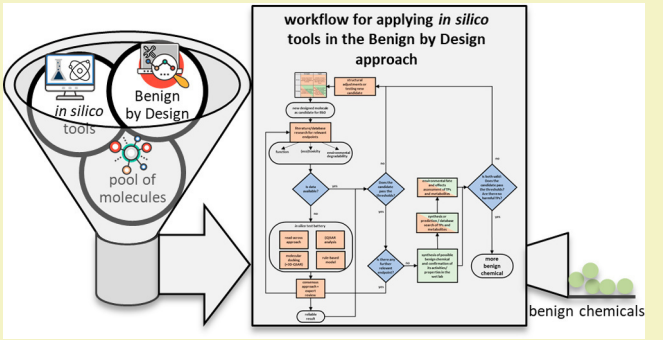
ABSTRACT: To avoid adverse side effects of chemicals, pharmaceuticals, and their transformation products (TPs) in the environment, substances should be designed to fully mineralize in the environment at their end-of-life while ensuring a degree of stability as needed for their application. These considerations should be implemented at the very beginning of chemical's and pharmaceutical's design (Benign by Design, BbD) to meet requirements set by planetary boundaries and upcoming legal frameworks (e.g., "Chemicals Strategy for Sustainability towards a Toxic-Free Environment" by the European Commission (EC)). *In silico* tools are already being implemented in the drug discovery process and the assessment of chemicals and pharmaceuticals. The advantage of which is avoiding or at least minimizing animal testing and chemical waste due to experimental testing as well as reducing the time to market. However, in the literature, there are just a few examples of how *in silico* tools could be implemented in the BbD process. Therefore, this study suggests a workflow supporting practitioners designing new environmentally mineralizing chemicals and pharmaceuticals. This would also result in a much faster and less expensive process than starting with repetitive synthesis and subsequent experimental testing to improve the compounds' properties.



KEYWORDS: *Benign by Design, (Q)SAR, In silico tools, Environment, Degradation, Mineralization, Toxicity, Toxic-Free, Planetary Boundary*

INTRODUCTION

The globally increasing chemicalization comes along with the increase of pollution in the environment. This also includes transformation products (TPs) of chemicals formed in treatment processes or in the environment.[1] Nowadays, almost the entire periodic table of elements is present in the production and use of consumer goods and technical products.[2] Even though there is a global movement toward a circular economy, many chemicals such as ingredients of household products, disinfectants, cosmetics or other personal care products, electronics, plastics, buildings, or pesticides and pharmaceuticals end up in the environment and cannot be recycled.[3] Chemicals, pharmaceuticals, and other products and materials have become increasingly complex due to new methods and pathways of synthesis, which have led to new properties that may promise advantages or new products for the market.[3,4] At the same time, manufacturers have improved the stability of chemicals and pharmaceuticals to allow for a more extended shelf life or to avoid any transformation during application, leading to increased persistence in effluent treatment processes and the environment. This translates into additional environmental fate and effect issues at present and in the future.

In 2020, the European Commission (EC) published the "Chemicals Strategy for Sustainability towards a Toxic-Free Environment", where several areas for taking action toward sustainable-by-design chemicals and a pollution-free environment have been identified.[5] The EC strategy includes the following areas: safe and sustainable-by-design chemicals, zero chemical pollution in the environment, and innovative tools for safety testing and risk assessment to reduce animal testing. In addition, the "European Union Strategic Approach to Pharmaceuticals in the Environment" strives for an environmentally benign design of pharmaceuticals.[6] What is needed are combined approaches and tools to improve Chemicals' and Pharmaceuticals' design and assessment regarding hazard, exposure, and risk under consideration of end-of-life issues from the very beginning (Benign by Design, BbD).[4,7] Chemicals,

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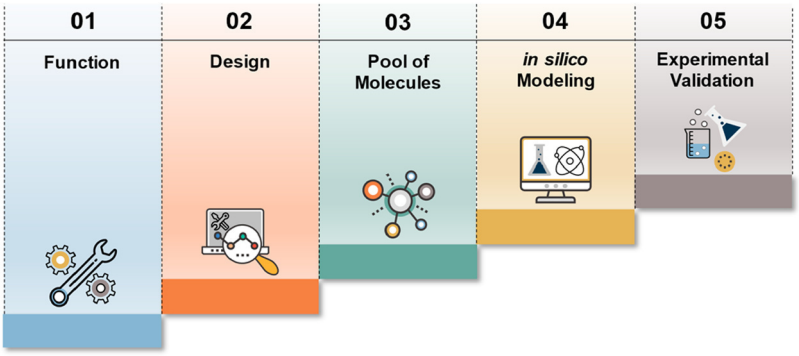

Figure 1. General procedure for the benign design of chemicals using *in silico* tools.

which necessarily end up in the environment because they cannot be circulated in a closed system, should be designed to mineralize completely either in water treatment processes or preferably even in surface water if they cannot be treated.[8] The BbD concept, based on the 10th principle of green chemistry,[9] intends to include environmental fate, (eco)toxicity, and stability during storage and application already at the beginning of the development of new chemicals depending on their anticipated life cycle while ensuring the desired function. Examples for its application range from product groups such as pharmaceuticals to fragrances, chelating agents, and ionic liquids as well as some general rules of thumb for such a design.[10−17]

In silico tools can play an essential role in the context of BbD. The general advantages of *in silico* tools compared to *in vivo* and *in vitro* testing are cost-effectiveness, speed, and reduction in animal testing.[18,19] Several authorities have recommended and some have even provided *in silico* tools to assess chemicals regarding hazard identification, risk assessment, and human health safety assessment.[5,20−26] Workflows on how to apply these *in silico* tools have been created for chemicals' risk assessment[27−29] and for computational toxicology,[30,31] but not for BbD.

A combination of *in silico* tools and experimental testing offers several advantages including priority setting and the selection of the most promising candidates for synthesis and testing.[32] Several studies have shown the feasibility of combining *in silico* and experimental testing to assess chemicals with unknown properties.[29,33−37] These studies revealed that *in silico* assessment is appropriate for assessing environmental fate and effects of chemicals and products of incomplete mineralization even before their synthesis.[29] *In silico* tools have also been successfully applied to design environmentally degradable chemicals in the context of the BbD concept.[11,13−15,38,39] Consequently, *in silico* methods allow for fast decisions whether the chemical is a promising candidate for further experimental testing while limiting the digital pool of possible chemicals. The assessment of chemicals under REACH,[40,41] the PBT assessment,[42] and the ICH M7 guideline[43] provide guidance on relevant end points in the context of BbD.

There is, however, still a lack of well-defined workflows that include the choice of end points, models, and an evaluation of the predictions when using *in silico* tools in a structured way for a molecule's design, including improved environmental degradation and mineralization while keeping or even improving desired properties. Additionally, such workflows should be developed in a way that practitioners who only have limited experience in the application of *in silico* tools can apply these models, interpret the predictions, and assess their accuracy.[44]

This study aims to contribute to the implementation of *in silico* tools in the design process of benign chemicals to support the topics of safe and sustainable-by-design, zero chemical pollution in the environment, and innovative tools for safety testing and risk assessment to reduce animal testing as outlined in the "Chemicals Strategy for Sustainability Towards a Toxic-Free Environment" by the EC.[5] As a first step, BbD approaches described in the literature were identified. Second, various *in silico* tools were studied to understand their application and limitations. On this basis, five preliminary steps have been identified, which can be used by practitioners when adapting the workflow presented here. Finally, a workflow for the application of *in silico* tools within the BbD framework was developed in order to help practitioners make faster and better-informed decisions in the selection of *in silico* tools and apply these successfully in a consistent and confident way.

GENERAL PROCEDURE FOR THE APPLICATION OF *IN SILICO* TOOLS FOR BbD

The design and redesign of molecules are often based on the assumption that even small changes in a chemical's structure may have tremendous effects on its properties and behavior.[45] These small changes combined with the significant difference in properties are called activity cliffs, a concept discussed in computational and medicinal chemistry.[46] Such activity cliffs can also be discussed in the context of the design for the degradability of the molecules. Molecules, which end up in the environment, should be readily mineralizable, while those that circulate and cannot leak from the system into the environment should be as stable and easily regainable as possible.[3] To implement this way of thinking into the chemicals' design process, the term stability needs to be redefined to consider the different physicochemical−biological conditions at each stage of life. The design process of benign chemicals can be divided into five steps (Figure 1). In the following sections, these five steps will be discussed in detail.

DIFFERENT APPROACHES OF THE BbD CONCEPT

Four approaches for the design of new molecules in the context of BbD have been derived from the literature: (i) nontargeted *de novo* design, (ii) targeted *de novo* design, (iii) nontargeted redesign, and (iv) targeted redesign (Table 1). In each case, a pool of molecules with potentially improved (environmental) properties is generated either by *in silico* tools or by experimental methods (Table 1). The most promising molecules out of this pool have to be identified according to their environmental fate and (eco)toxicity by employing *in silico* tools or experimental methods.

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ACS Sustainable Chem. Eng. XXXX, XXX, XXX−XXX

Table 1. Four Different Approaches of BbD to Generate a Pool of New Molecules with Potentially Improved (Environmental) Properties Either by *de Novo* or Redesign[a]

	Nontargeted	Targeted
De novo	Scanning a large amount of structures in databases for functionality, degradability and (eco)toxicity	Combination of molecular fragments which are known to favor a desired property or activity either *in silico* or by drawing
Redesign	Either nontargeted synthesis or *in silico* prediction of TPs and screening for functionality, degradability and (eco)toxicity	Either *in silico* or by synthesis: Integration of structural fragments in a known molecule that possibly improve functionality, degradability and decrease (eco)toxicity

[a]Green: experiments in the wet laboratories; amber: *in silico* tools. Transformation products (TPs).

De novo design is the process of designing a new molecule from scratch or discovering new functions or modes of action for already known and used chemicals and pharmaceuticals.[47] A redesign changes structures of already present chemicals by integrating new structural fragments while keeping the basic structure that is indispensable for its application. For the *de novo* design of substances, a lot of expert knowledge or high computational power for computing and scanning many different molecules is necessary. In contrast, in the redesign process, the target and mechanism of action are already known from the model compound. However, the redesign generates a limited set of candidates compared to the *de novo* design.[48]

For both, *de novo* design and redesign, two approaches, targeted and nontargeted, can be applied. The targeted approach refers to integrating structural fragments, which are known to enhance a specific desirable property or avoid the ones that favor unwanted effects systematically. This process is based on expert knowledge and the use of known design rules. The targeted approach can be very time-consuming due to the challenges that come with the optimization of the molecule targeting different end points. On the contrary, the nontargeted approach is characterized by an undirected, arbitrary way of generating molecules. It leads to a large pool of new molecules, which then are further evaluated regarding their properties to short-list them to some promising candidates.[8,48] In general, the nontargeted redesign is less time-consuming, comparably cheap, and offers the possibility of generating and screening a high amount of substances at once. The combination of *in silico* tools and experimental testing in the context of nontargeted design offers certain advantages including priority setting and selecting the most promising candidates for synthesis. The nontargeted approach requires less expert knowledge than the targeted approach as no candidates have to be developed from scratch based on known structure−activity relationships (Table 1).

Nontargeted *de Novo* Design. The typical drug discovery process presents an excellent example for a nontargeted *de novo* design since extensive databases are used to search for a promising structure ("hit structure"). More precisely, tens of thousands of compounds that contain the desired pharmacophore are tested in high-throughput screenings for their specific pharmaceutical activity. Identified active compounds are evaluated and optimized regarding an increased target site binding affinity, for example. Then, the leads are submitted to extensive testing and optimization, including pharmacokinetic properties (absorption, distribution, metabolism, excretion (ADME)), reduced toxicity, and chemical stability. *In silico* tools are essential to support these optimization cycles.[49,50] At the end of this process, the most promising and optimized candidates are selected and submitted to preclinical trials.[49,51] This procedure from the field of drug discovery can be adapted for the purpose of nontargeted *de novo* BbD if considerations like environmental mineralization and (eco)toxicity are implemented in the screening and optimization process.[52]

Targeted *de Novo* Design. The targeted *de novo* design approach combines low molecular weight fragments concerning their function to more complex molecules.[53] The generation of those molecules and their assessment are usually performed by *in silico* tools until the most promising candidates for synthesis are found. In drug design, the information about molecular fragments and their activities is used to combine them automatically into new molecules.[54] Such concepts could also be used to design new mineralizable molecules based on known rules and molecular fragments, which improve environmental degradability.[10,16,17,55] They may become apparent when using nontargeted approaches or scanning large databases and could subsequently be used for a rational benign design. In combination with mechanistic knowledge about the desired effects, experts may create molecules even from scratch for further *in silico* investigations. Marano[39] designed new molecules, which were inspired by the structure of 3-ß-D-galactopyranosyloxymethyl-4-sulfatomethylfuran (GSF). Different molecular fragments were combined and screened for activity via molecular docking and biodegradability via *in silico* models in parallel. The most promising candidates have been synthesized and have shown improved activity by a factor of >500, and its biodegradability nearly doubled compared to the starting compound GSF.[8] Furthermore, Zumstein and Fenner[17] showed certain possibilities for the design of peptide-based antibiotics, which are stable during treatment but rapidly deactivate by peptidases in wastewater treatment.

Nontargeted Redesign. Examples for the nontargeted redesign of pharmaceuticals are given by Rastogi et al.[13−15] They changed the structure of known compounds (β-blockers) via nontargeted synthesis. Therefore, photolysis in an aqueous medium was applied to generate many new derivatives (transformation products). Through a combination of biodegradability testing of the photolysis mixture and LC-MS/MS analysis, derivatives with improved environmental biodegradability were identified that still contained the indispensable lead structure for pharmaceutical activity. These were then further assessed by employing *in silico* tools (docking, (quantitative) structure−activity relationship models ((Q)SAR)) to identify the most promising candidates for activity, favorable ADME properties, and environmental biodegradability. Either experimental methods like the ones shown by Rastogi et al.[14,15] or *in silico* tools for the prediction of potential transformation products can be used for the nontargeted redesign.

Targeted Redesign. For the targeted redesign, structural fragments, which are known to improve the environmental degradation and mineralization[10] and/or to lower the toxicity,[55] are integrated into a known chemical structure. These fragments could, e.g., be ester-linkages[56] or biobased fragments like amino

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acids.[12,57] For the assessment of the possibly improved properties, either *in silico* or experimental methods can be used, depending on whether the structural fragments were incorporated into the parent compound's structure *in silico* or via targeted synthesis. Kümmerer et al.[38] used ciprofloxacin (an antibiotic from the group of fluoroquinolones) as a model compound. They changed its structure at well-defined points in a way that preserved the core structure being responsible for its pharmaceutical activity, while incorporating degradable or cleavable side chains (linked, e.g., via ester or amid bonds) based on knowledge about electronic and steric properties. The goal was to generate derivatives, which are stable enough for application but easily cleaved after excretion into less active and biodegradable fragments. Therefore, the molecular structure was designed to be stable under physiological conditions but degraded at lower pH values. The derivatives were first developed *in silico* by combining different fragments on the basis of expert knowledge with the model compound ciprofloxacin and the whole class of fluoroquinolone antibiotics. After the generation of different new molecules, they were screened via *in silico* tools and some promising candidates were further synthesized; their properties and activities were validated by experimental methods.

IN SILICO TOOLS: AVAILABILITY AND APPLICABILITY FOR BbD

The term *in silico* tools or *in silico* methods refers to (Q)SAR models, quantitative structure−property relationship (QSPR) models, expert rule-based models, grouping, docking, and read-across techniques[19,58] as well as modeling the fate and transportation of chemicals in the environment.[59,60] Models for transportation and fate (like the fugacity model Levels I, II, and III[61]) will not be discussed in this study, since they do not play a major role in the context of BbD.

(Q)SAR, expert rule-based and read-across models relate chemical representations of molecules to their properties or activities.[19] This leads to a (statistical) model that can find patterns in a given data set related to a specific property. The gathered information and correlations can further be used to predict unknown properties of chemicals. The parameters, which are used to represent the molecules *in silico*, are called (molecular) descriptors and are used as independent variables in the model development process.[20,62] A comparison of *in silico* tools based on (a) the algorithms and methods used to generate a prediction model and (b) the types of descriptors can be found in Tables S1 and S2.

Before the *in silico* assessment of the generated pool of molecules (also called "query chemicals"), some preliminary steps are necessary:

(1) Providing the structure of a candidate and a suitable chemical representation
(2) Defining the end points of interest
(3) Choosing the appropriate models with substances in the applicability domain (AD)
(4) Choosing the consensus approach for evaluation
(5) Preparing the (Q)SAR prediction reporting format and data storage

In the following section, they will be discussed in more detail against the background of BbD.

Chemical Structure. Basic requirements for the application of *in silico* tools are a known molecular structure and its representation, usually as a SMILES code. However, each computational representation of molecules lacks some information (like stereoisomerism) that could be important for the actual behavior of a molecule. Depending on how the structure of interest was derived (*in silico* or via, e.g., LC-MS analysis), information about possible enantiomers is sometimes missing. This should be kept in mind when it comes to the evaluation of biological activities or properties and when it comes to the synthesis and *in vitro* confirmation of the *in silico* predicted properties and activities of the query chemical.[48]

Defining End Points of Interest. Guidelines for REACH,[40,41] the PBT assessment,[42] and the ICH M7 guideline[43] discuss properties and activities of a chemical that should be considered when chemicals are assessed regarding their environmental fate, effects, and safety. Moreover, these discussed end points are also relevant in the context of BbD, like toxicity, ecotoxicity, degradation, and the bioconcentration factor. In addition, physicochemical properties like the *n*-octanol/water partition coefficient and soil adsorption coefficient give information about the behavior in the environment. The selection of the end points for BbD depends on the desired function and life cycle of the target substance (e.g., pharmaceutical or pesticidal activity, surfactant, flame retardant, plasticizer). Specifically, additional end points may be relevant. For example, ADME properties should be considered for a pharmaceutical's development.[63,64]

In the context of BbD, the assessment of the full mineralization by biotic or abiotic processes is crucial. If the substance is designed for rapid degradation into inorganic products (like water and carbon dioxide), end points for ecotoxicity become less important since the chemical or its TPs cannot elicit adverse side effects.[65] If a chemical is not mineralizable in the environment, ecotoxicity end points and bioaccumulation become relevant. Of course, during their application, all chemicals should be nontoxic. This applies in particular to carcinogenic, mutagenic, reprotoxic (CMR) activities and endocrine disruption. The relevant end points are listed in Table S3.

The prediction of the biodegradation of chemicals by *in silico* tools may be quite challenging. So far, models for physicochemical properties show a better performance than models for toxicity and biodegradation. It is much easier to represent physio-chemical properties in a model than more complex ones where uptake, transport within organisms and cells, the presence and activity of enzymes, and stereochemistry play a role.[58,66] Such complex end points are, e.g., (chronic) toxicity, teratogenicity, the prediction of drug metabolites in the human body,[67] and biotransformation pathways.[68]

The experimental (bio)degradation data for developing these models, if determined by a specific *in vitro* method, should be generally comparable and reproducible since the specified microorganisms are "prepared" according to the same protocol and added in a defined concentration to the test solutions.[66] However, these methods are also subject to uncertainty.[27] As for the understanding of biochemical pathways, including microbial biodegradation of test compounds, single-strain cultures selected by experimental conditions are often employed under optimum conditions. However, biodegradability in the environment depends on the absence or presence and competitiveness of such degraders. Biodegradability by microorganisms, for example, depends on their diversity, including the enzymatic diversity and number present. As such tests are biological systems, there is always some uncertainty compared, for example, to single-strain/single-substrate tests or physicochem-

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ACS Sustainable Chem. Eng. XXXX, XXX, XXX−XXX

ical end points. Furthermore, it is also noteworthy that biodegradability tests are performed with relatively high concentrations as they would not occur in the environment. Therefore, microorganisms involved in the degradation process may use other more easily degradable compounds as a carbon source.[41]

Hence, the development of reliable prediction models for environmental biodegradability of molecules is quite challenging due to the inhomogeneous raw data.[69] Biodegradation data used to build a model should be generated within a specific test guideline using the same inoculum source and should not be mixed. Such data sets are too scarce and sometimes consist of too few substances to build reliable models.[66] In other words, more experimental testing is needed to allow for better models and, therefore, better predictions.

Due to the discussed limitations, it is important to be aware of the challenges when it comes to the prediction of biodegradation and mineralization. This knowledge should be used in the selection of *in silico* models and the interpretation of their results. Until better models can be provided, confirmation by experimental testing is highly recommended. However, the *in silico* tools are extremely helpful for a preselection of promising candidates.

If the substance is fully mineralized in the environment, there may be no need to extensively check for its ecotoxicity and behavior in the environment. However, if the substance is not entirely eliminated but just deactivated by primary elimination, its transformation products and metabolites should be checked for their ecotoxicity and behavior in the environment.

Selecting Appropriate Models. Several models have been developed for the prediction of (eco)toxicity and (bio)-degradation as well as for physicochemical properties (Table S3). Factors that influence the choice of models are their availability, the straightforward interpretability of predictions, their AD, and reliability. Some models are freely available, while for others a license needs to be purchased. When a specific model is chosen, it is crucial to understand its characteristics (Tables S1 and S2) and how to interpret the predictions. For example, some models predict a classification or categories based on ordinal values (e.g., GHS classification), while others calculate continuous values.[58,70] In addition, some models are specialized for certain groups of substances (like pharmaceuticals) regarding their training set data. Therefore, the AD should be checked for every query substance to ensure the reliability of the prediction.[65,71] For specific substance groups, i.e., polymers, ionic liquids, salts, mixtures, nanoparticles, and organo-silicon compounds, it is difficult to find appropriate models.[27,72] This is partly due to the limited experimental data availability and quality.

Thus, it is recommended to avoid "black box" approaches, which give little information about the underlying methodologies and data basis.[73] Selected models should give detailed background information on the algorithms, training set data, weight of the descriptors, end point, and mechanistic rationale, and additionally, they should check the reliability of the prediction and if the query substance is within the AD.[23,27] If the model does not automatically check the AD, the ECHA provides some elements that can be reviewed to identify if the query substance falls within the AD of a certain model.[21] In addition, the validation criteria for (Q)SAR models can be checked for selecting appropriate models.[21,23] These are (1) a defined end point, (2) an unambiguous algorithm, (3) a defined applicability domain, (4) appropriate measures of goodness-of-fit, robustness, and predictivity, and (5) a mechanistic interpretation if possible.

Depending on the chosen design approach and the information needed, some models might be more helpful than others. Structural alerts, 3D-(Q)SAR, or docking may be beneficial if the gained insights will be directly used for further design decisions. If a high number of structures should be screened to identify the promising ones, descriptor-based (Q)SAR models or rule-based models may be helpful (Tables S1 and S2).

It is also important to keep in mind that different types of models result in different types of outputs. While descriptor-based (Q)SAR models provide a continuous, ordinal, or categorical result related to the whole molecule, they give only little insight into the underlying reaction or the mode of action. Therefore, they may be accurate while providing less helpful information when it comes to the molecular design. Fragment-based (Q)SAR, in contrast, can provide insights into the responsible fragments for a specific property. While interpreting the results of SA or rule-based models, it is important to know that the pure absence of a structural alert does not necessarily indicate that this substance is, e.g., nontoxic or biodegradable. If no alert is indicated by the model, this could also be due to a lack of structural alerts or rules in the model itself, i.e., a gap in the AD or low chemical diversity with respect to the feature searched for, which therefore will result in false negative predictions (Tables S1 and S2). Furthermore, it is important to mention that most models do not include alerts for nontoxic SAs.[31] This makes it even more important to find a well-balanced model, which is not too unspecific (because it could raise false-positive results) but also not too insensitive and therefore raises false-negative results.

The majority of free and commercially available models for biodegradability predictions rely on structural alerts or descriptor-based (Q)SARs (Table S3). Such models often try to find the most generalized relationship between the used training data and their given properties. Such generalizations can be misleading or unsatisfying if it comes to the optimization of molecules via small changes in the structure or the identification of so-called activity cliffs.[74,75] Matched molecular pair analysis (MMPA) is a particular case of (Q)SAR and is not based on the similarity principle.[76,77] Even though MMPA has only been used in drug development, not including environmental properties, so far, it could also be effectively used to design environmentally benign chemicals and pharmaceuticals. One of the most important applications of MMPA in the context of BbD could be the identification of outliers or activity cliffs since BbD approaches can be based on small changes in a molecule's structure, which lead to a significant change in its behavior. Such effects may be ignored in a generalized (Q)SAR model. It could also be used to obtain rules of thumb by scanning large databases, which then could be further used to guide structural improvements.

In the past few years, molecular docking for modeling biodegradability emerged.[78,79] The review of Liu et al.[78] demonstrated that molecular docking is a promising approach for *in silico* biodegradation investigations. Since it expresses the interaction between molecules and enzymes, it can help to analyze enzymatic reaction mechanisms as well as those for biodegradation in the environment, which play a crucial role in the context of BbD[78] (Table S2). Even though it can help to gain further insights into the enzymatic degradation reactions, it has some limitations. Since it is a highly specific investigation, it can be time-consuming. For each class of substances, which needs to

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ACS Sustainable Chem. Eng. XXXX, XXX, XXX−XXX

be predicted, the possible enzymes for degradation need to be known, making a high throughput screening or the investigation of new molecules challenging. As for the consideration of whether this will happen in the natural environment, knowledge on the abundance of specific enzymes and their specificity and activity have to be known. Nevertheless, there are microorganisms that are known to be able to convert and decompose several organic compounds due to the secretion of a variety of enzymes.[78] This could be used in a test battery if no detailed information is known. However, it is important to keep in mind that molecular docking predicts only the binding affinity of one molecule to a specific protein. A high binding affinity to a potentially degrading enzyme does not necessarily mean a high mineralization rate. Therefore, further studies are needed to fully understand the potential of molecular docking for biodegradability prediction.

Examples of selected models sorted by end point and software packages for the *in silico* assessment are given in Table S3. In addition to Table S3, the ECHA guideline for QSARs and grouping of chemicals offers an overview of specific software and models.[20] Furthermore, databases like the QSAR DataBank Repository or the Online Chemical Modeling Environment (OCHEM) contain different validated *in silico* models with detailed information about the input data, descriptors used, output formats, and ADs.[80,81] Available models for selected end points are also listed on the ANTARES web page.[82] Further information on models and their assessment is compiled in (Q)SAR model reporting formats (QMRFs), guiding practitioners in selecting the appropriate model. Some are accessible via the JRC QSAR model database.[83]

Many studies compare the performance of different models for specific end points.[69,84−92] Benfenati,[58] for example, illustrated the accuracy of four end points showing that models for carcinogenicity have a large error, while models for aquatic toxicity, mutagenicity (based on the Ames test), and the bioconcentration factor gave good results.

Even if models yield good predictive results for the discussed end points, they are still limited due to their characteristics (Tables S1 and S2) and their AD. Therefore, the ICH M7 guideline for predicting mutagenicity recommends using at least two independent models, in that specific case, one statistical and one rule-based model.[43] Similar approaches are needed for biodegradability prediction. Benfenati et al.[27] suggest overcoming this limitation by combining the predictions of different models and techniques. The ECHA recommends "run[ning] all (Q)SAR models available...especially when models are independent of each other".[21] Models count as independent if they are based on different algorithms, descriptors, or training sets. When different models are applied for the same end point ("*in silico* test battery"), a better reliability of the prediction results can be achieved.

There are several examples, which show that the combination of different *in silico* approaches, also called hybrid models, can lead to an even better understanding and prediction of certain properties.[58,90,93,94] Data mining approaches like clustering could be used to identify similar molecules[46] in a large data set for further usage of those molecules within one cluster for read-across approaches or the development of local (Q)SARs. There are also examples where the output of molecular docking with certain degrading enzymes was used to create 3D-(Q)SAR models.[93] Sushko et al.[94] used a combination of (Q)SAR and MMPA approaches to find significant single point transformations that lead to a change in behavior based on the (Q)SAR prediction of a large chemical data set. This so-called "prediction-driven MMP analysis" helped to improve the usually poor interpretability of (Q)SAR models.

Evaluation. After the *in silico* assessment, it is important to carefully evaluate the predictions of different models for the same end point as well as the results for different end points and their interplay. The combination of different prediction results for the same end point can avoid statistical problems as "it is hard to find the best model but it is more likely to find some good models",[27] and it may avoid over- or underestimation. As Valsecchi et al.[95] showed, consensus strategies seem to be more accurate and can cover the chemical space better than individual models. Such consensus approaches are already integrated into some software. Ballabio et al.[90] combined predictions from eight different models by different consensus approaches. They showed that the application of those consensus methodologies resulted in a reduction of uncertainty.

If there are multiple predictions for the same end point, several consensus approaches can be used. If the predictions of different models coincide, usually no further evaluation is needed. The only important factor to consider in that case is the reliability of the models. If one of the predictions is reliable, all others will contribute to the overall level of confidence.[27] If the predictions contradict, the results should be carefully assessed, including data (quality and source) of the various models and their AD. To gain a confident and robust conclusion based on those predictions, different strategies can be used:

(1) If it is possible to perform experiments for the same end point (maybe also mixture testing should be considered if the single substance is not available), those results could help to clarify the contradicting predictions.

(2) Statistical approaches or even hybrid models that automatically compare the outcome of different single predictions could be applied. There are different methods for integrating prediction results from individual models, like (a) voting methods; (b) weighting methods; (c) hybrid methods; (d) learning methods. All those methods are described in detail by Benfenati et al.[27] To choose one of those methods, it is important to decide (a) if all the results from all the used models should be treated as equally good; (b) if the models are equally reliable; (c) how conservative the prediction should be.

(3) If it is helpful to gain further insights and use the information for further design decisions, it is recommended to perform an expert review "by hand".[27,96] One important step is to incorporate expertise (e.g., on biodegradability) and data from analogs outside of the model, which may have, for example, the same SA but were already tested. This may also be relevant if there are no conflicting outcomes of different models because it can help to gain further mechanistic knowledge. There are some tools that can help search for analogs, like in CASE Ultra or the OECD QSAR Toolbox. For further information about the implementation of expert reviews, we recommend the work of Amberg et al.,[96,97] who discussed the use of expert reviews for the prediction of mutagenicity according to the ICH M7 guideline. They have shown that an expert review can improve *in silico* predictions and discussed procedures for integrating out-of-domain or indeterminate prediction results in such an expert review.

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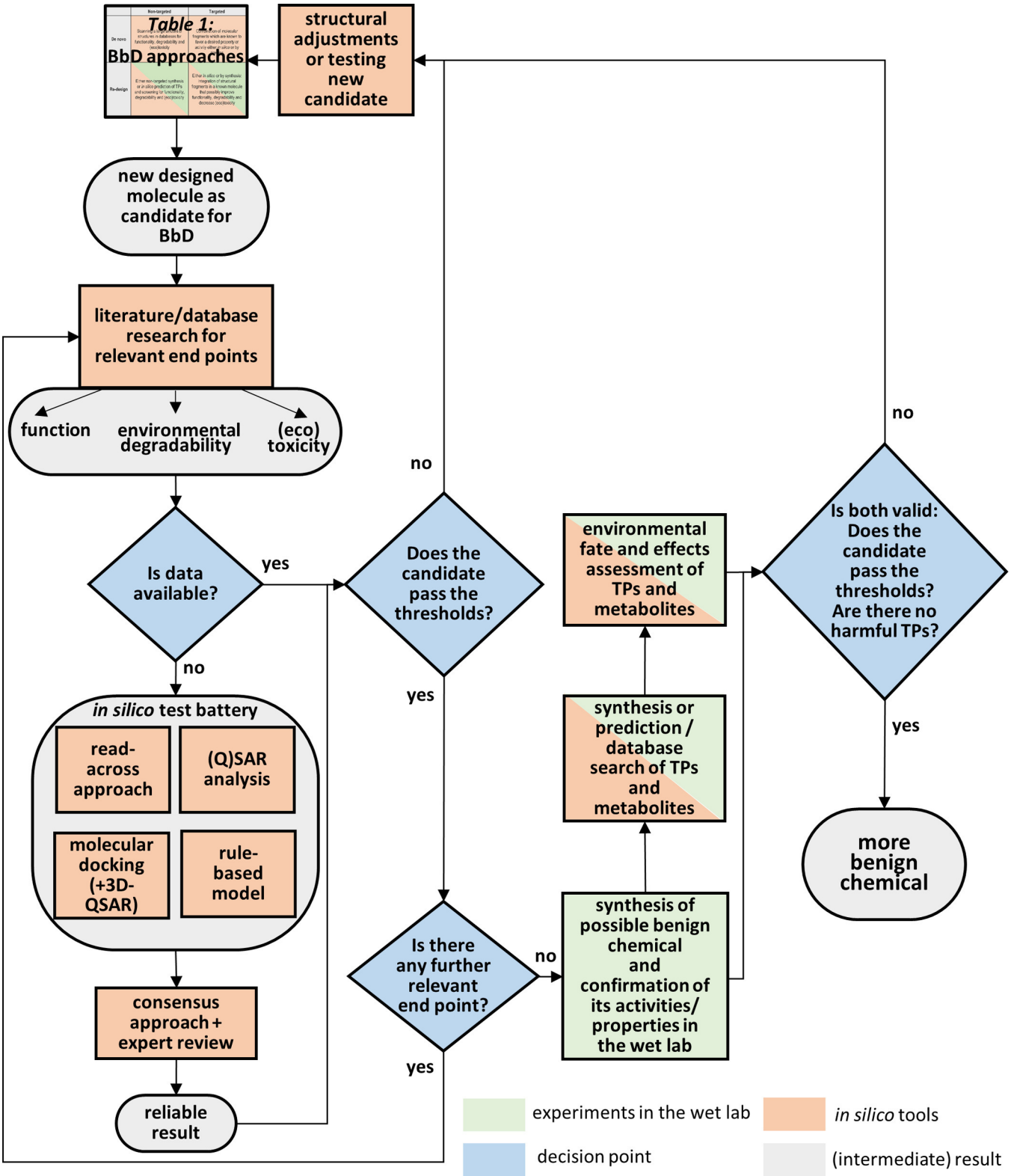

Figure 2. Workflow for the *in silico* assessment of newly or redesigned molecules according to the presented BbD approaches. It starts with the design of new molecules, followed by literature research and an evaluation with the help of a before defined *in silico* test battery. After the identification of promising candidates, they should be synthesized and the results should be verified by experimental methods also in regard to their possible transformation products (TPs). Boxes colored in both amber and green: practitioner needs to decide for *in silico* tools or experiments in the wet laboratories.

Data Management. After every prediction step in the *in silico* assessment and design process, the procedure and results should be recorded for transparency reasons, mainly, but not only, if they are later used for regulatory purposes. Therefore, the ECHA presented a QSAR Prediction Reporting Format (QPRF), which is used in the context of REACH registrations.[20]

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It is also recommended to store the results in an accessible database to get back to the results if needed or use them later for other purposes like filling data gaps or making different design decisions. Therefore, it is important to implement a clear data structure for storing the results internally. They will probably come from various models and software and will be in different data formats containing different kinds of information, which should be practically harmonized.

THRESHOLDS FOR MAKING DECISIONS: WHEN TO CONSIDER A NEW MOLECULE AS A POSSIBLE BENIGN CANDIDATE

Depending on the model type, the predictions can be classifications or continuous values. In both cases, the user needs to evaluate the prediction and draw conclusions if the candidate is possibly environmentally benign. Classifications could indicate, for example, if a substance is readily biodegradable or not. In either case, the meaning of the classification should be looked up in the model's manual. In order to make decisions whether a molecule can be considered as a promising candidate for a benign chemical, thresholds for specific end points, based on the ICH guideline[43] and ECHA guidelines,[40−42] are proposed. The thresholds have also been expanded regarding the principles of BbD.[45,48] In general, if the molecule is predicted to be fully mineralizable and nontoxic, it can be suggested as a candidate for synthesis to perform experimental tests in the wet laboratories for confirmation.

(1) The substance's degradation in the environment should not result in persistent products of incomplete degradation (transformation products). It should be fully mineralizable. Depending on the biodegradability test method used for generating the training set of the model, a substance is classified as readily biodegradable if it is degraded by ≥70% (removal of dissolved organic carbon (DOC)) or ≥60% (measured as theoretical oxygen demand (ThOD) or theoretical carbon dioxide ($ThCO_2$)) in ready biodegradability tests.[42,98] These pass levels indicate mineralization.[41]

(2) There should be no indications of CMR activities. The substance should be classified as class 4 or 5 according to the ICH M7 guideline.[43]

(3) The activity or functionality should be better or at least in the same range as comparable and already used substances.[45,48]

(4) The substance should show as much stability as needed for the storage and application under the existing conditions but not more.[45,48]

(5) The substance should not be bioaccumulative in the environment. Substances with a log $K_{ow} \leq 4.5$ are classified as not being bioaccumulative in aquatic organisms.[42] A substance is potentially bioaccumulative in air-breathing organisms if its log K_{oa} is >2 and log K_{ow} is >5.[42]

(6) The substance should not be toxic and should not show any chronic toxicity effects (in the environment). In the absence of chronic toxicity data, a substance is potentially toxic if its EC_{50} or LC_{50} for short-term aquatic toxicity (algae, daphnia, fish) is <0.1 mg/L.[42] It is also recommended to test for aquatic ecotoxicity for at least three trophic levels (algae, invertebrates, fish).[40]

APPLICATION OF *IN SILICO* TOOLS WITHIN BbD APPROACHES

After one or more molecular structures have been designed according to the presented approaches in Table 1, the workflow in Figure 2 can be applied to check if the molecules provide the intended features (i.e., biodegradability and no (eco)toxicity) while their function is ensured.

The newly designed molecular structure, no matter if it originates from targeted or nontargeted (re)design, be it experimentally or by computational structure variation, needs to be tested *in silico* against specific BbD end points, i.e., needed for function and environmental mineralization. These end points should be defined beforehand. The relevant end point categories are the function, (eco)toxicity, and environmental degradability (Figure 2). Concerning the context-dependent needs, the practitioner needs to decide on the order of the end points, whether function and (absence of) toxicity or environmental degradability should be applied as the first filter criteria.

Functions are usually handled as the most important and, therefore, the first decision criterion in *de novo* design. In the case of redesign, these are at least roughly known, and the question then is to optimize first the environmental profile and second the functional profile or vice versa. If a molecule does not present the necessary properties for function, there is usually no need to further assess its environmental properties but just adjust the molecule's structure until it fulfills the first requirements. However, in the context of BbD, biodegradability in the environment is of high importance and should be considered already in the early stages of the development as later adjustments may cause delays and additional costs. Furthermore, the inclusion of environmental biodegradation from the very beginning in the design may bring new functional groups into view, which could also improve functional properties but could have been overlooked before.

Regarding the targeted design of molecules and the purpose of getting more insights on designing greener chemicals, all the end point categories should be assessed in one course. Nevertheless, also here, the substance can be tested in a defined order of end points until a predefined break point is reached (i.e., a specific criterion was not fulfilled or not at the desired level). In such a case, the evaluation process could be stopped, and the molecule should be (slightly) adjusted according to the insights gained hitherto. After that, the *in silico* assessment of the adjusted molecule starts from the beginning. This process could be quite challenging since structural modifications, which lead to less toxic or better biodegradable molecules, could be unsatisfactory in other respects, such as performing less, being less potent or efficacious, or being relatively unstable at certain life stages where stability is needed. However, in the latter case, one should not think in terms of stability but in terms of half live needed, i.e., kinetics in dependence of the physical−chemical (e.g., pH, presence or absence of light or water) or microbiological constraints. Therefore, the process of adjusting the molecular structure and evaluating the properties of the new candidates might be repeated multiple times until a new molecule with well-balanced properties is found. Such an optimization circle could be applied several times, depending on the degree of improvement wanted and resources, such as time and money, available. At any point of this approach, the collected information about the structure−activity relationships related to the properties needed for application on the one hand and environmental degradation on the other can be used to gain new insights into

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the design rules of benign molecules and should be documented anyway. It should not be seen as a waste of time and money but rather as a basis or even a treasure for future activities.

For nontargeted design, a (large) pool of different new molecules is generated or accessed in databases, which should be screened for those with improved environmental degradation and decreased toxicity. In this case, specific criteria for end points can be used to exclude molecules from further assessment. Therefore, for nontargeted design, the workflow is less of a loop than a filter, which starts with many new molecules and results in some promising candidates, be it experimentally or computationally generated ones. However, even on the basis of rejected candidates for BbD, rules of thumb for the design can be derived from this and incorporated directly into the design decisions in targeted approaches.

Starting with a newly designed molecule as a candidate for a benign chemical, the first step in the workflow (Figure 1) is to check the literature and databases[99,100] for relevant information regarding the end points that cover function, (eco)toxicity, and environmental degradability. If data for one or more end points are available in the literature, the above-summarized thresholds need to be checked, and either the candidate is rejected to make structural adjustments or the practitioner has to decide if additional end points need to be considered.

If no data is available in the literature and databases, the next step could be searching for analogs of the query chemical and available information regarding their biodegradability and other relevant end points. There are several databases available that contain experimental data for (bio)degradability (like the OCHEM[81] or Biodegradation NITE[101] databases) and (eco)-toxicity[99,100] end points. They could be used to gather data about similar substances to perform a read-across analysis.[102,103]

If enough analogs with known properties were found, those read-across results could be further used to incorporate them with other results later in the consensus approach and expert review. It would also be possible to perform a MMPA with the gathered information (with tools like OCHEM). Even though those results would not necessarily be used to assess a specific query chemical, the MMPA can provide further insights into responsible groups for biodegradability, and therefore, the results can be later used for rational design decisions.

If information about potential degradation enzymes is available, it would also be an option to perform molecular docking to assess the potential biodegradability of the query chemical, as shown by Han et al.,[93] who also incorporated the docking results of similar compounds into a local 3D-(Q)SAR model to gather even more information.

The next step, or if no analogs have been found, is the analysis by (Q)SAR models and rule-based models. To incorporate all the predictions into one final result, a consensus approach and/or an expert review should be performed, as already explained, to improve the confidence of the predictions and gain even more information about the behavior of the query chemical. The whole process ends up in one reliable result, which decides if the query chemical will be omitted or further evaluated or if its structure will be adapted.

If all relevant end points have been assessed and comply with the defined thresholds, i.e., the molecule is biodegradable, neither bioaccumulative nor (eco)toxic, and its function is ensured, it should be synthesized to perform experimental tests in the wet laboratories to confirm the predicted activities and properties. In addition, if the designed molecule will enter the aquatic environment after the intended use (e.g., pharmaceuticals), it is important to identify and assess (human) metabolites as well as any other (environmental) TPs by either *in silico* tools or *in vitro* tests in order to prevent the accumulation of harmful TPs in the environment.[33,34,36]

There are no case studies published yet that applied the whole workflow as presented in Figure 2. However, there are some studies, which served as a basis for the development of the herein presented workflow. Boethling[11] has shown how predictive models can be included in the rational design of musks with improved environmental attributes. Boethling incorporated experimental data from databases and predicted values for the likelihood of biodegradation, the octanol/air partition coefficient, bioaccumulation factors, and acute ecotoxicity (PBT properties) to compare different musks and get insights about their structure−property relationships. Those insights lead to the identification of musk-types with the best environmental properties overall and can be further used for the rational design. This case study did show how useful *in silico* tools can be to screen substances in regard to their environmental attributes and that the biodegradation data and related structures are consistent with already known rules of thumb.[10] Rastogi et al.[13−15] used different *in silico* tools for the development of their β-blocker alternatives. After their nontargeted redesign in the wet laboratories and the screening of the substances for biodegradability, they applied QSAR models for biodegradation to identify the responsible structural alterations in those molecules. Furthermore, they applied tools for the prediction of ADME properties, mutagenicity, and docking tools to assess the biodegradable derivatives of the redesigned β-blockers further. Leder et al.[104] published a study about a fluoroquinolone with improved environmental properties, based on the redesign of ciprofloxacin. They started with the *in silico* targeted redesign based on expert knowledge as described in Table 1. After the generation of different possible molecules, they evaluated them further via *in silico* tools (QSAR models for biodegradation, ADME properties, toxicity, molecular docking) and synthesized the most promising candidates for experimental evaluation. Even though none of them published their *in silico* workflow in its entirety, they have shown that *in silico* tools can support the successful development of derivatives with improved environmental properties and help to learn more about the underlying structure−activity relationships.

LIMITATIONS AND CHALLENGES IN THE APPLICATION OF THE *IN SILICO* WORKFLOW FOR BbD

When it comes to the interpretation and usage of different *in silico* tools, some challenges and limitations appear.[105] Regarding the model selection, the practitioner could sometimes be faced with nontransparent information about descriptors, algorithms, predictive power, applicability domains, or external validations ("black boxes"). Depending on how the training data is presented, it can become quite challenging to overview the used data. In any such cases, it is important to decide if a model still can be applied in a certain context and how reliable the predictions can be.

The results of any *in silico* assessment should never just rely on pure statistics since the reasoning should not be based on correlation alone.[106] The same holds true for rules as new rules could be hidden and a molecule is always more than just the sum of its functional groups or structural alerts. Statistical models, for example, try to identify fragments (often functional groups) found in most of the molecules that display a particular property

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ACS Sustainable Chem. Eng. XXXX, XXX, XXX−XXX

related to them from the training set. However, these fragments may also be "coincidental features", which do not describe the actual mechanism for the activity. Other fragments modulate them as for the strength of an effect of assertion of a property wanted or not wanted, e.g., by steric or electronic effects.[96] This makes a fundamental mechanistic understanding of the practitioner and an expert review indispensable.

After a successful *in silico* assessment and the investigation of promising candidates, it may still be challenging to synthesize these substances for further testing. Such considerations should also be kept in mind during the design process.

The discussion above reveals that, for the BbD process, knowledge in the application of *in silico* tools, function, properties and activities of chemicals in the environment, and chemical synthesis is needed. This emphasizes an interdisciplinary approach to incorporate knowledge from different fields.[107] Furthermore, it becomes more and more important to train young professionals in sustainable chemistry and application of *in silico* tools for the chemicals' assessment in the context of BbD.[108−110]

Nevertheless, it is essential to keep in mind that there are no green chemicals per se since there are no absolute rules on how benign a chemical should be. To find a more benign and more sustainable solution, it is always necessary to compare different alternatives and their properties in a given context to find the best solution. In addition, green chemistry metrics could also support the decisions for greener alternatives.[111,112]

CONTRIBUTION TO THE CHEMICALS STRATEGY FOR SUSTAINABILITY

The EC's chemicals strategy for sustainability foresees to act in accordance with safe and sustainable-by-design, zero chemical pollution in the environment, and innovative tools for safety testing and risk assessment to reduce animal testing.[5] The presented workflow for BbD combines both the application of innovative tools such as *in silico* methods and the development of safe and environmentally friendly chemicals, consequently supporting the implementation of this strategy and sustainable chemistry. In this regard, a higher priority for environmentally mineralizing chemicals prevents the generation of TPs. Therefore, the savings of subsequent extensive *in silico* or *in vitro* or even *in vivo* experiments for risk assessments of a large number of (possible) TPs is also saving money and time to market.

The great advantage of *in silico* tools is that only the chemicals' structure is needed for the fast assessment of newly designed chemicals without synthesizing them. This eliminates the unnecessary use of resources and waste related to the synthesis and experimental testing of compounds that could fail. In addition, *in silico* tools are less time-consuming and cheaper than experimental testing and animal testing, including workforce and regulations to be met. This contributes to the aims of the chemicals strategy for sustainability as well.[5] The generated data can even be used to register chemicals under REACH, if certain conditions are fulfilled[21] or at least to fill existing data gaps of chemicals and TPs.

CONCLUSION

The development of mineralizing chemicals according to BbD is of importance to minimize the environmental pollution with chemicals, which necessarily end up in the environment since they cannot be circulated in a closed system. These chemicals should be designed to mineralize completely either in water treatment processes or preferably even in surface water in the absence of effluent treatment. For the first time, a generic workflow has been developed to combine BbD approaches with *in silico* tools to enhance the successful application of these tools. The implementation of this workflow will help practitioners who have no, little, or less experience in applying *in silico* tools to better understand how to evaluate properties and activities of newly designed chemicals to implement those insights into the design of benign and green molecules. As of its character, BbD is an interdisciplinary approach and needs the cooperation of chemists, pharmacists, computer scientists, and the chemical and pharmaceutical industry. The workflow will help all involved parties understand which decisions need to be made and how *in silico* tools should be applied for assessments in the design process. Thereby, this study demonstrates how to implement BbD in the context of the new "Chemicals Strategy for Sustainability Towards a Toxic-Free Environment" developed by the EC. It contributes significantly toward a toxic- and pollution-free environment. The systematic investigation of new molecules, developed either by target or by nontarget design, could generate a lot of data, mainly if *in silico* tools are used. Therefore, new insights into designing rules for benign molecules may be obtained. Those may help in the target design and the extension of already known rules of thumb. In addition, the regulatory acceptance of the generated data for the registration of chemicals according to, e.g., REACH after their design is supported when the assessment considers the mentioned QPRF and specified criteria for models and the prediction results. Hence, effort and time for collecting the needed data for registration are decreased when considering these points directly in the assessment. To fully develop the potential of *in silico* tools for the assessment of chemicals in the context of BbD, future research should address improving the software and the data quality, number, and availability for the models' training sets, especially for biodegradation models, as well as the easy interpretability of the predictions. Further research should come up with best practice examples to demonstrate the feasibility and advantages of the BbD workflow.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c03070.

Additional information on *in silico* tools, models, and relevant end points for BbD (PDF)

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ACS Sustainable Chem. Eng. XXXX, XXX, XXX−XXX

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ACS Sustainable Chem. Eng. XXXX, XXX, XXX−XXX

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■ ABBREVIATIONS

AD, applicability domain; ADME, absorption, distribution, metabolism, and elimination; BbD, Benign by Design; CMR, cancerogenic, mutagenic, reprotoxic; DOC, dissolved organic carbon; EC, European Commission; GSF, 3-ß-D-galactopyranosyloxymethyl-4-sulfatomethylfuran; MMPA, matched molecular pair analysis; PBT, persistent, bioaccumulative, toxic; QPRF, QSAR prediction reporting format; (Q)SAR, (quantitative) structure−activity relationship; ThOD, theoretical oxygen demand; TPs, transformation products; ThCO2, theoretical carbon dioxide

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Inventory of biodegradation data of ionic liquids

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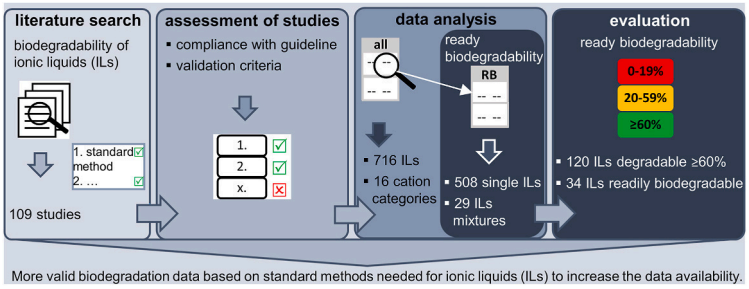
HIGHLIGHTS

- Review delivered 109 ILs biodegradation studies that applied standardised methods.
- Methods were evaluated on compliance with the guidelines, validity of the data.
- Biodegradation data on 716 different ILs including 29 ILs mixtures were provided.
- 34 ILs classified as readily biodegradable in accordance with the guidelines.
- More valid ILs biodegradation data based on standard methods needed.

GRAPHICAL ABSTRACT



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ABSTRACT

Ionic liquids (ILs) are increasingly of interest for environmentally open applications. Therefore, completely mineralising ILs are highly desirable. We reviewed the current state of knowledge on ILs' environmental biodegradability and identified research needs. Literature data were evaluated as for applied standard methods (e.g. OECD, ISO, APHA) for biodegradation of ILs in order to get an overview on the validity of the test results received and ILs' biodegradability. 109 studies were evaluated. The ILs were categorised based on the cation's core structure. The biodegradation data was classified according to a traffic light system (red: 0–19% degradation, amber: 20–59% degradation, green: ≥ 60% degradation). Not all studies could be assessed for compliance with the test guidelines due to missing test parameters. Moreover, no study discussed all validation criteria as defined by the test guidelines. Consequently, the reliability and quality of the existing biodegradation data is restrained. With regard to the different cations classified for ≥ 60% biodegradability, phosphonium ILs are the least biodegradable, followed by imidazolium ones. The most ILs that were biodegradable are cholinium ILs. The results indicate the need for more and qualitatively better testing according to standard methods including application and reporting of all validation criteria in order to get reliable data that enables the comparison of the test data and a comprehensive understanding of ILs' biodegradability. Moreover, reliable data allows the selection of sufficiently environmentally biodegradable ILs if an introduction into the environment during use cannot be excluded.

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1. Introduction

Ionic liquids (ILs) are generally defined as salts composed of an anion and cation with melting points below 100 °C (Gutowski, 2018; Ludwig et al., 2012). Often used cations are quaternary alkyl ammonium, imidazolium, phosphonium or pyridinium. Inorganic anions comprise halides, sulphates or hexafluorophosphate. Organic anions are alkyls ulphates, trifluoromethanesulphonate, bis(trifluoromethylsulphonyl) amide, tosylate or dicyanamide (Gutowski, 2018). In addition to single ILs compounds that consist of one specific cation and anion, the interest in binary and ternary mixtures of ILs, e.g. combinations of different cations and anions, has risen as they provide different physico-chemical properties than the single ILs compounds and can therefore be designed or adapted to the specific application (Chatel et al., 2014; Niedermeyer et al., 2012). Since recently, applications are under discussion that do not solely include closed-loop industrial processes, where ILs could be reused, but applications that allow ILs to enter the environment unintentionally during use or at the end-of-life, such as applications as active pharmaceutical ingredient (API), herbicide, surfactant, antistatic agents in resins, coatings, and cleaning agents and as ingredient in personal care products and household cleaning agents (Choudhary et al., 2017; Czuryszkiewicz et al., 2019; Ferraz et al., 2011; Gutowski, 2018; Moshikur et al., 2019; Niemczak et al., 2017; Pernak et al., 2018; Wust et al., 2019). It is known that most ILs are generally not readily (bio) degradable and are also considered to display (eco)toxicological effects (Amde et al., 2015; Costa et al., 2017; Jessop, 2018; Pham et al., 2010; Siedlecka et al., 2011). However, recently biodegradable ILs have been developed in order to prevent the accumulation of ILs in the environment (Haiβ et al., 2016; Harjani et al., 2008; Hou et al., 2013; Morrissey et al., 2009; Peric et al., 2013; Suk et al., 2020). To meet the requirement that both the anion and the cation should mineralise in waste water treatment processes or the environment (Kümmerer et al., 2019), it is first necessary to obtain an up-to-date overview of the state of knowledge on the biodegradability of ILs currently in use and available for future applications.

A few studies already reviewed applied standard test methods and experimental data to identify good biodegradable ILs and deduced rules of thumb for the biodegradability based on structure-biodegradability relationships (Coleman and Gathergood, 2010; Costa et al., 2017; Jordan and Gathergood, 2015; Stolte et al., 2011, 2015). Furthermore, the data needed for the assessment of persistence, bioaccumulation and toxicity including data for biodegradability has been discussed (Kowalska et al., 2021). However, biodegradation studies were not systematically analysed and efforts made to meet validity criteria were neither reported nor discussed.

Accordingly, the aim of this study was to get an overview on applied standard methods, the compliance with the related guidelines and tested ILs in order to understand which standard tests were mostly applied, the reliability of results and to what extent ILs are biodegradable. A systematic literature search was conducted by applying specified search strings. The collected studies were limited to the ones that specified the standard test name, allowed to extract the biodegradation rate and made a clear specification of the IL's structure. Biodegradation data of tests following standard methods, e.g. according to Organisation for Economic Co-operation and Development (OECD) 301 guidelines or standards of the International Organisation for Standardisation (ISO) was collected. The collected biodegradation data refers to the whole IL including anion, cation and side chains attached to the cation. To better compare the biodegradation rates, the biodegradation data was limited to ready biodegradability test methods in order to identify readily biodegradable ILs. Finally, the reviewed data was evaluated regarding knowledge and data gaps, which have to be considered in future testing in order to get reliable data of ILs' biodegradability to design them for environmental biodegradability (Lorenz et al., 2021).

2. Methods

2.1. Approach for the literature search

In order to gather available biodegradability data for ILs, a literature search was conducted by applying defined search strings. The search was done via the well-established scientific databases SciFinder, Reaxys, Scopus and Web of Science and limited to studies until the end of 2020. The search strings were as follows: "ionic liquids AND biodegrada*", "ionic liquids AND OECD", "ionic liquids AND ISO 14593". Instead of "ionic liquids AND biodegrada*" the search string "ionic liquids AND biodegradation" was used for SciFinder as this database searches automatically for strongly connected terms like "biodegradable" and "biodegradability".

In order to understand which efforts were made for biodegradation testing of ILs, the collected studies were limited to only the ones that followed standardised methods developed by e.g. the OECD, the ISO or the American Public Health Association (APHA). The focus lies on standardised methods as these are valid and enable to better compare the biodegradation data. No restrictions were made regarding both the conditions and the environmental compartment (e.g. soil or water under aerobic or anaerobic conditions) of the biodegradation test as long as the study referred to a standard method. Further criteria to be appropriate for the literature evaluation are if the standard test name was specified, the biodegradation rate was extractable and no contradictory but clear specification was made for the ILs structure, e.g. when comparing structure and name. Studies were also included that applied modified versions of the standard methods, e.g. studies that measured the biodegradability based on primary elimination of the substances or used a substance concentration deviating from the standard guideline. Studies were excluded if the IL's cation consists of a mixture of different cation core structures in order to enable categorisation of the ILs according to their cation core structure and compare their biodegradability.

2.2. Examination of the literature

The applied test parameters in the methods' descriptions of reviewed studies (duration, concentration of inoculum and substance, source of inoculum, temperature, blank control, negative control, quality control, toxicity control, number of determinations, measurement of degradation and validation criteria) were collected if indicated. Furthermore, the studies were checked if all validation criteria defined in the guidelines (see Table A.1 for validation criteria) were applied.

The tested ILs were categorised according to the core structure of the cations such as e.g. imidazolium, cholinium, pyridinium. However, the collected biodegradation rates for every IL refer to the whole IL including anion, cation and side chains attached to the cation. In general, the cholinium ILs could also be classified as quaternary ammonium compounds (QACs), but they were assigned to a separate cholinium cation category because of the high number of different substances that were tested. Derivatives or structural related substances are included as well in the cholinium cation category, e.g. betainium, carnitinium or other structural variations in the side chains at the cation. Within each cation category the different ILs were listed, their biodegradation rate (integer number) and the respective study. If studies specified decimals for biodegradation rates, they were rounded to an integer number. If the biodegradation rate was not precisely extractable from a figure and the value was smaller than 10%, the rate was equalled zero. The list of ILs needed to be checked for duplicates by comparison of the structures since the Chemical Abstracts Service (CAS) registry number could not be used as not every ILs is registered in the CAS database and for compound names abbreviations or different nomenclatures were used. Therefore, the Simplified Molecular Input Line Entry Specification (SMILES) code either was looked up in databases or generated by drawing the structures in ACD/ChemSketch (version 2018.2.1) in order to search for

duplicates by inserting the ILs' structures in the software package Canvas by Schrodinger (version 4.3.013).

2.3. Evaluation of the ready biodegradability data

Due to their charged nature, ILs are most likely to be found preferentially in aqueous environments. Therefore biodegradation rates were compared, which were measured by methods for testing the biodegradation in water under aerobic conditions in order to better compare the biodegradation rates. Since the standard methods vary with regard to the testing conditions (e.g. concentration of inoculum and substance, aerobic or anaerobic), we examined biodegradation data based on the OECD 301 guidelines as these are ready biodegradability tests and more stringent compared to the OECD 302 series. In Table A.2 the different test parameters for the OECD 301 guidelines are summarised. In addition, we included ISO 7827, ISO 9408 and ISO 14593, since these are very similar to OECD 301A, OECD 301F and OECD 310, respectively (OECD, 1992, 2014) as can be seen in Table A.2. Depending on the standard test method, the biodegradation of organic substances is measured as dissolved organic carbon (DOC) (OECD 301A, 301E, ISO 7827), the evolution of carbon dioxide (OECD 301B, OCED 310, ISO 14593) and calculation of the biochemical oxygen demand (BOD) (OECD 301C, 301D, 301F, ISO 9408). Biodegradation studies that applied these methods were appropriate for this evaluation, where tests lasted 28 d, the correct inoculum source was used according to the guideline and where the mineralisation was measured and not the primary elimination. Furthermore, the biodegradation data of single ILs compounds and ILs mixtures consisting of a cation and a mixture of different anions was separated. For each IL the mean value of the found biodegradation data was calculated in case that more than one biodegradation test was reported. Beforehand negative values were zeroed, because they indicate that no biodegradation occurred. These could arise since in the calculation of the biodegradation rate the blind control is subtracted from the test substance. The biodegradation data was classified according to the Traffic Light System that has been already applied by Gore et al. (2013) and Jordan et al. (2016). IL's biodegradability was classified green, amber and red, if the biodegradability was $\geq$ 60%, 20–59% and 0–19%, respectively. The threshold of $\geq$ 60% was chosen since the removal of $\geq$ 60% of theoretical oxygen demand (ThOD) within 10 days starting from a degradation level of 10% ThOD is used to differentiate between readily and non-readily biodegradable substances (OECD, 1992). The interval of 20–59% was chosen since it indicates in ready biodegradability tests that the substance could be inherently biodegradable (OECD, 2006). Degradation by 0–19% means that no or minimal biodegradation occurred.

3. Results and discussion

3.1. Evaluation of the literature search

The literature search yielded in 3256 references without duplicates. In order to understand which efforts were made for biodegradation testing, reviews, books and book chapters, patents and conference transcripts were removed to focus on research articles that possibly contain detailed experimental biodegradation data. Out of these 2965 research articles finally 109 articles were identified that contained experimental biodegradation data of ILs obtained by a standard test method. The other articles were excluded because they just mentioned that ILs could be biodegradable without further specifying or measuring biodegradation and therefore not offering new experimental data. Some of the articles that contain experimental biodegradation data were excluded as they measured the biodegradation by a specific microorganism or the IL contained a mixture of different cation categories, why it was not possible to classify the ILs according to the cation core structure. One article focused on the biodegradability of selected anions in combination with inorganic cations and was therefore excluded as well. Finally, the selected 109 articles were appropriate to be reviewed and evaluated regarding applied standard methods and tested ILs (chapter 3.2 and 3.3). For the evaluation of ready biodegradability data the review of applied methods indicated that only 67 out of these 109 articles were appropriate to ensure comparability of the data as the tests lasted 28 d, the correct inoculum source according to the guideline was used and the mineralisation was measured (chapter 3.4 and 3.5).

All 109 articles were published between 2002 and 2020. Since 2002 the interest in biodegradation data of ILs had been growing with the highest number of studies in 2015, 2016 and 2019 (Fig. 1). We focused on standard methods as they enable a better comparison of biodegradation rates and, like the OECD biodegradability test methods, can be used for chemical's registration under REACH (ECHA, 2017a). There are many studies concerning the biodegradability of quaternary ammonium compounds (QACs) or cationic or anionic surfactants without naming them ILs, which are not considered in this study as the search strings showed. Indeed, these missing substance classes show the need to broaden the perspective on which substances can be considered as ILs and not just focusing on the ILs that are described in this review.

A limitation of the literature search was, that the biodegradation data for an IL could sometimes not easily be extracted since the required value was either not mentioned in the text or in a table but visualised in a dot plot only and could therefore lead to reading errors. In some cases it was not possible to assess the readily biodegradability of ILs according to the OECD pass levels by means of the presented data in the studies. The OECD (1992) states a pass level of 70% removal of DOC or 60% removal of ThOD within 10 days starting from a degradation level of 10% DOC or ThOD. Therefore, in order to assess the ready biodegradability it was necessary to check the course of biodegradation, which was not possible if the final biodegradation rate was only presented in a table or in the text. Moreover, the intervals on the scale of dot plots were sometimes too large, why following the course of biodegradation and evaluating the ready biodegradability was accompanied by too many reading errors. Hence, the classification according to OECD (1992), whether or not an IL, which is biodegradable by $\geq$ 60%, is readily biodegradable could not be done for all ILs, why the information on this classification is missing for some ILs in chapter 3.4 and 3.5.

3.2. Evaluation of the methods used for biodegradation testing

The evaluation of the 109 studies shows that biodegradation data was presented either under aerobic conditions in water, seawater or soil or anaerobic conditions in water (Fig. 2). Some of the studies reported experimental data for two different standard methods, which is why the number of all studies in Fig. 2 is higher than 109. Some studies named the applied method BOD. For these methods two types were applied, one according to APHA and the other type without referring to a specific standard. Therefore, the BOD methods were divided into BOD_modified and BOD_APHA. Concerning the methods named OECD 301_modified (Fig. 2) no specific guideline of the OECD 301 series was mentioned in the studies and the primary elimination was measured.

Most of the studies applied tests under aerobic conditions in water and only a few under aerobic conditions in seawater and soil and under anaerobic conditions in water (Fig. 2). The closed-bottle test (OECD 301D), the manometric respirometry test (OECD 301F) and the CO_2 headspace test (ISO 14593) were the most frequently used standard methods.

The evaluation of the standard methods (Fig. 2) comprised the comparison of the method's description in the respective study with the test guidelines cited there to get an overview of the compliance. Due to no access to ASTM D 5988–96 of the American Society for Testing and Materials (ASTM), ISO 11734, the Chinese standard GB/T 15818–2006 and CEC L-33-A93 of the Coordinating European Council (CEC), it was not possible to compare the methods' descriptions in the studies with the guidelines. Regarding the other guidelines, some methods' descriptions in the studies were incomplete and important parameters of the test

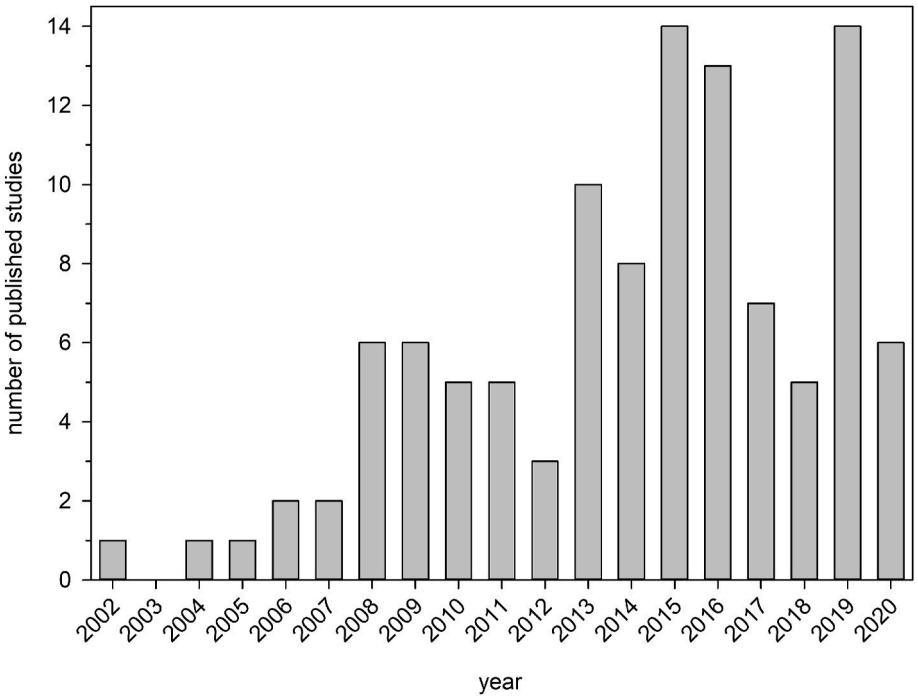


Fig. 1. Historical trend in published studies that contain experimental data on the biodegradability of ILs generated by standard methods.

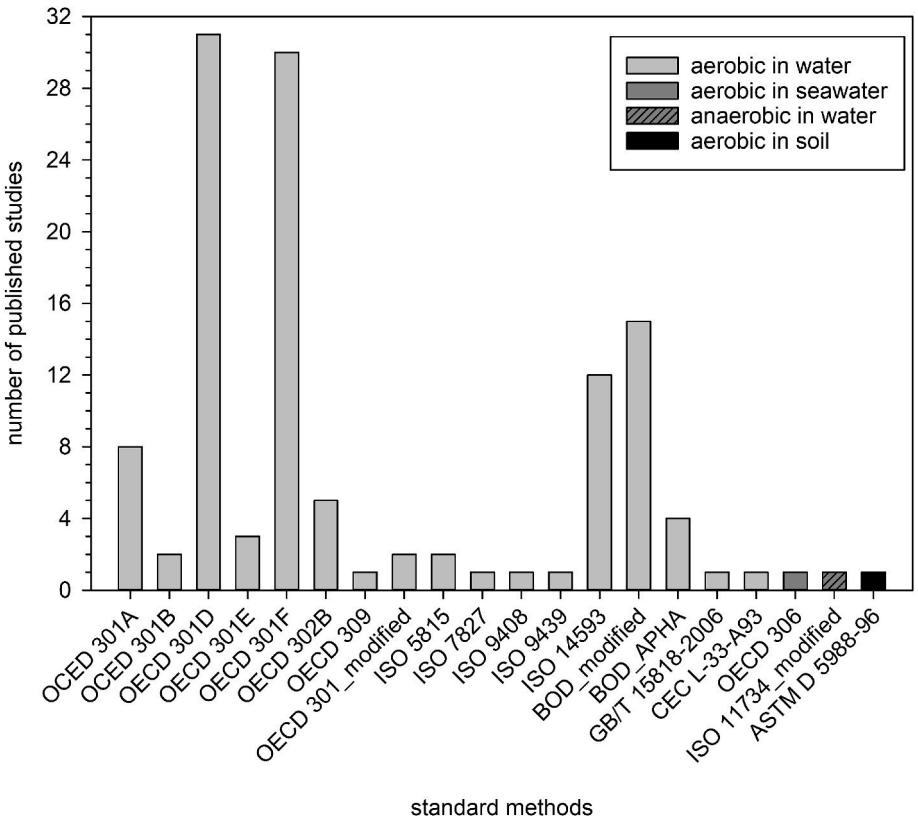


Fig. 2. Number of published studies using standard methods (including modified ones) applied for biodegradability testing of ILs under different conditions. _modified indicates that the test was not completely applied according to the test guideline.

conditions were not stated e.g. inoculum source, concentration of inoculum or substance, reference control or toxicity control. In addition, sometimes substance concentrations were higher than recommended in the guidelines and in some cases the specific unit for the concentration (e.g. mg ThOD L^{-1} for OECD 301D, see Table A.2) was not indicated. Therefore, the concentration could not be compared directly to the specification in the guideline. In these cases, the method could not be checked for compliance with the guidelines. However, the studies were

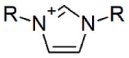
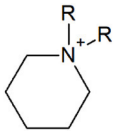
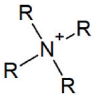
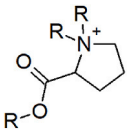
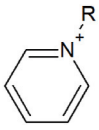
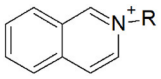
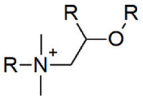
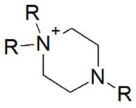
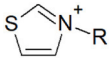
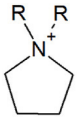
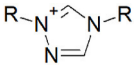
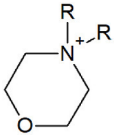
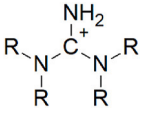
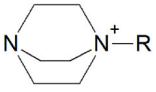
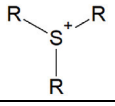
included in Fig. 2, but not separated from the other studies. Furthermore, the validation criteria of the methods were not complete or discussed not at all. Just in 20 studies one or more validation criteria were clearly stated as validation criteria and determined but never all. Some studies described validation criteria, e.g. the positive control, in the text without naming it validation criteria and discussing compliance with them. Such studies are not included in the before-mentioned 20 studies. Therefore, it is not possible to divide the data in Fig. 2 in valid and non-valid biodegradation data. It would be necessary to assess the validity retrospectively by going through the methods' descriptions and results. However, this was often limited due to missing test parameters in the descriptions. Hence, the studies considered could not all be checked for compliance with the standard guideline and validation criteria why it cannot be ruled out that studies applying a modified version of the standard guideline and non-valid biodegradation data are included in Fig. 2.

The standard methods need to be considered when comparing and interpreting the biodegradation data of ILs as they stipulate different conditions. The test conditions for ready biodegradability, e.g. OECD 301A-F and ISO 14593, are more stringent compared to the OECD 302 series (inherent biodegradability) because of lower microorganism density and diversity (up to 1000 colony forming units in ready biodegradability tests compared to several grams dry mass per litre in 302 series). Within the OECD 301 series a higher ratio between substance concentration and microorganism density in the inoculum is used based on a very low bacteria concentration and diversity. The closed-bottle test (OECD 301D) is the most stringent of the OECD 301 series regarding the ratio of substance concentration and microorganism density and thereby their diversity (Table A.2). The classification of inherent biodegradability is not related to a specific pass level (OECD, 2006). A substance that is biodegradable by 20–69% in one of the tests of the OECD 302 series is inherently biodegradable and primary degradation to transformation products has occurred showing a partially degradation of the parent compound (OECD, 2006). The substances degraded by 20–69% are potentially persistent in the environment (ECHA, 2017b). A substance that is biodegradable by $\geq$ 70% in one of the tests of the OECD 302 series is also classified as inherently biodegradable, but are mineralised and therefore not persistent (ECHA, 2017b). However, inherently biodegradable classified substances need a higher inoculum density such as present in wastewater treatment for biodegradation, which does not reflect environmental conditions (OECD, 2006). In contrast, substances classified as readily biodegradable degrade completely in the aquatic environment (OECD, 1992). Furthermore, the comparability of the biodegradation data is limited due to the inoculum that is used. Depending on the standard test methods the inoculum source varies (e.g. activated sludge, effluent of wastewater treatment plants, surface waters, soils) leading to different biological conditions regarding the microorganism species and density and enzymatic diversity (Rücker and Kümmerer, 2012). The inoculum not only varies between sources, but also between locations and seasons, which influences the species community and density making the inoculum a variable factor and restrains the reproducibility of biodegradation tests (Rücker and Kümmerer, 2012; Stolte et al., 2011). Consequently, the biodegradability of a specific ILs may vary by seasons and locations worldwide.

3.3. Evaluation of the data availability of ILs

In the 109 studies 716 different ILs in total including 29 ILs' mixtures were tested by the above described standard methods. These ILs were classified according to the cation core structure (Table 1) and differ in the anion or side chains at the cation or both. Altogether 16 different cation categories have been identified in the 109 studies, which are imidazolium, QACs, pyridinium, cholinium, phosphonium, pyrrolidinium, morpholinium, octanium, piperidinium, prolinium, quinolinium, piperazinium, thiazolium, triazolium, guandinium, and sulphonium (Table 1).

Table 1
Cation categories for ILs. R = H or side chain. Quaternary Ammonium Compounds (QACs).

cation category	Structure	cation category	structure
imidazolium		piperidinium	
QACs		prolinium	
pyridinium		quinolinium	
cholinium		piperazinium	
phosphonium		thiazolium	
pyrrolidinium		triazolium	
morpholinium		guandinium	
octanium		sulphonium	

In Fig. 3A the number of different substances per cation core structure tested for biodegradability under different conditions is summarised. Most of the ILs were tested only under aerobic conditions in water. Only imidazolium ILs were tested under all conditions. Pyridinium ILs were tested under aerobic and anaerobic conditions in water. Regarding tests in water under aerobic conditions, ILs incorporating an imidazolium cation were the most frequently tested substances followed by QACs and pyridinium cations. 245 different imidazolium ILs were tested, 134 QACs and 109 pyridinium ILs. For piperidinium, prolinium, quinolinium, piperazinium, thiazolium, triazolium, guandinium and sulphonium cations less than 9 different substances were tested for biodegradability.

The results show that there is the need for testing ILs, especially of the cations morpholinium, octanium, piperidinium, prolinium, piperazinium, quinolinium, triazolium, guandinium, sulphonium and thiazolium since the data availability for tests under aerobic conditions in water is very scarce compared to the imidazolium ILs and not existent under aerobic or anaerobic test conditions in water, seawater and soil. Since some ILs will potentially occur in the environment in water due to their water solubility a positive trend in testing is that aerobic conditions in water were the most applied for all cation categories. However, ILs that contain a non-polar group, e.g. linear alkyl chains, could accumulate in the sediment (Mrozik et al., 2009; Stepnowski, 2005).

The 716 ILs are either commercially available or were synthesised for research. Theoretically and unlinked to the 716 ILs, around 10^6 single ILs compounds and when considering additionally binary and ternary

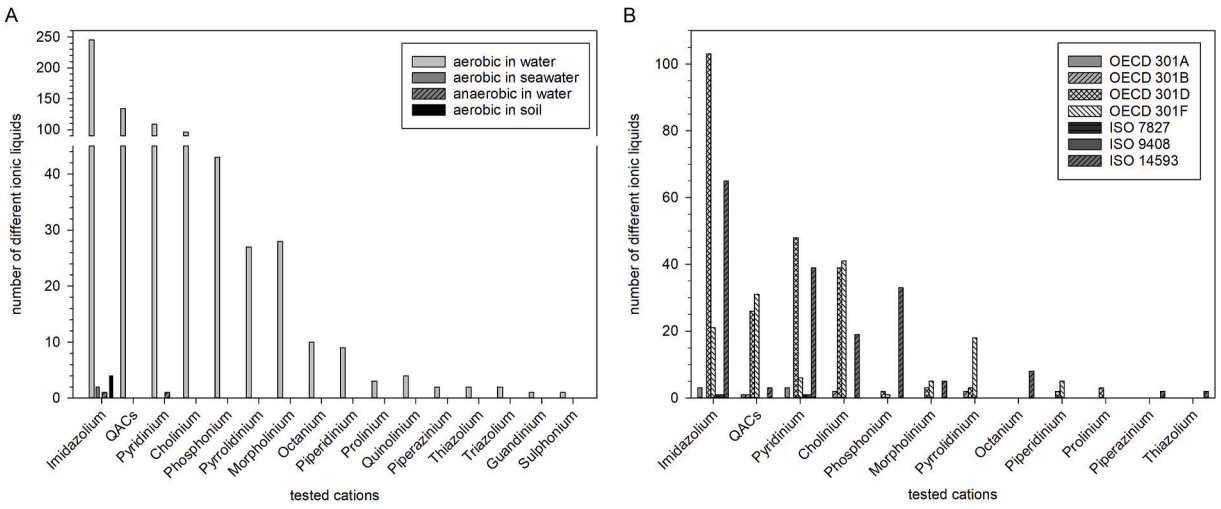


Fig. 3. A) Number of different ILs per cation tested for biodegradability by (modified) standard methods under aerobic and anaerobic conditions in water, aerobic conditions in seawater and aerobic conditions in soil. B) Number of different single ILs compounds per cation tested for biodegradability according to OECD 301A, OECD 301B, OECD 301D, OECD 301F, ISO 7827, ISO 9408 and ISO 14593. Quaternary Ammonium Compounds (QACs).

mixtures in total up to 10^{18} ILs are possible, resulting from the different combinations of cation, anion and side chains at the cation core and combinations of ILs in case of mixtures (Plechkova and Seddon, 2008). No publication was found that states the number of all described ILs in the literature or all ILs per cation core structure without focusing on specific properties or activities. No study did this for biodegradability. Therefore, a precise number of all ever described ILs is missing. The "IL Thermo" (v2.0) ionic liquids database on physico-chemical properties contains data for 2482 ILs based on literature (Dong et al., 2007; Kazakov et al., 2020). It is not clear if all 716 ILs are in this database, as in the search options the SMILES code is not used. The name, CAS number or chemical formula can be used as input for the search. Since the reported names in the studies were not standardised, not all 716 ILs are assigned to a specific CAS number and the chemical formula was not generated for all ILs, the ILs were not looked up in this database. Even though it is not clear if all 716 ILs are also in this database, comparing the number of 716 ILs with the number of ILs in this database, reveals that many more ILs have been described in the literature regarding their physico-chemical properties than have been tested for biodegradability. Therefore, a huge gap exists for biodegradation data of ILs. In order to better access biodegradation data of ILs and to get an overview on the diversity of tested ILs a database should be developed. The database would also facilitate making better-informed decisions as to which IL should be applied in a given situation that requires environmental biodegradability after the function of the IL is fulfilled to minimise environmental pollution.

3.4. Ready biodegradability data of single ILs compounds

For the evaluation of the biodegradability data based on standard methods, which assess ready biodegradability in water under aerobic conditions, from the 109 studies 67 were only considered since they complied with the criteria specified in the method's chapter 2.3. Due to the reasons described in chapter 3.2 it was not possible to differentiate between modified methods and total compliance with the guidelines. Nevertheless, the studies considered for this evaluation applied tests for 28 d, used the allowed inoculum source and measured the mineralisation and not the primary elimination.

The number of different ILs per cation tested according to the selected methods is visualised in Fig. 3B. In case a substance was tested in two different studies by the same method, it was counted as single substance. OECD 301C and E are not listed since they were not applied or the data did not comply with the selection criteria to be appropriate for the data analysis. Likewise, the cations quinolinium, triazolium, guandinium and sulphonium were not included since they were either not tested according to these methods or the methods did not comply with the selection criteria to be appropriate for this evaluation. In total, 508 different ILs were tested according to ready biodegradation test methods. 468 ILs were tested according to one standard method. For 39 ILs two different standard methods were applied and one IL was tested according to three different methods. The most prevalent method was OED 301D as 229 ILs were tested according to this method, followed by ISO 14593 (176 ILs) and OECD 301F (128 ILs) (Fig. 3B).

The most frequently tested ILs contained an imidazolium, QACs, pyridinium or cholinium cation (Fig. 3B). For the imidazolium ILs and pyridinium ILs the method OECD 301D was mostly applied, since 103 and 48 ILs respectively were tested according to this method (Fig. 3B). In contrast, most of the QACs and cholinium ILs were tested according to OECD 301F (31 and 41 ILs respectively) (Fig. 3B). For piperazinium and thiazolium cations two substances were tested according to ISO 14593. A lack of data exists in particular for the cations quinolinium, triazolium, guandinium and sulphonium.

A positive trend is that at least 508 of 716 ILs were tested for ready biodegradability as these are the most stringent tests. Nevertheless, the results show that not every study on ready biodegradability could be used for this evaluation as important test parameters were not met (measuring primary elimination instead of mineralisation, duration of test). Therefore, more testing under stringent conditions, like the OECD 301 series, and compliance with the guidelines is needed to close the data gap at least for the remaining 208 ILs. Due to the reasons discussed in chapter 3.2 and that 229 ILs were already tested according to OECD 301D, this method is recommended for testing to obtain data according to one specific method for numerous ILs that allows to better compare their biodegradability.

The biodegradation data per cation was classified according to the traffic light system in order to visualise the difference in the biodegradability between the tested ILs (Fig. 4). The green classification refers only to biodegradability and does not consider all 12 principles of green chemistry, which should be fulfilled to call an IL green (Anastas and Eghbali, 2010). As explained in chapter 3.2 the data for Fig. 4 also contains test results which were not assessed for validity. From Fig. 4A it is apparent that most of the imidazolium single ILs compounds were not biodegradable or only limited since the red and amber classification are prevalent. Only 17 of 181 imidazolium ILs were classified as green (biodegradability $\geq$ 60%). The same applies for QACs, pyridinium, pyrrolidinium, morpholinium and piperidinium ILs. 25 of 60 QACs ILs,

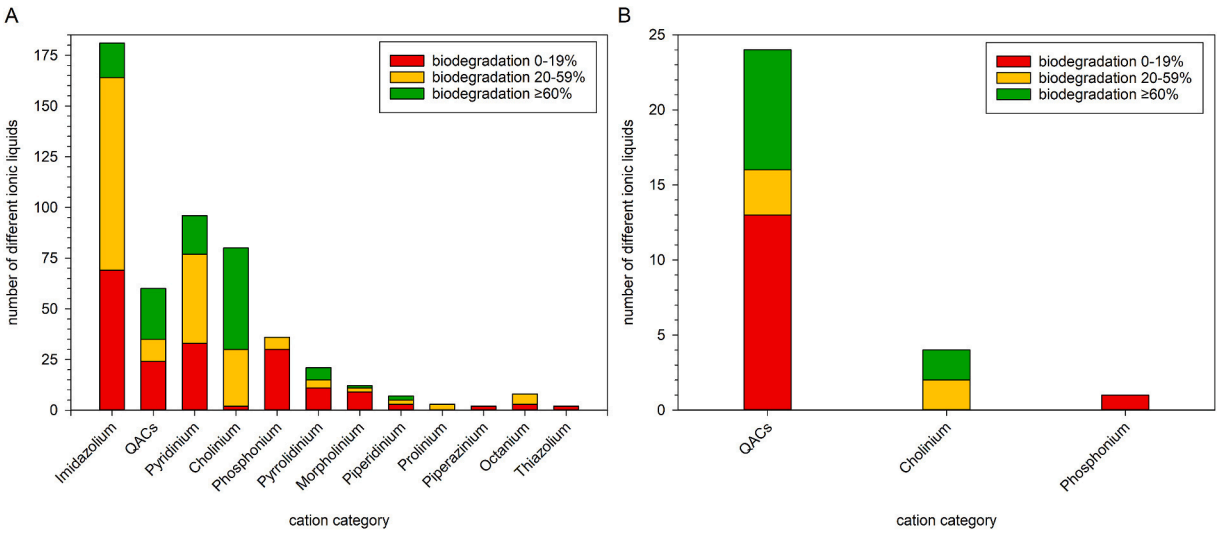


Fig. 4. A) Classification of biodegradation data of single ILs compounds measured according to OECD 301A, OECD 301B, OECD 301D, OECD 301F, ISO 7827, ISO 9408 or ISO 14593. B) Classification of biodegradation data of ILs mixtures measured according to OECD 301A and OECD 301F. The number of different ILs per cation category results from the different combinations of side chains attached to the cation core structure and the different anions. The biodegradation classification is based on the biodegradability of the whole IL including side chains and anion. Quaternary Ammonium Compounds (QACs).

19 of 96 pyridinium ILs, 6 of 21 pyrrolidinium ILs, 1 of 12 morpholinium ILs and 2 of 7 piperidinium ILs were classified as green (Fig. 4A). None of the ILs that contain either the phosphonium, prolinium, piperazinium, octanium or thiazolium cation was biodegradable by ≥ 60% (Fig. 4A). Indeed, most of them were classified as red. The group of cholinium ILs was the only one in which more ILs were classified as green than the sum of red- and amber-classified ILs. In total, 50 out of 80 cholinium ILs were biodegradable by ≥ 60% (Fig. 4A). With regard to the different cations and comparing how many ILs were classified as green from the total number, phosphonium ILs were the least biodegradable ones followed by imidazolium and pyrrolidinium. Most ILs that were biodegradable were cholinium ILs. Pyridinium and QACs ILs were in the middle.

Regarding the green-classified ILs no imidazolium ILs, ten QACs, three pyridinium ILs, 16 cholinium ILs and five pyrrolidinium ILs were classified as readily biodegradable in accordance with the guidelines. For the remaining ILs that were biodegradable by ≥ 60% it was either not mentioned in the studies that they were readily biodegradable or the pass levels were not applied correctly. In some cases the pass level was equated with a degradation rate of 70% removal of DOC or 60% removal of ThOD. The reason for not classifying the ILs as readily biodegradable could be that the ISO guidelines do not indicate a pass level (see Table A.3). Based on the available data in the studies an assessment of the pass level was not possible afterwards since it was either not possible to follow the course of biodegradation or it was accompanied by to many reading errors as pointed out in chapter 3.1.

A closer look at the 17 imidazolium ILs in Fig. 5 reveals that one IL contained a cation incorporating a phenylalanine moiety connected to a leucine moiety via a peptide bond and the anion was bromide (Coleman and Gathergood, 2010). Four ILs were dicationic incorporating a side chain with an ester bond attached to either a dodecyl or tetradecyl or hexadecyl chain and the anion was chloride or bromide (Wang et al., 2019). One imidazolium IL contained a 1-butyl-3-methylimidazolium cation and lauroyl sarcosinate as anion (Mustahil et al., 2019). One imidazolium IL was characterised by an ester linkage in the alkyl side chain of the cation and a saccharin derived anion (Harjani et al., 2009a). Three ILs had in common that the imidazolium cation was substituted with side chains that contained ether groups and a nitrile group in meta-position and the anion was bis(trifluoromethylsulphonyl)amide (Raj et al., 2019). Seven ILs consisted of a cation with an ester linkage in the alkyl side chain and five of them incorporated additionally at least one ether bond. The anions were either dodecylsulphate or octylsulphate (Gathergood et al., 2006; Harjani et al., 2009a; Morrissey et al., 2009).

The green-classified QACs include the following 25 ILs, which are illustrated in Fig. 5. Six ILs were characterised by a phenylalanine moiety attached to the cation and a chloride anion (Suk et al., 2020). Six ILs contained one or two hydroxyethyl chains in the cation and a carboxylic acid anion (Peric et al., 2013). Two ILs had two or three ethyl chains in the cation and an acetate anion (Lotfi et al., 2017). Five substances were characterised by a dibutyldimethylammonium cation and contained either an acetate or citrate or lactate or succinate or levulinate anion (Boissou et al., 2014). Five QACs contained different alkyl side chains in the cation and a carboxylic acid anion (Müller et al., 2018; Pernak et al., 2015b, 2018). One QAC contained a didecyldimethylammonium cation and a nitrite anion (Zabielska-Matejuk et al., 2016).

Regarding the 19 green-classified pyridinium ILs (Fig. 5), one IL contained a carboxymethyl group in the cation and a bromide anion, another IL an octyl and methyl chain in the cation and a bromide anion (Docherty et al., 2010, 2015; Haiβ et al., 2016). Four pyridinium ILs had an ester group in the cation and a halogenide or hexafluorophosphate anion (Harjani et al., 2008, 2009b). Three ILs contained a pyridinium or 3,4-dimethylpyridinium cation with a butyl or hexyl or octyl chain and a lauroyl sarcosinate anion (Mustahil et al., 2019). One IL had a hydroxyl group in the cation and an iodide anion (Neumann et al., 2014). Three ILs contained an ester group in the cation and an octylsulphate anion (Harjani et al., 2008, 2009b). One IL contained both a hydroxyl group and an ether group in the cation and a saccharinate anion (Stasiewicz et al., 2008). Five ILs contained a cation that incorporated either a hydroxyl or ester group or a combination of a butyl chain and a carboxyl group or a combination of a hydroxyl and ester group and a bis(trifluoromethylsulphonyl)amide anion (Ford et al., 2010, 2015; Harjani et al., 2008, 2009b).

The 50 green-classified cholinium ILs in Fig. 5 were characterised by a cholinium cation or a cholinium derivative. The ILs with a cholinium cation were combined with a methylsulphonate anion (one ILs) or different types of carboxylic acid anions (13 ILs) or amino acid anions (19 ILs) or an indole-3-butyrate anion (one ILs) or a desoxycholate anion (one ILs) or a litocholate anion (one ILs) (Hou et al., 2013; Kaczmarek et al., 2020; Klein et al., 2013; Markiewicz et al., 2016; Mustahil et al., 2019; Pisarova et al., 2012; Stolte et al., 2012; Wu et al., 2019; Yazdani et al., 2016; Yu et al., 2008). Some of the green-classified cholinium derivatives contained one ester group in the cation (acetylcholine or

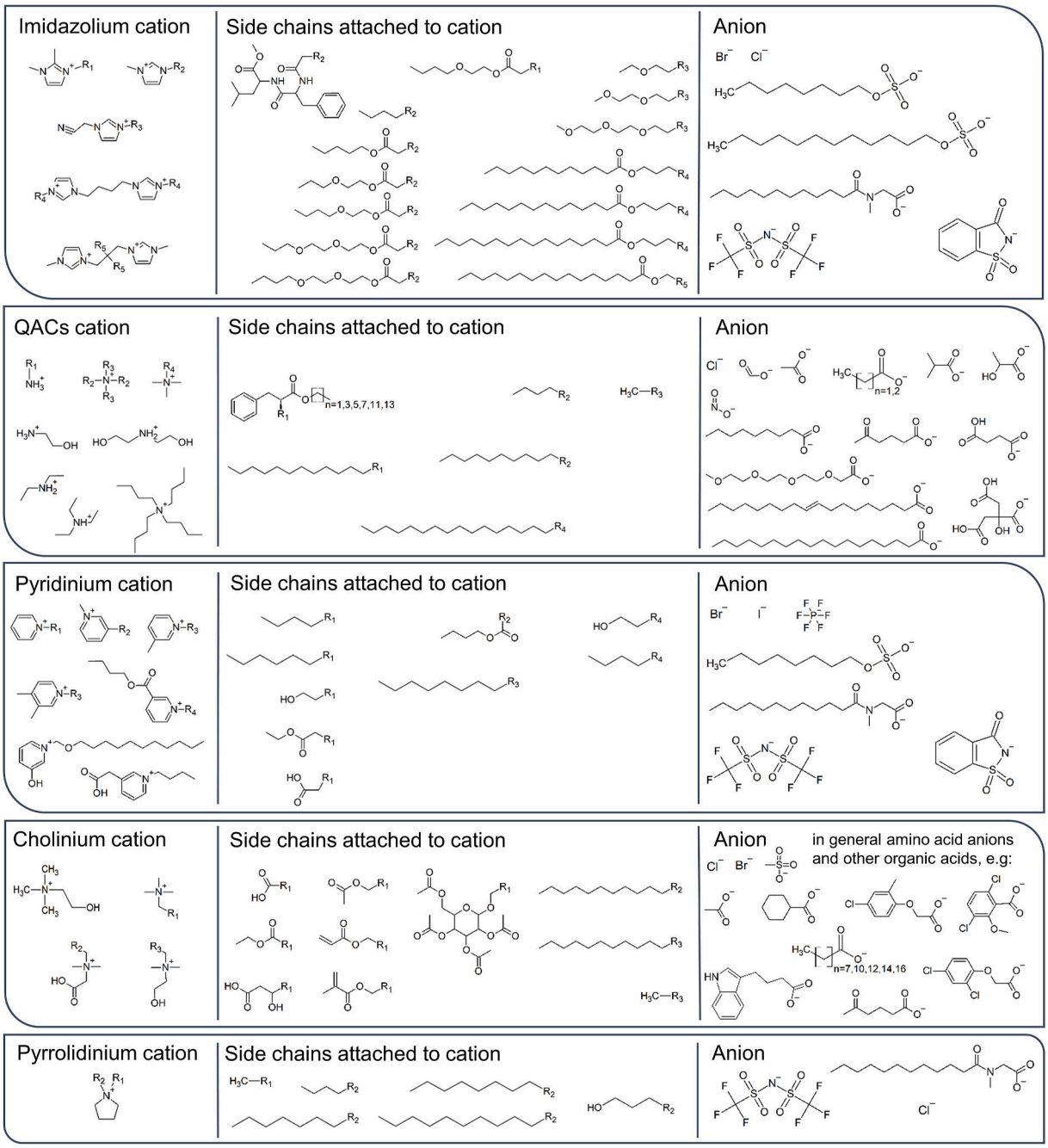


Fig. 5. Structural fragments of the imidazolium, QACs, pyridinium, cholinium and pyrrolidinium single ILs compounds that were biodegradable by ≥ 60%. The anions for the cholinium ILs are not fully listed but examples only.

2-ethoxy-N,N,N-trimethyl-2-oxoethanaminium) and a carboxylic acid anion (one ILs) or (2,4-dichlorophenoxy)acetate (one ILs) or (4-chloro-2-methylphenoxy)acetate (one ILs) or 3,6-dichloro-2-methoxybenzoate anion (one ILs) (Czuryszkiewicz et al., 2019; Markiewicz et al., 2016). One IL contained a carnitine cation in combination with a (2,4-dichlorophenoxy)acetate anion (Pernak et al., 2016). Two ILs contained a betaine cation and a (2,4-dichlorophenoxy)acetate or (4-chloro-2-methylphenoxy)acetate anion (Pernak et al., 2016). Two cholinium ILs contained a dodecylbetainium cation and a nonanoate or (2,4-dichlorophenoxy)acetate anion, respectively (Niemczak et al., 2017; Pernak et al., 2018). Two cholinium derivatives contained one ester group and an ethylene group ([2-(acryloyloxy)-ethyl]-trimethylammonium or [2-(methacryloyloxy)-ethyl]-trimethylammonium) in the cation and a chloride anion (Pernak et al., 2015a). One cholinium derivative contained a bromide anion and a D-glucose moiety in the cation (the cholinium and D-glucose are sharing an oxygen atom) (Erfurt et al., 2020). Two derivatives contained an indole-3-butyrate anion and an ethyl (2-hydroxyethyl)dimethylammonium or dodecyl (2-hydroxyethyl)dimethylammonium cation (Kaczmarek et al., 2020).

A pyrrolidinium IL of the six ILs showing a biodegradability by ≥ 60% in Fig. 5 contained a 1-butyl-1-methylpyrrolidinium cation and a lauroyl sarcosinate anion (Mustahil et al., 2019). Three ILs contained a bis(trifluoromethylsulphonyl)amide anion and a 1-alkyl-1-methylpyrrolidinium cation (Eshetu et al., 2017). The three ILs differed in the alkyl chain length in cation (heptyl or octyl or decyl). One pyrrolidinium IL consisted of a 1-methyl-1-octylpyrrolidinium cation and a chloride anion (Neumann et al., 2014). One IL contained a hydroxyl group in the side chain at the cation (1-(3-hydroxypropyl)-1-methylpyrrolidinium)

and a chloride anion (Neumann et al., 2014).

From the twelve morpholinium ILs only morpholinium lauroyl sarcosinate was biodegradable by ≥ 60% (Mustahil et al., 2019). The two green-classified piperidinium ILs were 1-(3-hydroxypropyl)-1-methylpi peridinium chloride and piperidinium lauroyl sarcosinate, which were biodegradable by 79% in the MRT (OECD 301F) (Neumann et al., 2014) and by 100% in the CBT (OECD 301D) (Mustahil et al., 2019), respectively.

Against the background of the discussion on comparability of biodegradation data in chapter 3.2, for the ILs that were tested twice or more the range in biodegradation rates is small, which was 0–23%. However, for two imidazolium ILs the range was 47–59%, for one QAC 84% and for one pyrrolidinium IL 79%.

For 1-hexyl-3-methylimidazolium bromide biodegradation rates of 7% and 54% were available measured by OECD 301D and OECD 301A respectively (Docherty et al., 2007; Liwarska-Bizukojc and Gendaszewska, 2013). The large range between both biodegradation rates could be explained by the conditions in the tests. OECD 301A is less stringent regarding substance concentration and microorganisms' diversity and density compared to OECD 301D (Table A.2), why 1-hexyl-3-methylimidazolium bromide was better biodegradable under less stringent conditions. The range in biodegradation rates for 1-octyl-3-methylimidazolium chloride is 59%. The IL was degraded by 60%, 60% and 1% in tests according to OECD 301F (Markiewicz et al., 2014, 2015; Peric et al., 2013). Butyl-trimethyl-ammonium methylsulphonate was tested according to OECD 301B and two times according to OECD 301F (Pisarova et al., 2012; Stolte et al., 2012). The IL was biodegraded by 4% and 88% under conditions according to OECD 301F. In the test, where butyl-trimethyl-ammonium methylsulphonate was biodegradable by 88%, the IL was classified as readily biodegradable (Stolte et al., 2012). In the test according to OECD 301B the IL was degraded by 61% (Stolte et al., 2012). Due to the difference in rates and the mean value of 51%, the IL was classified as amber. The reason of the difference in biodegradation rates of 1-octyl-3-methylimidazolium chloride and butyl-trimethyl-ammonium methylsulphonate could be that the inoculum was sourced from different waste water treatment plants why it differed in species' diversity. 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulphonyl)amide was tested according to OECD 301D and OECD 301F (Juneidi et al., 2015; Samorì et al., 2015). In the first test the IL was degraded by 79% and in the second test it was not biodegraded. As OECD 301D is more stringent compared to OECD 301F regarding substance concentration and microorganisms diversity and density (Table A.2), a reason that the IL was biodegradable under conditions according to OECD 301D but not to OECD 301F could be that the inoculum was taken from different locations and therefore differed in species and their density.

Although the ranges in biodegradation rates were for most of the ILs appropriate, the different standard methods and their related testing conditions make it difficult to compare the data. In addition, the results show that it is not straightforward to identify readily biodegradable cations, anions or structural fragments since the biodegradability of ILs in total strongly depends on the anion, cation and the side chains at the cation. To identify if a specific fragment of an IL is crucial for its biodegradability, a counterpart as reference of this IL with a similar structural composition is needed that just differs in the specific fragment. Since inorganic cations and anions do not contribute to the biodegradability rate as they do not contain any carbon that could be used for microorganisms' growth, these could be used in an IL to assess the actual biodegradability. Therefore, data for the organic anions combined with inorganic cations and for organic cations combined with inorganic anions would be needed to have a reference when assessing the biodegradability of ILs consisting of an organic cation and anion.

3.5. Ready biodegradability data of ILs mixtures

In addition to single ILs compounds, mixtures of ILs were measured according to ready biodegradability test methods. These mixtures contained either a combination of cations of the same cation category or different anions. Most of the ILs mixtures belonged to QACs (24 ILs). Four ILs mixtures contained a cholinium cation core structure and one ILs mixture the phosphonium cation core structure (Fig. 4B). The prevalent standard test methods was OECD 301F. 22 QACs, four cholinium and one phosphonium ILs were tested according to this method. Two QACs were tested according to OECD 301A.

Fig. 4B shows that the biodegradability of most of the QACs mixtures was classified as red (13 ILs) and amber (three ILs). Eight QACs mixtures were biodegradable by ≥ 60%, which are illustrated in Fig. 6 regarding their structural fragments. Seven of them were classified as readily biodegradable. Two readily biodegradable QACs mixtures contained a di (tallowoyloxyethyl)dimethylammonium cation, where tallow stands for a mixture of dodecyl, tetradecyl, hexadecyl and octadecyl chains. The anion is either chloride or 2-(4-chloro-2-methylphenoxy)acetate (Pernak et al., 2015a). One readily biodegradable QAC mixture was didecyldimethylammonium canolate, which contained a mixture of anions based on different fatty acids (Pernak et al., 2015b). Two QACs mixtures contained a mixture of benzalkonium cations that differed in the alkyl side chain, which was either dodecyl or tetradecyl. The anions were oleate or canolate (Pernak et al., 2015b). Readily biodegradable were also hexadecyltrimethylammonium canolate and hexadecyltrimethylammonium cocoate, for which the anions were a mixture of different fatty acids (Pernak et al., 2015b). Furthermore, bis (2-hydroxyethyl)methyloleylammonium pelargonate was biodegradable by ≥ 60%. The side chains in the cation were a mixture of saturated and unsaturated dodecyl, tetradecyl, hexadecyl and octadecyl chains (Pernak et al., 2018). Regarding the cholinium ILs mixtures, two were biodegradable by ≥ 60% and one of them was classified as readily biodegradable. The four cholinium ILs mixtures contained different cations of a cholinium derivative and one specific anion. The cations were all characterised by a betainium core structure containing an amide bond in different alkyl side chains, which is the cocoamidopropylbetainium cation (Fig. 6). Coco stands for a mixture of linear octyl, decyl, dodecyl, tetradecyl, hexadecyl and octadecyl chains (Niemczak et al., 2017). The green classified cholinium ILs mixtures contained a 2, 4-dichlorophenoxyacetate or 4-chloro-2-methylphenoxyacetate anion (Fig. 6). The combination of the cocoamidopropylbetainium cation and the 2,4-dichlorophenoxyacetate anion was readily biodegradable (Niemczak et al., 2017). The phosphonium ILs mixture was not biodegradable. It consisted of a trihexyl (tetradecyl)phosphonium cation and two anions, which were 2-[(phosphonomethyl)amino]-acetate and 3, 6-dichloro-2-methoxybenzoate (Choudhary et al., 2017).

Compared to the single ILs compounds, where 508 ILs of 12 different cation categories were tested for ready biodegradability, less data was available for ILs mixtures. Only 29 ILs mixtures of three cation categories were tested for ready biodegradability. Comparing the number of ILs mixtures tested for biodegradability with the number of ILs mixtures available in the "IL Thermo" (v2.0) ionic liquids database on physicochemical properties reveals a lack of data. The database contains 2063 ILs mixtures (Dong et al., 2007; Kazakov et al., 2020), while only 29 ILs mixtures, which are not necessarily in this database, were tested for ready biodegradability. Therefore, more testing of ILs mixtures is needed to close the gap and enable better-informed decisions in selecting the most appropriate ILs for a given application when biodegradability is needed to prevent the accumulation in the environment.

3.6. Challenges and requirements of future biodegradation testing

The approach of the literature search and the chosen criteria to select appropriate articles (chapter 2.1) excluded studies that investigated the biodegradation pathway without applying a standard method nor identified transformation products or measured the biodegradability using specific microorganisms as inoculum. Of course these studies are also of importance to understand the biodegradability of ILs and the risk

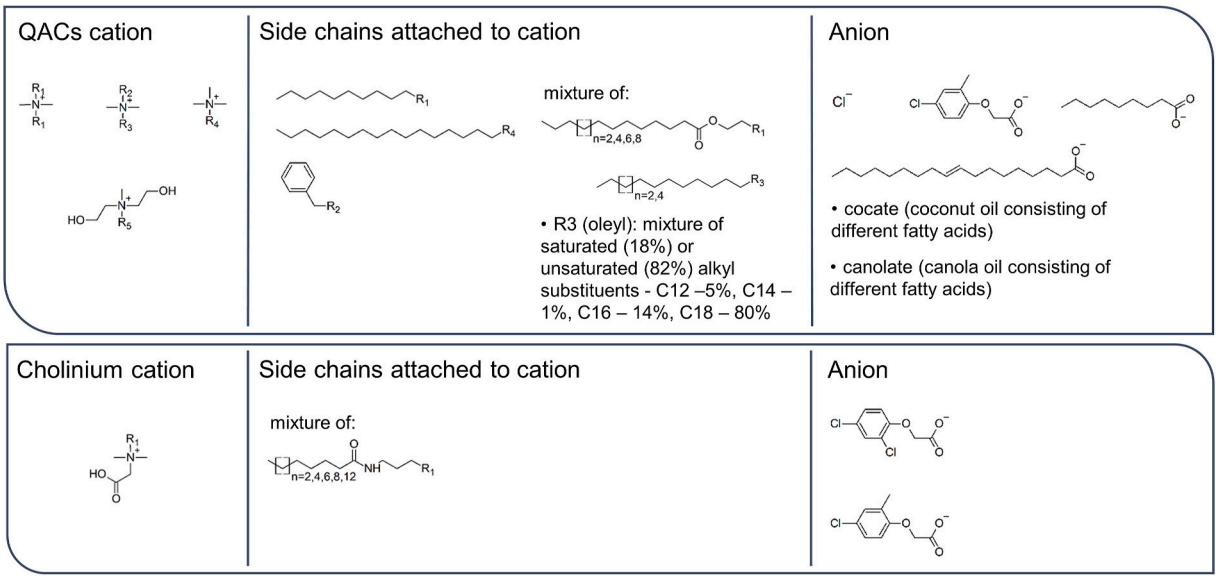


Fig. 6. Structural fragments of the ILs mixtures that were biodegradable by ≥ 60%.

to human and the environment since e.g. transformation products can be more toxic than the parent compound (Gutowski et al., 2015; Hensen et al., 2020; Kümmerer et al., 2019). Therefore, studies investigating the biodegradation pathway or identifying transformation products give further insights into the biodegradation or even mineralisation of the cation and anion, which are complementary to the biodegradation rates measured according to standard methods.

Since the reliability and quality of the data is restrained due to the unclear compliance of the biodegradation tests with the guidelines, incomplete validation criteria as well as limited comparability of the data because of the testing conditions, the availability of ILs' biodegradation data deteriorates. Hence, the methods' descriptions urgently need to be improved to better assess whether the tests are in compliance with the guidelines and if the validation criteria were fulfilled. This would help to better interpret and compare the reported data of the studies. Prospectively, studies should indicate all test conditions and validation criteria that have to be met. Special emphasis should be placed on a correct assessment of the ready biodegradability according to the stated pass levels in the OECD guideline in order to avoid the dissemination of misleading information on readily biodegradable ILs. Just referring to a biodegradation rate by ≥ 60% leaves it unclear for the reader if the 10-day-window was met or not.

Only if data was measured according to one specific method and that is valid, it can be used for a dataset to compare chemicals, to derive structure-biodegradability relationships and build reliable predictive models such as (quantitative) structure-biodegradability relationship models ((Q)SBR). Both structure-biodegradability relationships and (Q)SBR models for biodegradation are a first starting point for the design of mineralisable ILs and preventing environmental pollution (Grabitz et al., 2021; Jordan and Gathergood, 2015; Lorenz et al., 2021). Therefore, more data related to one test method is needed allowing to generate a dataset on biodegradation of ILs. Since the closed-bottle test (OECD 301D) is the most stringent of the OECD 301 series and is the most applied for testing ILs' biodegradability, this test method is recommended for testing to increase the data availability of ILs for this specific method in order to better compare the data and make it appropriate for further applications that have been mentioned above.

In order to better access biodegradation data of ILs and get an overview on the diversity of tested ILs a database should be developed. The database would also facilitate to make better-informed decisions which IL should be applied in a given situation that requires environmental biodegradability after the function of the ILs is fulfilled to minimise environmental pollution.

4. Conclusion

For the first time, ILs' studies were systematically collected and evaluated regarding applied methods and biodegradability of ILs. The systematic literature search enabled a comprehensive overview on applied standard methods and the tested ILs regarding the different cation categories. The evaluation of the methods' descriptions and the assessment of the compliance with the guidelines revealed that more testing according to standard methods, especially OECD 301D, meeting validation criteria is required to get valid data that allows for the comparison of the biodegradation data and a better understanding of the biodegradability of ILs. Furthermore, valid data offers the opportunity for future applications such as evaluation of structure-biodegradability relationships including (Q)SBR model development and registration under REACH. Moreover, the test method should be fully described regarding all important test parameters even if not requested by test respective guidelines in order to better compare the data of different studies and understand the possible variance in biodegradation rates. Although, many efforts have been made for biodegradation testing of ILs in the past 18 years, this review shows that just the tip of the iceberg of ILs has been tested. The classification of the ready biodegradability data of each cation category based on test results of sufficient quality reveals that most of the ILs were biodegradable less than 60%. Further testing is needed especially of the cations morpholinium, octanium, piperidinium, prolinium, piperazinium, quinolinium, triazolium, guandinium, sulphonium and thiazolium since just a few ILs were tested for biodegradability.

Author contributions

Ann-Kathrin Amsel: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft **Oliver Olsson**: Conceptualization, Methodology, Supervision, Writing - review & editing **Klaus Kümmerer**: Conceptualization, Methodology, Funding acquisition, Project administration, Supervision, Resources, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Anhang der Publikation 2

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Publikation 3

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Identification of structure–biodegradability relationships for ionic liquids – clustering of a dataset based on structural similarity

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Identification of structure–biodegradability relationships for ionic liquids – clustering of a dataset based on structural similarity†

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Environmentally open applications as herbicides or active pharmaceutical ingredients are discussed for ionic liquids (ILs). Since most of the ILs are not readily biodegradable in the environment, they may persist there. To prevent the accumulation of persistent and toxic ILs, both the cation and anion need to be designed to completely mineralise in the environment. Several studies summarised structure–biodegradability relationships (SBRs) and gained rules of thumb for ILs' biodegradability based on the available literature data. However, no study systematically analysed a dataset using an *in silico* tool. Therefore, to identify SBRs a dataset on the ready biodegradability of 508 ILs was clustered according to IL similarity by using the software Canvas by Schrödinger. The biodegradability was divided into three classes (biodegradation rates 0–19%, 20–59% and ≥60%). The identified SBRs were compared with the available rules of thumb from the literature. The results show that the cholinium cation and its derivatives acetylcholine, betaine and carnitine are promising candidates for designing environmentally mineralising ILs if a good biodegradable anion is chosen. Imidazolium and phosphonium ILs should be avoided. For pyrrolidinium and quaternary ammonium compounds cations containing ester or carboxyl groups in side chains and alkylsulphate anions should be tested to close gaps in SBRs and possibly design a mineralising IL. Due to the limited data of morpholinium, 1,4-diazabicyclo[2.2.2]octanium (DABCO), piperidinium, prolinium, piperazinium and thiazolium ILs, SBRs could not be clearly identified. Further research is needed on whether structural adjustments according to the findings can increase the biodegradability of not yet fully degrading (20–59%) ILs.

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Introduction

Established applications of ionic liquids (ILs) include closed-loop industrial processes, either commercialised or on a pilot scale, where ILs could be reused, *e.g.* as acid scavengers or solvents.[1–3] Recently, ILs have been applied as solid phase mercury adsorbents and antistatic additives and discussed as electrolytes in dye-sensitized solar cells, active pharmaceutical ingredients (APIs), herbicides, surfactants and ingredients in personal care products and household cleaning agents.[4–13] Therefore, ILs can enter the environment directly, during use or at the end-of-life of the mentioned products or through spills resulting from incidents in closed-loop processes. Quaternary ammonium compounds (QACs), pyridinium and phosphonium ILs were detected in surface water, sediment and wastewater effluents.[14,15] Many ILs are (eco)toxic and not readily biodegradable.[16–20] Since the planetary boundary for novel entities is exceeded, the accumulation of persistent and toxic compounds in the environment needs to be avoided by safe and sustainable-by-design ILs according to the EU "Chemicals Strategy for Sustainability towards a Toxic-Free Environment".[21,22] Therefore, ILs should be designed from the very beginning to fully mineralise in the environment after they have fulfilled their function in order to comply with one criterion of many to be safe and sustainable-by-design.[23–25] It is crucial that full mineralisation is reached and no metabolites are formed in the environment since there is a risk of metabolites (*i.e.* transformation products, TPs) being more (eco)toxic than their parent compounds.[23,26] Metabolites need to be included in a parent compound's risk assessment, which is demanding in resources (time, money, staff), in order to prevent harmful and persistent metabolites from accumulating in the environment.[26,27]

Because of the versatile combinations of the IL's anion and cation, IL properties can be tuned to the desired application,

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that's why ILs are also called designer solvents.[9] Considering the different combinations of the anion, cation and side chains of the cation core, around 10^6 different ILs are possible.[9] This huge variety of ILs offers the chance to consider mineralisation in the design of ILs. The design of ILs includes both the anion and the cation, *i.e.* both should mineralise in the environment.[23,28]

In order to support the design of mineralising ILs, it is crucial to understand the underlying structure–biodegradability relationships (SBRs) as shown by Lorenz *et al.*[24] General rules of thumb (RoTs) by Boethling *et al.* for designing biodegradable compounds give a first reference point (Table 1).[29] Many experimental studies compared a small number of ILs within an individual series of analogues and their biodegradation data to identify SBRs, sometimes without calling it SBR identification, *e.g.*, studies by Haiß *et al.*, Suk *et al.*, Harjani *et al.*, and Gathergood *et al.*[30–33]

In addition, several studies reviewed experimental data of selected ILs to identify SBRs and to deduce RoT for this specific substance class (Table 1).[19,28,34–36] The evaluation of available ready biodegradation data following *e.g.* the OECD 301 series showed that 508 ILs of 12 cation categories based on the cation's core structure (*i.e.* imidazolium, pyridinium, cholinium) were tested for ready biodegradability.[37] However, this dataset was not evaluated for SBRs. Thus, no large dataset based on available studies on the biodegradability of ILs was systematically analysed regarding SBRs. Since ILs differed in many structural fragments, manually clustering to group ILs according to their structures is difficult. Accordingly, this study seeks to identify SBRs by applying *in silico* clustering to the dataset ILs_RB, which was based on the review of Amsel *et al.*[37] To support the environmentally benign design of future ILs, SBRs regarding cation, anion and side chains attached to the cation were identified for each of the 12 cation categories. Furthermore, the SBRs were compared with the available RoTs[19,28,34–36] to discuss their applicability. To support the large-scale elucidation of structure–activity relationships, *in silico* clustering is commonly used and was applied to IL's cytotoxicity.[38–41] Therefore, *in silico* clustering according to structural similarity will be applied for the first time to group ILs, which provides the basis for the comparison of structures and biodegradability.

Methods

Data sources for the dataset ILs_RB and pre-assessment of the data

Data of ILs' biodegradation studies from the literature review of Amsel *et al.*[37] following ready biodegradability test methods (OECD 301A-F, ISO 7827, ISO 9408 and ISO 14593) were utilised for the dataset ILs_RB. The data in a study by Amsel *et al.* comprised results of ready biodegradability test methods (OECD 301A-F, ISO 7827, ISO 9408 and ISO 14593), which were classified using colour codes into biodegradable (≥60% biodegradation, green), possibly inherently biodegradable (20–59% biodegradation, amber) and non-biodegradable (0–19% biodegradation, red) ILs.[37] The colour coding is only related to biodegradability and does not indicate if an IL is green according to the 12 principles of green chemistry.[42] Due to the missing information on validation criteria in some studies, it was not possible to clarify whether the collected data were valid or not in such cases.[37] The ready biodegradability test methods were chosen as they are the most stringent for biodegradability ("readily biodegradable") compared to other tests (*e.g.* OECD 302 series "inherently biodegradable"). Within the available pre-assessment in the literature review 3256 references were limited to 68 studies according to the following criteria to be appropriate for the evaluation of the ready biodegradability of ILs and the dataset ILs_RB: (a) original experimental biodegradation data was presented, (b) ready biodegradability was measured, (c) tests lasted 28 d, (d) the allowed inoculum source was used, and (e) mineralisation was measured.[37] In this study the data of single IL compounds was used in order to deduce SBRs. Single IL compounds consist of one specific cation and anion and are different from IL mixtures, which contain different combinations of different anions or cations and were therefore excluded. Hence, this study used from Amsel *et al.* data of 508 ILs sorted according to the cation core structure (181 imidazolium ILs, 60 QACs, 96 pyridinium ILs, 80 cholinium ILs, 36 phosphonium ILs, 21 pyrrolidinium ILs, 12 morpholinium ILs, 8 1,4-diazabicyclo [2.2.2]octanium (DABCO) ILs, 7 piperidinium ILs, 3 prolinium ILs, 2 piperazinium ILs and 2 thiazolium ILs) (Table 2).[37] These data contained 605 biodegradation test results considering that for some ILs more than one biodegradation result was available. Therefore, the mean value of the biodegradation rate was calculated for some ILs to enable the evaluation of the cluster analysis in the post-assessment. The used biodegradability data are available in the ESI in Tables S1 to S12.†

Post-assessment: clustering of ILs and identification of structure–biodegradability relationships

In the post-assessment the dataset ILs_RB was analysed by clustering the ILs within each cation category according to the structural similarity using the software package Canvas by Schrödinger (version 4.3.013). Clustering was chosen since it enables the classification of compounds in datasets according to specified parameters. In Canvas different clustering methods were available regarding the similarity metrics, the clustering method and the cluster linkage method. We calculated the similarity by applying the Tanimoto coefficient, which ranges from zero to one, while a value of one indicates that the molecules are very similar but not necessarily identical.[43] The Tanimoto coefficient proved to be suitable as a metric for the similarity of molecules represented by binary fingerprints.[43] Furthermore, the Tanimoto coefficient minimizes the bias towards larger molecules, which consist of more bits than smaller molecules.[38,44] We chose Ward's hierarchical clustering using Molecular ACCess System (MACCS) structural keys as descriptors.[45,46] Hierarchical clustering methods organise compounds into clusters having a strict

Table 1 Rules of thumb for improved biodegradability of ILs based on the literature. Inorganic anions are not listed as they do not contain any oxidisable carbon. RoT = Rule of Thumb. ⬇: hindering biodegradability, ⬆: promoting biodegradability, and ⬇⬆: effect not clear

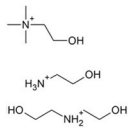
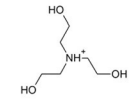
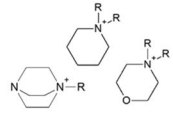
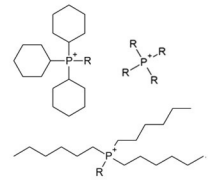
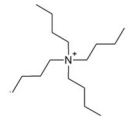
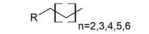
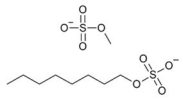
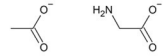
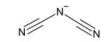
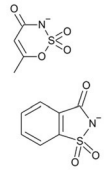
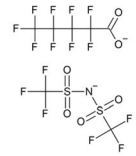
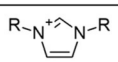
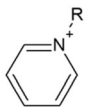
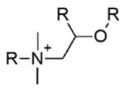
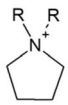
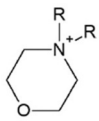
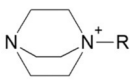
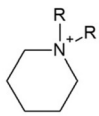
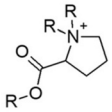
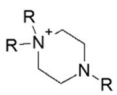
Fragment of IL	RoT #	Structure	Effect	Lit.	RoT #	Structure	Effect	Lit.	RoT #	Structure	Effect	Lit.
Cation	**C1**		⬆	19, 28, 34 and 35	**C4**		⬇ ⬆	19, 35 but 28	**C7**		⬇	19, 28, 35 and 36
	C2		⬆	19 and 35	**C5**		⬇	19, 28 and 35	**C8**		⬇	35 and 36
	C3		⬆	19, 28, 34 and 36	**C6**		⬇	19, 28 and 35	**C9**	Dicationic	⬇	35
Side chain	**S1**	R–OH	⬆	28, 29 and 35	**S6**	Amino acid moieties	⬆	19 and 35	**S11**	R–F halogens	⬇	28 and 29
	S2		⬆	19, 28, 29 and 34–36	**S7**		⬆	29	**S12**		⬇	19
	S3		⬆	28, 29 and 35	**S8**		⬇ ⬇⬆	19, 28, 35 but 29	**S13**		⬇	28
	S4	 medium n-alkyl chain	⬆	19, 28, 29, 35 and 36	**S9**		⬇	36	**S14**	 branched alkyl chains	⬇	19, 29 and 35
	S5	R–C≡N	⬆	35	**S10**		⬇ ⬆	19, 28, 29, 35 but 34				
Anion	**A1**	 in general linear alkylsulphates	⬆	19, 28 and 34–36	**A3**	 in general organic acids (e.g. amino acids)	⬆	19, 28, 35 and 36	**A5**	 dicyanamide	⬇	19
	A2	 in general linear alkylsulphonates	⬆	19, 35 and 36	**A4**		⬆	34	**A6**	 in general perfluorinated	⬇	19, 28, 35 and 36

Table 2 Number of ILs per cation core structure and per biodegradability class. R = H or side chain. The colour coding indicates the class of biodegradability (red: 0–19%, amber 20–59%, and green: ≥60%). Imidazolium (Imid), pyridinium (Pyri), quaternary ammonium compounds (QACs), cholinium (Chol), phosphonium (Phos), pyrrolidinium (Pyrr), morpholinium (Morph), 1,4-diazabicyclo[2.2.2]octanium (DABCO), piperidinium (Piperi), prolinium (Prol), piperazinium (Pipera), and thiazolium (Thia)

Cation	Structure	No.	Red	Amber	Green	Lit.
Imid		181	69	95	17	30, 31, 33 and 51–86
Pyri		96	33	44	19	30–32, 53–55, 58, 59, 61, 64, 70, 78, 81, 83–85 and 87–92
QACs		60	24	11	25	7, 31, 55, 59, 61, 71–73, 79, 90 and 93–99
Chol		80	2	28	50	6, 7, 30, 31, 57, 67, 70, 73, 79, 81, 83, 85, 96, 97 and 100–107
Phos		36	30	6	0	55, 59, 73 and 108
Pyrr		21	11	4	6	64, 68, 79, 85, 96 and 109–111
Morph		12	9	2	1	64, 77, 81, 85, 112 and 113
DABCO		8	3	5	0	90 and 112
Piperi		7	3	2	2	64, 79 and 85
Prol		3	2	1	0	30 and 81
Pipera		2	2	0	0	90
Thia		2	2	0	0	87

hierarchy resulting in a dendogram.[44,47] The Ward's method for cluster linkage has the advantage of minimising the total variance within each cluster and balancing the cluster levels leading to more reliable clustering of similar compounds.[38,44,47] Different fingerprints are available in the software package Canvas (version 4.3.013).[48] The most appropriate allocation of ILs per cluster was achieved by MACCS structural keys compared to Radial or MOLPRINT2D fingerprints that were available in Canvas (version 4.3.013). Furthermore, MACCS structural keys were proven to be appropriate for Ward's clustering.[44,47,49] The Simplified Molecular Input Line Entry Specification (SMILES) code was used as an entry format to calculate MACCS structural keys.

Canvas (version 4.3.013) automatically used the Kelley criterion to limit the number of clusters. The Kelley criterion measures the optimal distribution of clusters at a given cluster level.[44,50] An accurate examination of the ILs per cluster revealed that for the phosphonium and pyrrolidinium ILs a manual adjustment of the number of clusters was necessary to move some ILs in the appropriate cluster by lowering the merging distance. For imidazolium, pyridinium, QACs and cholinium ILs the Kelley criterion was used. The settings for generating clusters for each cation category are summarized in Table 3.

In the next step the environmental biodegradation data and the biodegradability class were assigned to the respective ILs in the clusters. By showing how many ILs were assigned to which class of biodegradability in each cluster and by comparing the clusters, SBRs were deduced. Within a cluster the ILs were compared regarding their structure and biodegradability to identify why an IL is better biodegradable than others. The available RoTs (Table 1) were compared with the clustering results.

Results and discussion

Clustering ILs according to structural similarity

The ready biodegradation data for single IL compounds used for the dataset ILs_RB were analysed in the study by Amsel *et al.* regarding applied methods and tested ILs.[37] Therefore, only the most important characteristics will be presented here. Of the total number of 508 ILs, 188 ILs were biodegradable by ≤19% (red), 200 ILs were biodegradable by 20–59% (amber) and 120 ILs were biodegradable by ≥60% (green). The number of ILs per cation category and their classification of biodegradability are summarized in Table 2. Canvas could not process some ILs due to their high molecular size, which is why in total two imidazolium ILs were excluded from the data analysis. Morpholinium, DABCO, piperidinium, prolinium, piperazinium and thiazolium ILs were not clustered since for each

Table 3 Settings for the number of clusters. Quaternary ammonium compounds (QACs)

Cation	No.	Number of clusters	Setting for the number of clusters	Merging distance
Imid	179[a]	27	Kelley criterion	0.05
Pyri	96	12	Kelley criterion	0.11
QACs	60	10	Kelley criterion	0.10
Chol	80	13	Kelley criterion	0.10
Phos	36	13	Manual adjustment	0.06
Pyrr	21	11	Manual adjustment	0.04

[a] 2 excluded due to their molecular size.

cation core structure, ready biodegradability data for less than 12 ILs were available only, whereby no appropriate clusters could be generated. The reasons were that, on the one hand, Schrödinger generated one cluster for each IL of the morpholinium and piperidinium ILs, when applying the Kelley criterion, which did not help to obtain an overview of similar and non-similar ILs. On the other hand, the dataset contained only eight DABCO ILs, three prolinium ILs, two piperazinium ILs and two thiazolium ILs, which could be handled manually and did not need further grouping into clusters to identify similarities in the structure, *e.g.* functional groups and alkyl chains. Therefore, the SBRs for the cations morpholinium, DABCO, piperidinium, prolinium, piperazinium or thiazolium were derived without clustering. The ILs for every cation core structure were clustered according to their structural similarity.

179 imidazolium ILs were divided into 27 clusters (Fig. 1A). In Fig. 1A the red and amber classification is prevalent showing that most of the imidazolium ILs were not biodegradable or only limited. Only 6 of 27 clusters contained imidazolium ILs, which were biodegradable by ≥60%. In total, 17 imidazolium ILs were classified in the green category, which was allocated to clusters 7, 13, 15, 22, 23 and 26 (Fig. 1A).

For analysing the biodegradation, 96 pyridinium ILs were divided into 12 clusters (Fig. 1B). In total, 19 green classified pyridinium ILs were allocated to 8 of 12 clusters (Fig. 1B).

The 60 QACs were divided into 10 clusters (Fig. 1C). Fig. 1C shows that 6 of 10 clusters contained QACs that were biodegradable by ≥60% (clusters 1, 2, 5, 7, 8, 9), which were in total 25 of 60 QACs. No biodegradable QACs were found in 4 of 10 clusters.

The 80 cholinium ILs and their derivatives were separated into 13 clusters (Fig. 1D). As can be seen in Fig. 1D, 12 of 13 clusters contained in total 50 ILs that were biodegradable by ≥60%. In clusters 3, 4, 8 and 9 all ILs were biodegradable by ≥60%. Only in cluster 6 no cholinium IL was classified as green (Fig. 1D).

Clustering of 36 phosphonium ILs resulted in 13 clusters (Fig. 1E). No cluster contained ILs that were biodegradable by ≥60% (Fig. 1E). Only six ILs allocated to clusters 4, 5 and 6 were classified as amber (Fig. 1E).

The 21 pyrrolidinium ILs were divided into 11 clusters (Fig. 1F). Biodegradable pyrrolidinium ILs were found in 4 of 11 clusters (clusters 4, 6, 7, 10). Only red classified ILs were assigned to clusters 1, 2, 3, 5, 8 and 9 (Fig. 1F). The structural features of the ILs in every cluster are summarised in the ESI in Tables S13–S18.†

Imidazolium ILs

The imidazolium ILs, which were biodegradable by ≥60%, were characterised by the structural fragments presented in Fig. 2. The examination of the biodegradable imidazolium ILs in clusters 22 and 26 suggests that biodegradability was achieved by either incorporating side chains with ester bonds attached to the cation or anions like octylsulphate or dodecylsulphate or saccharinate, which complied with RoT S2, A1 and A4 (Table 1). The increased biodegradability of 1-butyl-3-methylimidazolium lauroyl sarcosinate and 1-ethyl-3-methylimidazolium lauroyl sarcosinate (cluster 15, Fig. 2 and 3) compared to the reference ILs with a bromide anion (clusters 2 and 4, Fig. 4) could result from the anion since sodium lauroyl sarcosinate was biodegradable by 82% in the closed-bottle test (OECD 301D).[85]

A contradiction to RoT A6 was found in cluster 23 in Fig. 2 since three ILs with the bis(trifluoromethylsulphonyl)amide anion were biodegradable. The biodegradable ILs in cluster 23 (Fig. 2) contained cations with cyano and ether groups. The influence of an ether group is not clear according to RoT S10 (Table 1). However, most of the studies in Table 1 found a hindering effect for the ether group. RoT S5 indicates a promoting effect of the cyano group. The bis(trifluoromethylsulphonyl) amide anion is known to be non-biodegradable.[114] Furthermore, the carbon content of the bis(trifluoromethylsulphonyl)amide anion is small compared to the cations in cluster 23 in Fig. 2 (2 carbons in the anion compared to 9, 10, 12 carbons in the cations) and accordingly did not contribute equally to the overall biodegradation rate. Therefore, the cation must have been degraded even though it contained ether groups, which contradicts RoT S10 (Table 1).

Another discrepancy in cluster 23 is that an IL containing an ester group in the cation (Fig. 3), which should increase biodegradability according to RoT S2 (Table 1), was less biodegradable than the ILs mentioned before in cluster 23 (Fig. 2). All ILs in cluster 23 were measured according to OECD 301D, which is why the reason for this discrepancy could be related to different test conditions (*e.g.*, test concentrations, inoculum source) that are allowed by this method. Hence, the ILs in cluster 23 did not follow the available RoT S2, S10 and A6 (Table 1).

In general, the ILs with an alkylsulphate anion (clusters 25, 26 and 27, Fig. 2 and 3) were biodegradable or only slightly, which is in line with RoT A1 (Table 1).

RoT A3 (Table 1) suggests organic acid anions to enhance the biodegradability. These anions were allocated to cluster 6 (amino acid derived anions) and cluster 16 (lactate, levulinate, acetate anions). The cations of these clusters contained only short *n*-alkyl chains (≤C6) (Fig. 3 and 4). The ILs were not or only slightly biodegradable and classified as red and amber, respectively (Fig. 1A). Comparing these ILs to similar ILs with a halogenide anion (one IL of cluster 2 and three ILs of cluster 4, Fig. 3 and 4), of which three are classified red and one as amber, a slight increase in biodegradability could be observed due to the levulinate anion (cluster 16, Fig. 3) and amino acid derived anions (cluster 6, Fig. 3). However, cysteinate (cluster 6, Fig. 4), lactate and acetate anions (cluster 16, Fig. 4) did not increase the biodegradability of the ILs.

For each of the anions, dicyanamide (cluster 4, Fig. 4 and cluster 15, Fig. 3), perfluoropentanoate (clusters 16 and 17, Fig. 4) and methylsulphonate (cluster 24, Fig. 4 and cluster 26, Fig. 3) two ILs were available that differ in the side chains. The effect of the anions on biodegradability was not clear due to the limited data availability.

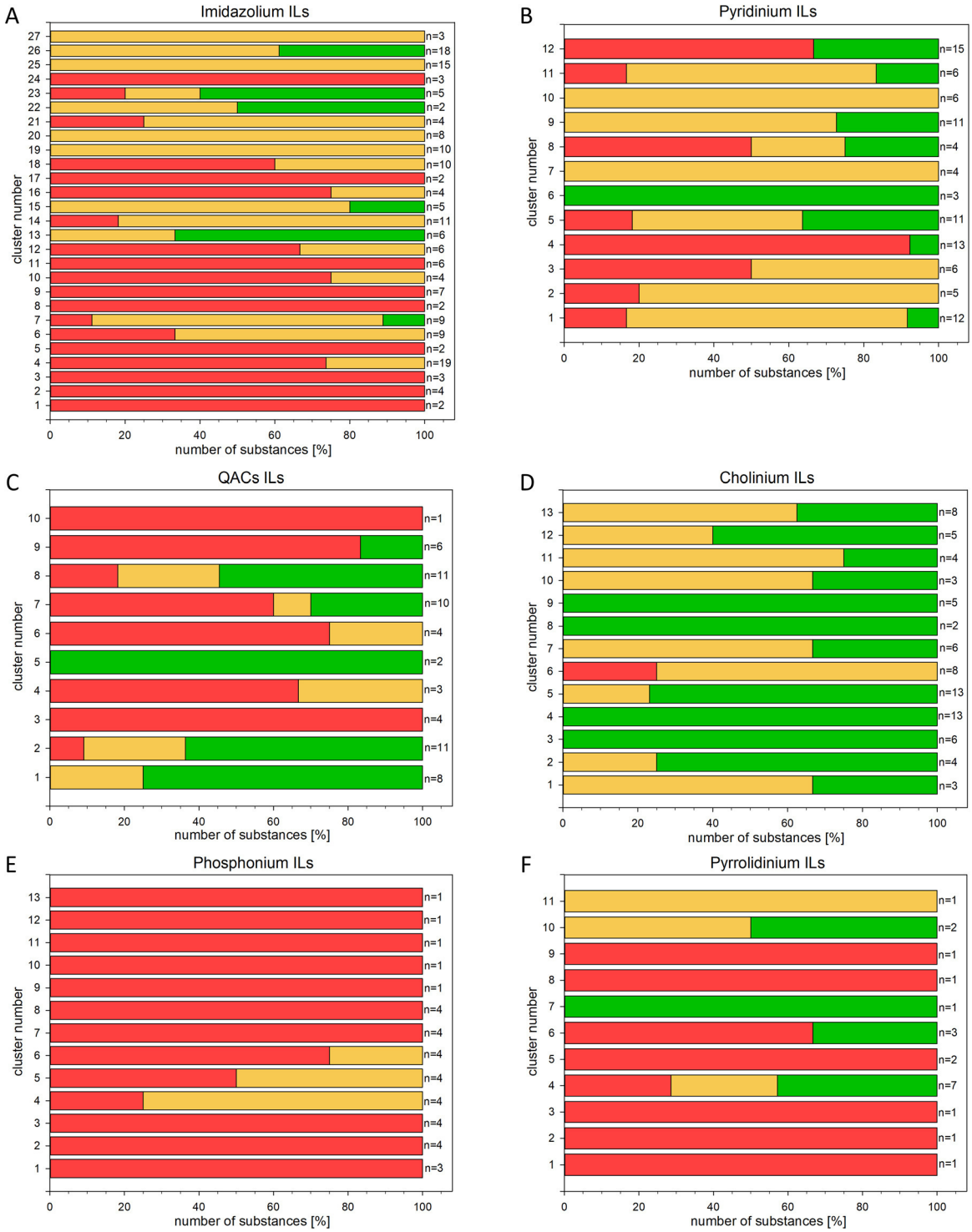


Fig. 1 Biodegradability of (A) imidazolium, (B) pyridinium, (C) QACs, (D) cholinium, (E) phosphonium and (F) pyrrolidinium ILs per cluster. Biodegradability was classified according to the Traffic Light System. Red means 0–19% degradation, amber means 20–59% degradation and green means ≥60% degradation. *n* = number of compounds per cluster.

Two ILs contained a saccharinate anion (cluster 22, Fig. 2 and 3) and were better biodegradable than the reference ILs that contained the same cation and a halogenide anion (clusters 4 and 14, Fig. 3 and 4). The results indicated a positive effect of the saccharinate anion on the biodegradability of the IL as a whole in accordance with RoT A4 (Table 1) and Harjani *et al.*[56]

ILs, that contained mainly esters or a carboxyl group in the side chains of the cation and inorganic anions, were allocated

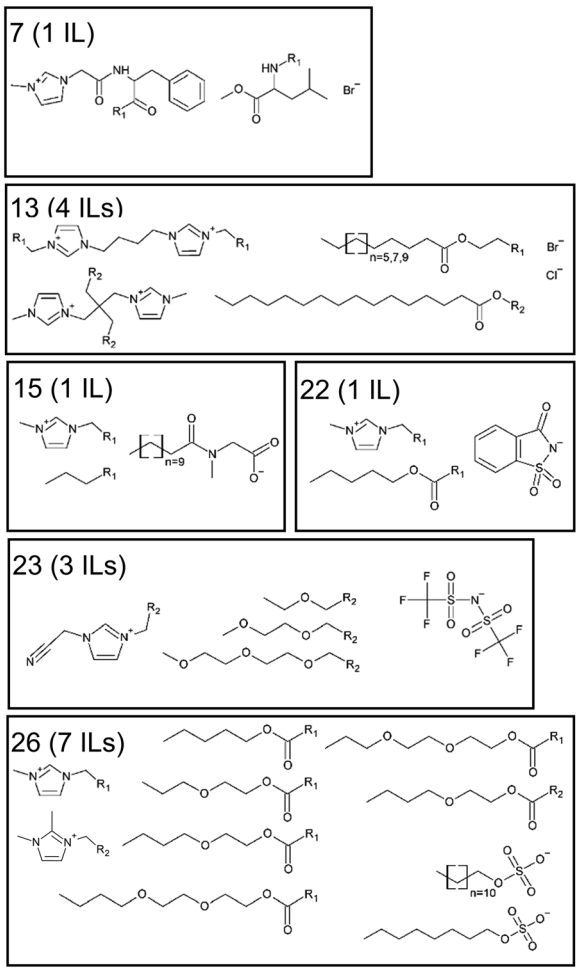


Fig. 2 Imidazolium ILs, which were biodegradable by ≥60% per cluster.

to clusters 12, 14 and 18 (Fig. 3 and 4). None of these ILs was classified as green (Fig. 1A). Compared to clusters 12 and 18, cluster 14 contained the most amber classified ILs (Fig. 1A). The structural difference was that in cluster 14 the cations were only two- or threefold substituted and harboured longer linear alkyl chains (up to C8) than in clusters 12 and 18 (Fig. 3 and 4). According to RoT S2 and S4 (Table 1) ester as well as medium linear alkyl chains (≤C8) increase biodegradability, which would be in line with these findings.

The monocationic 1-alkyl-3-methylimidazolium halogenide ILs in cluster 4 were slightly biodegradable or non-biodegradable (Fig. 3 and 4). Since the biodegradability increased with an increase in the alkyl chain length up to decyl and longer alkyl chains decreased biodegradability in cluster 4, RoT S4 and S9 (Table 1) were confirmed.

Regarding multiple substituents, some of the ILs in clusters 3, 4, 8, 12, and 18 contained an imidazolium core structure that was four- or fivefold substituted by different side chains (Fig. 3 and 4). Most of them were not biodegraded or only slightly even though they included ester groups (Fig. 1A). The findings indicate that multiple (>2) substituents did not promote biodegradability, which is confirmed by Liwarska-Bizukojc *et al.*[65]

Furthermore, amino acid moieties in side chains should increase biodegradability (RoT S6, Table 1). The respective ILs were allocated to cluster 7, which were mostly classified as amber and showed increased biodegradability compared to alkyl-imidazolium ILs in clusters 4 and 10, where the most ILs were classified as red (Fig. 1A). However, only the IL with a phenylalanine moiety connected to a leucine moiety *via* a peptide bond in the cation was biodegradable by ≥60% (Fig. 2). The other red and amber classified ILs contained a phenylalanine moiety connected to different alkyl side chains or a tyrosine moiety. The red classified IL was characterized by a phenylalanine moiety connected to a dodecyl chain, which is the longest alkyl chain in this cluster compared to the other ILs with a phenylalanine moiety (Fig. 3 and 4).

Amide bonds were included in ILs in clusters 5, 8, 9 and 27. While in clusters 5, 8, and 9 all ILs were classified as red, in cluster 27 all were classified as amber (Fig. 1A). Inconclusive trends regarding the effect of amide bonds on biodegradability were described in RoT S8 (Table 1). The results showed that only the ILs with an octylsulphate anion (cluster 27, Fig. 3) were slightly better biodegradable than the ones with a halogenide or tetrafluoroborate anion (clusters 5, 8 and 9, Fig. 4) showing that the better biodegradability could be attributed to the octylsulphate anion (RoT A1, Table 1) and not to the amide bond.

Ether groups were incorporated in two ILs in cluster 15 (Fig. 3), in ten ILs in combination with ester groups in the same side chain in cluster 26 (Fig. 2 and 3) and one IL in cluster 27 in combination with an amide group in the same side chain (Fig. 3). Cluster 23 was discussed before regarding its contradictory data, why this cluster will not be considered in the discussion on the influence of the ether group. Due to the different organic anions (dicyanamide and trifluoroacetate in cluster 15, octylsulphate in clusters 26 and 27) and missing reference compounds that contain an inorganic anion, it could not be assessed whether ether groups promote or hinder biodegradability. At least, with regard to cluster 26 ether groups in combination with an ester did not have a strong negative effect on biodegradability as these ILs were classified as amber or green (Fig. 1A), which is in agreement with the findings of Coleman and Gathergood for RoT S10 (Table 1).[34]

Dicationic imidazolium ILs were not biodegradable according to RoT C9 (Table 1). In cluster 4 five ILs containing only alkyl chains were not biodegradable (Fig. 4), which confirmed RoT C9 (Table 1). In contrast, in cluster 13 the dicationic ILs contained ester groups and were classified as amber or green (Fig. 2 and 3). Furthermore, tris- and tetrakis cations, containing ester groups in side chains and some additionally alkyl chains (≥C4) (clusters 19, 20, and 21, Fig. 3 and 4), were predominantly classified as amber (Fig. 1A). Therefore, functional groups in side chains of polycationic cations influenced biodegradability and the polycationic character did not hinder biodegradability.

In this dataset there was only one IL with a carboxylic acid moiety, which was not biodegradable (cluster 18, Fig. 4). Therefore, RoT S3 (Table 1) could not be confirmed. Based on

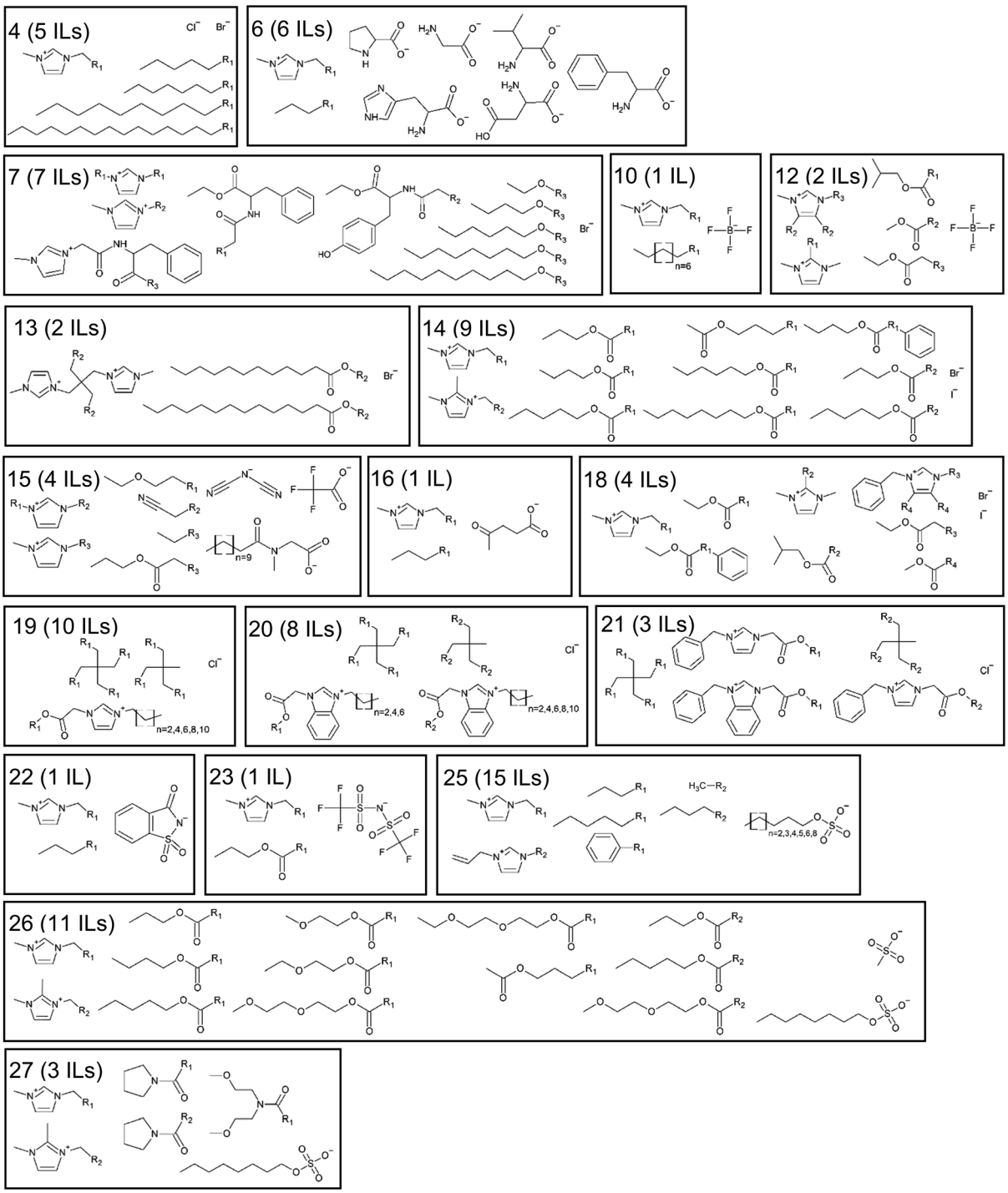


Fig. 3 Imidazolium ILs, which were biodegradable by 20–59% per cluster.

the limited data regarding the carboxylic acid moiety further testing is needed, to identify the influence on biodegradability.

Cations possessing hydroxyl groups were also less tested than the ones with a carboxylic acid moiety. Only three ILs were included in this dataset in clusters 17 and 24 (Fig. 4), which were not biodegraded. All contained a 1-(2-hydroxyethyl)-3-methylimidazolium cation and a bromide, perfluorobutanesulphonate or perfluoropentanoate anion (clusters 17 and 24, Fig. 4). In general, the hydroxyl group should increase biodegradability (RoT S1, Table 1). However, even the IL with the bromide anion was not all degraded. Therefore, RoT S1 (Table 1) was not valid for these three imidazolium ILs supporting Vieira *et al.*'s findings.[70]

Six Ils contained the cyano group in a side chain attached to the cation (clusters 2, 15, and 23) and five of them additionally one to three ether groups in a second side chain (clusters

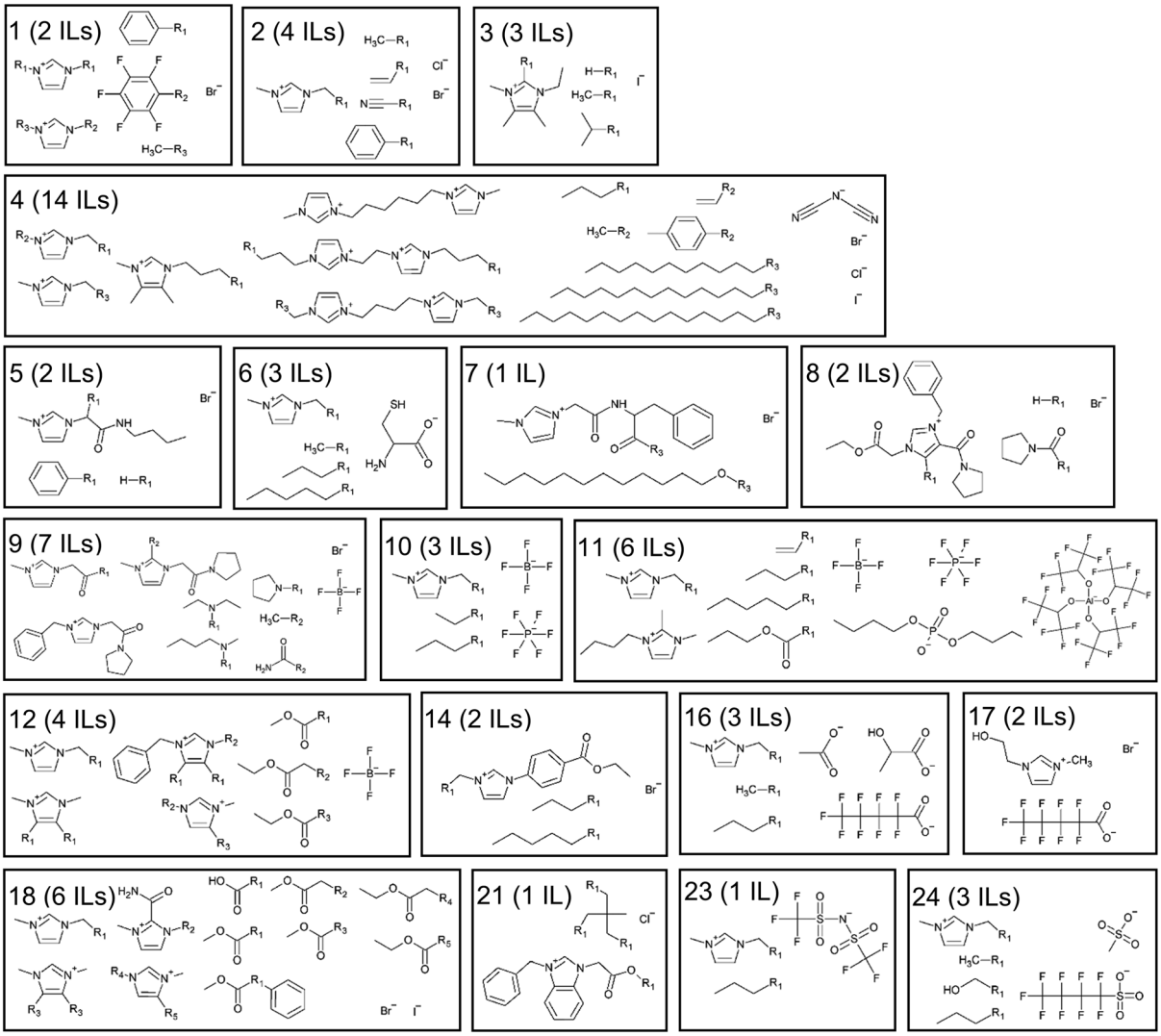


Fig. 4 Imidazolium ILs, which were biodegradable by ≤19% per cluster.

15 and 23). The anions were chloride (cluster 2, Fig. 4), dicyanamide or trifluoroacetate (cluster 15, Fig. 3) and bis(trifluoromethylsulphonyl)amide (cluster 23, Fig. 2). The contradiction with RoT A6 (Table 1) in cluster 23 was discussed before. Two ILs in cluster 15 that contained a cyano and ether group in two different side chains were slightly biodegradable (Fig. 3). The IL that contained only the cyano group was not biodegradable (cluster 2, Fig. 4). A reference compound was missing to identify whether the ether or cyano group promoted the biodegradability of the two ILs in cluster 15. Due to the IL in cluster 2, it is assumed that the cyano group did not promote biodegradability. However, since the reason for the increased biodegradability of the ILs in clusters 15 and 23 was not identified, the effect of the cyano group is not clear. Therefore, RoT S5 (Table 1) was neither confirmed nor objected.

The effect on the biodegradability of allyl or vinyl groups was not clear, too. Both groups were incorporated in a side chain attached to the cation in four ILs (clusters 2 and 11, Fig. 4, cluster 25, Fig. 3). The anions were bromide (cluster 2), tetrahexafluoroisopropoxyaluminate (cluster 11) and octylsulphate (cluster 25). Only the IL containing the octylsulphate anion was slightly biodegradable, and the others were not. The octylsulphate anion was most likely the reason for this result since it is biodegradable (RoT A1, Table 1). Due to the IL in cluster 2 it is assumed that the allyl group did not promote biodegradability. Therefore, more data are needed on ILs that incorporate a good biodegradable group, *e.g.* an ester, and an allyl or vinyl group in the cation to clarify if the allyl and vinyl group hinder biodegradability and to confirm RoT S13 (Table 1).

Ten ILs contained the benzyl or phenyl group in one or two side chains attached to the cation and a bromide anion (clusters 1, 2, 4, 8, 9, 14, and 18, Fig. 3 and 4) or an octylsulphate anion (cluster 25, Fig. 3). Excluded were the ones with amino acid (cluster 7) or mandelic acid moieties (clusters 5, 14, and 18) or that were polycationic (clusters 19, 20, and 21). The ILs

with twofold substituted cations and one or two side chains containing a benzyl or phenyl group (clusters 1, 2, 4, 9, and 14) and three- or fourfold substituted cations (clusters 8 and 18) were not biodegradable (Fig. 4). Only the IL containing a cation, which was fourfold substituted by three side chains with an ester group and one side chain with the benzyl group and 3-benzyl-1-methylimidazolium octylsulphate, was slightly biodegradable and classified as amber (clusters 18 and 25, Fig. 3). Therefore, the benzyl and phenyl groups did not promote biodegradability, which confirmed RoT S12 (Table 1). RoT S7, which was meant for chemicals in general, could not be confirmed based on these data.

Pyridinium ILs

All 15 ILs in cluster 12 contained a bis(trifluoromethylsulphonyl)amide anion. Ten ILs were classified as red, while 5 ILs as green (Fig. 1B). The cation's side chains of the red classified ILs were based on *n*-alkyl residues or contained an amide bond, ether, hydroxyl, allyl, benzyl or tertiary amine group (Fig. 5A). One IL of the 10 red classified ILs was dicationic (Fig. 5A). The green classified IL cations contained ester or hydroxyl or carboxyl groups (Fig. 5C). On the one hand the reason for the biodegradable ILs in cluster 12 could be that good biodegradable side chains were attached to the cation as determined by Ford *et al.* and Harjani *et al.*[32,87] On the other hand a reason could be that the carbon content of the anion is small compared to the cations of the green classified ILs (2 carbons compared to 7, 9, 11, and 12 carbons, respectively) and accordingly contributed less to the overall biodegradation rate than the cation. Therefore, the bis(trifluoromethylsulphonyl)amide anion did not promote biodegradability, which is in line with RoT A6 (Table 1).

ILs with an octylsulphate anion and a cation with an ester group in the side chain, like 3-(butoxycarbonyl)-1-butylpyridinium octylsulphate and 3-(butoxycarbonyl)-1-methylpyridinium octylsulphate were biodegradable by ≥60% (cluster 9, Fig. 5C). The ILs containing only *n*-alkyl chains, like 1-butyl-3-methylpyridinium octylsulphate and 1-butylpyridinium octylsulphate, were classified as amber (cluster 9, Fig. 5B).

The octylsulphate anion had a promoting effect on biodegradability when comparing amber classified ILs with 1-butylpyridinium bromide and 1-butyl-3-methylpyridinium bromide (cluster 4, Fig. 5A), which were classified as red. Therefore, the octylsulphate anion enhanced biodegradability, which is in line with RoT A1 (Table 1).

Three ILs contained a lauroyl sarcosinate anion and were characterised by *n*-alkyl chains in the cation (C1, C4, C6, C8) (cluster 6, Fig. 5C). All ILs were biodegradable (Fig. 1B). There were only two reference ILs for *n*-butylpyridinium lauroyl sarcosinate in the dataset combining the same cation and a halogenide anion (*n*-butylpyridinium chloride, *n*-butylpyridinium bromide, cluster 4, Fig. 5A). Since the reference ILs were not biodegradable, the lauroyl sarcosinate anion had a promoting effect on biodegradability.

In the dataset there was only one pyridinium IL with a dicyanamide anion (cluster 4, Fig. 5A) and one with a perfluoropentanoate anion (cluster 5, Fig. 5B). 1-Butyl-3-methylpyridinium dicyanamide was classified as red and 1-ethyl-3-methylpyridinium perfluoropentanoate as amber. There was no reference IL for 1-ethyl-3-methylpyridinium perfluoropentanoate containing an inorganic anion, which is why it could not be clarified which part of the IL was biodegradable. The cation and anion in 1-butyl-3-methylpyridinium dicyanamide were both not biodegradable, since 1-butyl-3-methylpyridinium bromide as a reference (cluster 4, Fig. 5A) was not biodegradable.

Acesulfamate (cluster 10, Fig. 5B) and saccharinate (cluster 11, Fig. 5A and B) had a promoting effect on biodegradability according to RoT A4 (Table 1). However, in combination with cations incorporating ether groups in the side chain, most of the ILs were only classified as red or amber (Fig. 5A and B). Only one IL containing the saccharinate anion was biodegradable (cluster 11, Fig. 5C). Stasiewicz *et al.* suggested that the reason could be that this IL contained a longer alkyl chain compared to the other ILs in cluster 11.[91] For assessing the influence of the acesulfamate and saccharinate anion biodegradation data of the anions combined with an inorganic cation and data of ILs combining the anions with a cation that do not contain any ether groups would be needed since these can have a negative effect on biodegradability according to RoT S10 (Table 1).

The negative effect of the ether group was identified in cluster 9 (Fig. 1B). The ILs contained an octylsulphate anion and differed in the side chains attached to the cation (cluster 9, Fig. 5B and C). The ILs incorporating a cation with only ester and eventually a second side chain with *n*-alkyl groups (Fig. 5C) were better biodegradable than those that contained an ether group or both ether and ester (Fig. 5B). The same effect was observed in cluster 12. The ILs without ether groups, but with ester or hydroxyl or carboxyl groups in side chains, were biodegradable by ≥60% (Fig. 5C), while the other compounds with ether groups were not (Fig. 5A). Hence, the ether group decreased the biodegradability, which is in line with RoT S10 (Table 1).

Besides, the foregoing discussion showed that the promoting effect of the hydroxyl group, ester group and carboxyl group according to RoT S1, S2 and S3 (Table 1) was found for pyridinium ILs.

The amide group was incorporated in amino acid derived ILs with a tyrosine, phenylalanine or alanine moiety and a mandelic acid derivative combined with a bromide anion (clusters 1, 2, and 3, Fig. 5A and B), in one IL with an octylsulphate anion (cluster 9, Fig. 5B) and with a bis(trifluoromethylsulphonyl)amide anion (cluster 12, Fig. 5A). Due to the many different structural fragments of the ILs, the influence of the amide bond on biodegradability could not be assessed. The ILs based on amino acids (clusters 1–3, Fig. 5A and B) were not biodegradable by ≥60%. Based on these findings, amino acid moieties (cluster 1–3, Fig. 5A and B) did not necessarily increase biodegradability and contradicts RoT S6 (Table 1).

Furthermore, RoT S4 (Table 1) was shown in clusters 2, 7, 10 and 11. In cluster 2 increasing *n*-alkyl side chains of the

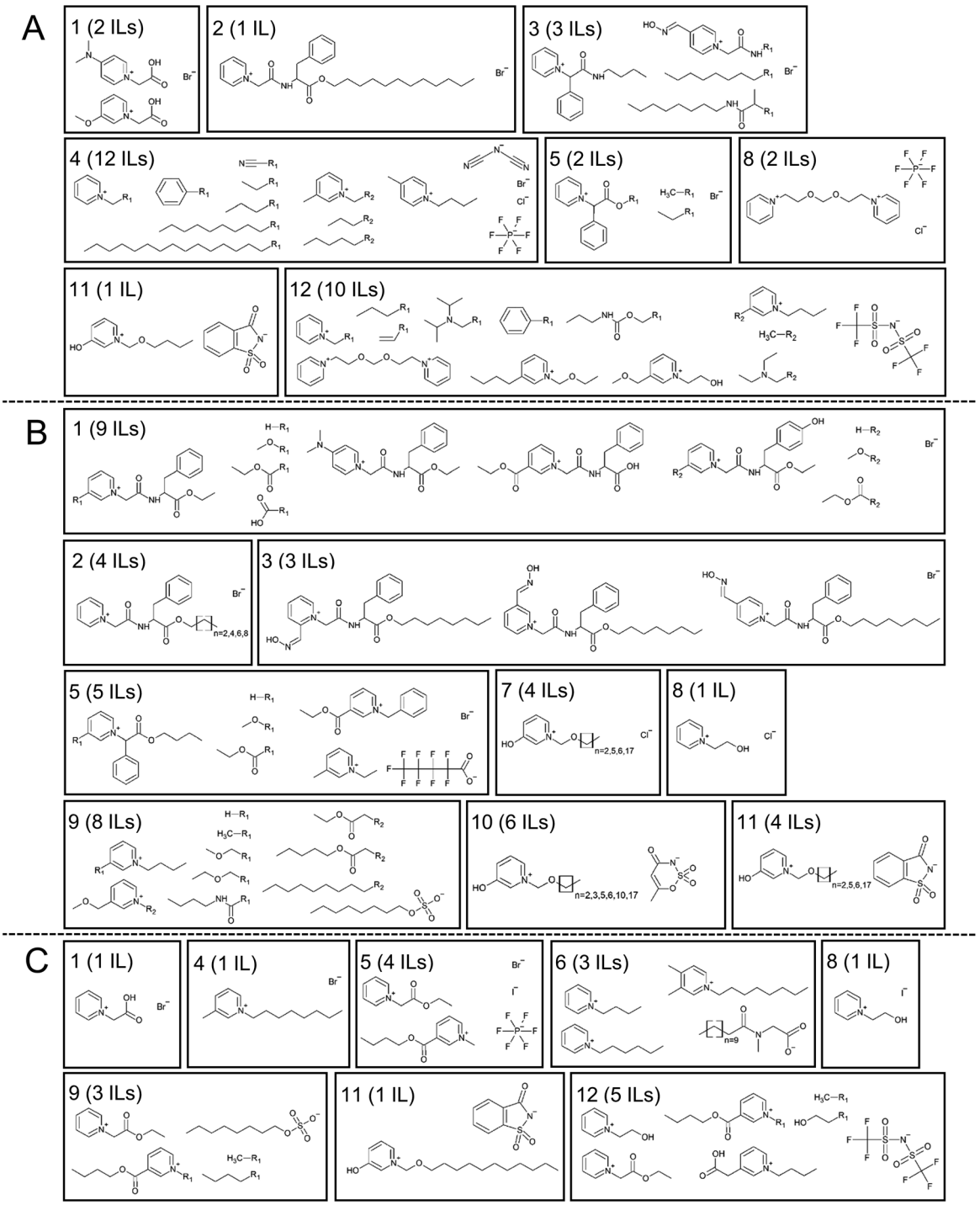


Fig. 5 Pyridinium ILs, which were (A) biodegradable by ≤19%, (B) biodegradable by 20–59% and (C) biodegradable by ≥60%, per cluster.

cation enhanced biodegradability in the order C4 < C6 < C8, in clusters 7, 10 and 11 in the order C3 < C7 < C11 (Fig. 5). However, in clusters 7, 10, and 11 an enhancing effect of the increasing alkyl chain was observed until undecyl deviating slightly from RoT S4 (Table 1). In clusters 2, 7, 10 and 11 RoT S9 (Table 1) was shown, too. In cluster 2 the ILs with a decyl (Fig. 5B) and dodecyl (Fig. 5A) chain were less biodegradable than similar ILs containing a butyl, hexyl or octyl chain (Fig. 5B) as shown by Suk *et al.*[31] A decreasing effect was shown by Stasiewicz *et al.* for an octadecyl chain in the ILs in clusters 7, 10 and 11 (Fig. 5B).[91]

The dicationic ILs in clusters 8 and 12 incorporated the same cations that were connected *via* ether bonds and differed only with regard to the anions (Fig. 5A). The dicationic ILs were not biodegradable and their cation's structural characteristics hindered biodegradability, which is in line with RoT C9

(Table 1). Ford *et al.* concluded that the acetal group was not hydrolysable under the test conditions.[87]

In the dataset there were three ILs with a benzyl group in the cation (cluster 4 in Fig. 5A, cluster 5 in Fig. 5B, and cluster 12 in Fig. 5A), one IL that contained a cyano group in the cation (cluster 4, Fig. 5A) and one IL with an allyl group (cluster 12, Fig. 5A). All ILs except one with a benzyl group in cluster 5 were not biodegradable. Due to the limited data it was not possible to assess the influence of the benzyl, cyano or allyl group on biodegradability. The ILs with a mandelic acid moiety or amino acid derived ILs with a tyrosine or phenylalanine moiety in clusters 1, 2, 3 and 5, which contained a benzyl group, too, were not considered in the discussion of the influence of the benzyl group. These cations included other functional groups like amide or ester bonds, which influenced the biodegradability, too, and hindered the assessment of the relationship between biodegradability and the benzyl group.

Pyridinium ILs, which incorporated anions based on linear alkylsulphonates and salts of organic acids (*e.g.* acetate, lactate, salts of amino acids), were not tested for biodegradability. A combination of these anions with a good biodegradable pyridinium cation like 1-(2-hydroxyethyl)pyridinium should be tested for biodegradability since it could be a candidate for a benign IL.

Quaternary ammonium ILs

Regarding the influence of fluorinated anions according to RoT A6 (Table 1), the available data for QACs showed that all ILs with a bis(trifluoromethylsulphonyl)amide anion were not biodegradable (cluster 3, Fig. 6A). There was no reference IL containing the same cations and an inorganic anion. However, the bis (trifluoromethylsulphonyl)amide anion is known to be non-biodegradable,[114] which is why it must have had a hindering effect.

Alkylsulphate and alkylsulphonate anions were allocated to cluster 4 (Fig. 6A and B). The QACs triethyl-methylammonium methylsulphate and tributyl-methylammonium methylsulphonate were not biodegraded (Fig. 6A), whereas trimethylbutylammonium methylsulphonate was slightly biodegraded and classified as amber (Fig. 6B). Methylsulphate and methylsulphonate anions did not promote biodegradability of the QACs (cluster 4, Fig. 6A and B). Other alkylsulphates or alkylsulphonates or references containing the cations and an inorganic anion were not tested, which would be needed to assess the anion's influence on biodegradability and compliance with RoT A1 and A2 (Table 1) precisely.

Furthermore, QACs with a tetraalkylammonium cation (ethyl or butyl) and anions based on amino acids (cysteinate, prolinate, hydroxyprolinate), were classified as red and amber (cluster 6, Fig. 6A and B), which contradicts the promoting effect of amino acid anions according to RoT A3 (Table 1).

In addition, QACs containing different side chains in the cation and anions based on carboxylic acids were allocated to clusters 2, 5, 7 and 8 (Fig. 6A–C). Some of them were biodegradable (Fig. 6C), and some not (Fig. 6A). Since the available data did not contain a reference IL combining the cations in clusters 2 and 5 and 7 cations in cluster 8 with an inorganic anion, the effect of the anions could not be assessed without investigation of the cation's influence.

Enhanced biodegradability compared to tetramethylammonium bromide (cluster 10, Fig. 6A) as a reference was achieved in cluster 2 by the introduction of one hydroxyethyl group in the cation (monoethanolammonium) in combination with the anions formate and butanoate and two hydroxyl groups in the cation (diethanolammonium) in combination with the anions acetate, propionate, butanoate and isobutanoate (Fig. 6C). These QACs were better biodegradable than the diethanolammonium pentanoate and triethanolammonium butanoate and triethanolammonium pentanoate, which were classified as amber (cluster 2, Fig. 6B). Nevertheless, these combinations of cation and anion enhanced biodegradability compared to tetramethylammonium bromide (cluster 10, Fig. 6A) as RoT S1 and A3 (Table 1) suggested. However, the increasing number of hydroxyl groups did not correspond to an increasing biodegradability. Diethanolammonium formate was classified as red and contradicts RoT S1 (cluster 2, Fig. 6A). The hydroxyl group did not *per se* increase biodegradability since the QACs consisting of the cations *n*,*n*-diethyl-*N*-(2-hydroxyethyl)hexylammonium and *n*-butyl-*n*,*n*-bis(2-hydroxyethyl)pentylammonium combined with a bis(trifluoromethylsulphonyl)amide anion were not biodegradable (cluster 3, Fig. 6A). Therefore, the effect of hydroxyl groups on biodegradability was not clear.

A good biodegradable QAC in cluster 2 was dodecylammonium 2,5,8,11-tetraoxatridecan-13-oate (Fig. 6C). In general, ether groups should hinder biodegradability according to RoT S10, but in this anion the ether groups were promoting.

A good biodegradable combination for a QAC was also shown in cluster 5, where the QACs were composed of an acetate anion and a diethylammonium or a triethylammonium cation (Fig. 6C).

Compared to clusters 2 and 5 fewer QACs were classified as green in clusters 7 and 8 (Fig. 1C). The cations in clusters 7 and 8 were fully alkylated and the anions were different alkyl carboxylic acids, hydroxyl acids, keto acids, dicarboxylic and tricarboxylic acids as well as saturated and unsaturated fatty acids (Fig. 6A–C). To sum up, carboxylic acid anions in 2, 5, 7 and 8 increased the biodegradability compared to tetramethylammonium bromide (cluster 10), which is in line with RoT A3 (Table 1). However, the biodegradability depended on the side chains of the cation, too. For favouring biodegradability, the ammonium cation should have only one or two side chains, while the two other positions should be hydrogen atoms like in clusters 2 and 5, where most of the QACs were classified as green (Fig. 6C). This finding is supported by Pernak *et al.*'s conclusion recommending the use of alkyltrimethylammonium cations and long alkyl chains to increase biodegradability compared to dialkyldimethylammonium cations.[94]

In cluster 1 most of the QACs were classified as green (Fig. 1C). The QAC cation contained only one phenylalanine moiety and differed in the alkyl chain, which was connected *via* an ester bond to this moiety (Fig. 6B and C). This cluster indicated a positive influence of the amino acid moiety on bio-

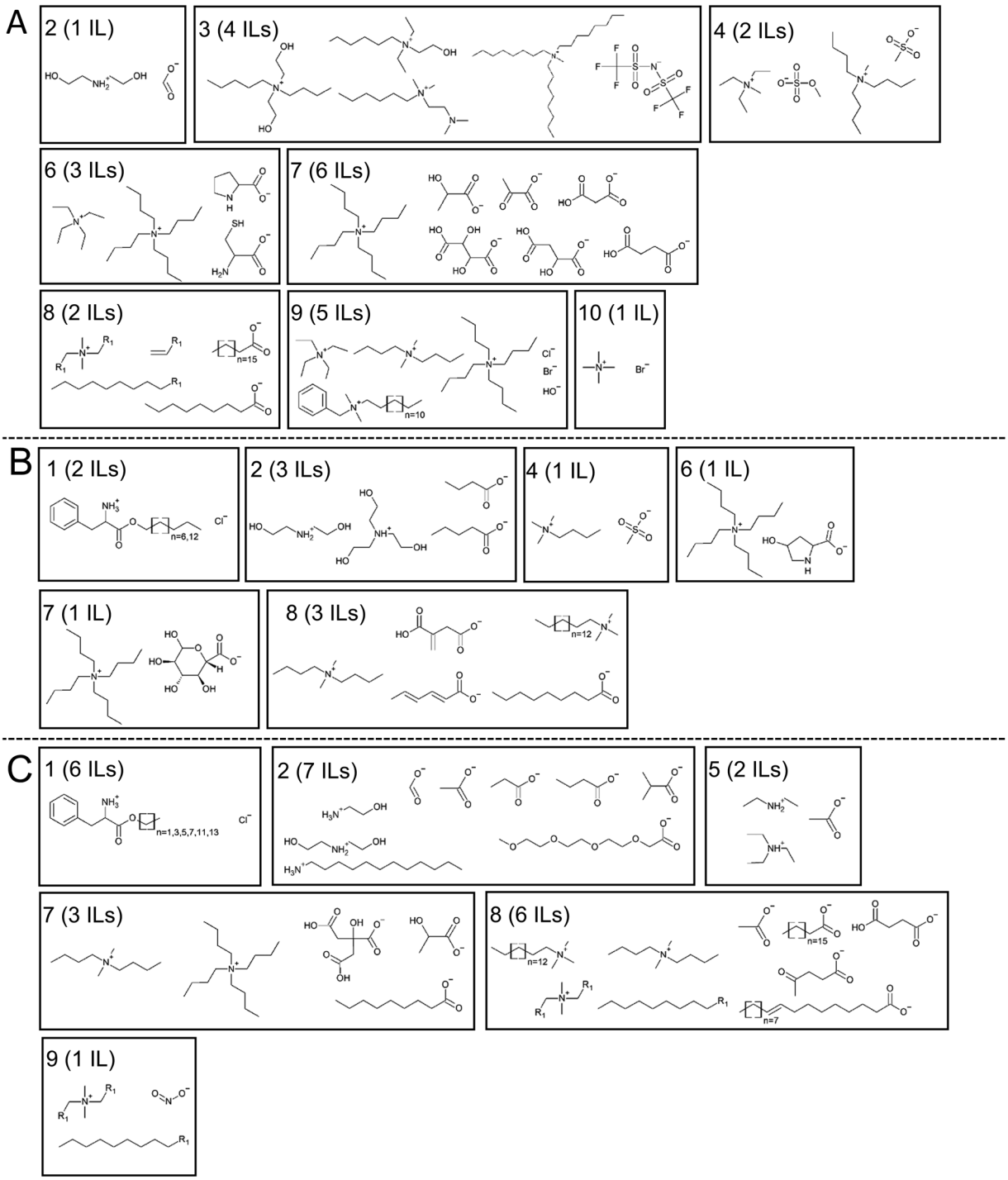


Fig. 6 QACs, which were (A) biodegradable by ≤19%, (B) biodegradable by 20–59% and (C) biodegradable by ≥60%, per cluster.

degradability if compared to a fully methylated QAC like tetramethylammonium bromide in cluster 10 (Fig. 6A). In contrast, the increasing alkyl chain length bonded to the phenylalanine moiety decreased the biodegradability as shown by Suk *et al.*[31] Hence, RoT S6 and S9 (Table 1) were true in this case, but RoT S4 (Table 1) was not proven here.

Comparing the QACs in clusters 9 and 10 that had a halogenide, hydroxide or nitrite anion and different *n*-alkyl side chains (C1, C2, C4, C10, C14) in the cation, the available data illustrated that an increasing alkyl side chain did not promote biodegradability as most of them were classified as red (Fig. 6A). Only didecyldimethylammonium nitrite was classified as green (cluster 9, Fig. 6C). Therefore, RoT S4 was not valid for these cations. Further testing of QACs with different alkyl side chains of the cation and inorganic anions is needed to assess the effect of the alkyl chains.

In the dataset there was only one QAC that contained an allyl group in the cation (cluster 8, Fig. 6A), which was not biodegradable. Since diallyldimethylammonium pelargonate (cluster 8) was less biodegradable than tetrabutylammonium pelargonate (cluster 7, Fig. 6C), the allyl had a hindering effect in this QAC. Based on the limited data regarding allyl groups and that no reference compound for diallyldimethylammonium pelargonate without an allyl group, like dipropyldimethylammonium pelargonate was tested, further testing is needed, to identify the influence on biodegradability.

Furthermore, in the dataset there was only one QAC with a benzyl group in the cation (cluster 9, Fig. 6A), which was not biodegradable. Due to no reference compound the effect of the benzyl group on biodegradability could not be assessed.

The clustering showed, that QACs incorporating ester or ether in *n*-alkyl chains or carboxyl groups attached to the cation were missing in the dataset (except for the ester group in the phenylalanine moiety in cluster 2). For designing biodegradable ILs testing these kinds of QACs could give further insights on SBRs regarding ester or ether groups.

Cholinium ILs

From the data of cholinium ILs it is not straightforward to determine the influence of the anions or structural variations at the cation on the biodegradability as in this dataset there is no IL containing the unmodified cholinium cation and an inorganic anion, like chloride, which would indicate the biodegradability of the cation to compare this data with the ILs in Fig. 7. Nevertheless, some trends could be observed.

ILs with the cholinium cation and anions based on organic acids were allocated to clusters 3, 4, 5, 8 and 9 (Fig. 7B and C). As can be seen in Fig. 1D, in these clusters, except cluster 5, all ILs were classified as green. Therefore, RoT A3 (Table 1) is true for cholinium ILs as long as the anions are based on amino acids, alkyl carboxylic acids, lauroyl sarcosinate, cyclopentane and cyclohexane carboxylic acids, indole-3-butyrate, benzoate, salicylate, desoxycholate and lithocholate.

In cluster 5 three ILs were classified as amber indicating that the anions had a hindering effect on the biodegradability if compared to the green classified cholinium ILs in clusters 3, 4, 8 and 9 (Fig. 7B and C). The anions were perfluoropentanoate, naphthoxyacetate and anthracene-9-carboxylate (Fig. 7B). Furthermore, the perfluorobutanesulphonate anion in cluster 1 hindered biodegradability since the biodegradability of the cholinium IL decreased as the methylsulphonate anion (Fig. 7C) was replaced with a perfluorobutanesulphonate anion (Fig. 7B). The findings indicate that fluorinated anions and polycyclic aromatic carboxylic acids hindered biodegradability as shown by Vieira *et al.* and Yu *et al.*, respectively.[57,70]

Therefore, the results confirmed the hindering effect of fluorinated anions in RoT A6 (Table 1) and the promoting effect of the methylsulphonate anion in RoT A2 (Table 1).

Furthermore, in clusters 11, 12 and 13 the ILs incorporated cholinium derivatives as cations and four different herbicidal anions based on carboxylic acids with an aromatic ring, which appeared in every cluster, except 2-(4-chloro-2-methylphenoxy) propionate (only clusters 11 and 13) (Fig. 7B and C). Many of them were classified as green, but some as amber (Fig. 1D). Because of the structural variations in the cation it was not possible to clarify if the anion or the cation in the amber classified ILs had a hindering effect on biodegradability (clusters 11, 12, and 13, Fig. 7B and C). Only for two ILs in cluster 12 with a (4-chloro-2-methylphenoxy)acetate anion and a cholinium derivative that contained instead of the hydroxyethyl chain an ethylprop-2-enoate or ethyl-2-methylprop-2-enoate chain (cluster 12, Fig. 7B) a chloride analogue was available in cluster 2 (Fig. 7C). While the two ILs in cluster 12 were classified as amber, the chloride analogues in cluster 2 were classified as green (Fig. 7B and C). Therefore, Pernak *et al.* concluded that the (4-chloro-2-methylphenoxy)acetate anion had a hindering effect on biodegradability.[105] A vinyl group was present in these four ILs in combination with an ester in the same side chain (cluster 2, Fig. 7C and cluster 12, Fig. 7B). Since the (4-chloro-2-methylphenoxy) acetate anion had a hindering effect and the ILs in cluster 2 were classified as green (Fig. 7B and C), the vinyl group had no hindering effect on biodegradability. Therefore, RoT S13 (Table 1) was not found in clusters 2 and 12.

With regard to the cholinium derivatives in clusters 1 and 2 in Fig. 7B the ether group instead of the hydroxyl group in the cholinium cation decreased the biodegradability compared to the ILs containing the same anion but the unmodified cholinium cation (clusters 1 and 5, Fig. 7C). Stolte *et al.* and Markiewicz *et al.* concluded that the cholinium derivative containing the ether group is not biodegradable.[67,96] Therefore, RoT S10 (Table 1) proved true.

In cluster 7 the cations contained a linear alkyl chain (C2, C4, C6, C8, C10, C12) instead of one methyl chain, while the anion was indole-3-butyrate (Fig. 7B and C). In this cluster the highest biodegradability was observed for the cation with the ethyl chain (Fig. 7C), while for the alkyl chains butyl, hexyl and octyl it was decreasing (Fig. 7B) and then for decyl (Fig. 7B) and dodecyl (Fig. 7C) slightly increasing. RoT S4 (Table 1) was in this case not retrieved, but elongated alkyl side chains were hindering as found by Kaczmarek *et al.*[103]

Cluster 6 was the only cluster that contained red classified ILs. Amino acid moieties were inserted in the cation instead of one methyl chain (cluster 6, Fig. 7A and B). Therefore, RoT S6 (Table 1) was not true for the cholinium cation.

The introduction of an ester group instead of the hydroxyl group (cluster 2, Fig. 7C and cluster 12, Fig. 7B and C), a betaine or carnitine cation (cluster 13, Fig. 7B and C) and a dodecylbetainium cation (cluster 3, Fig. 7C and cluster 11, Fig. 7B and C) did not have a strong hindering effect on biodegradability as some of them were classified as green. However, only in cluster 2 two ILs contained an inorganic anion, which is why the cation was responsible for the biodegradability. For the other ILs a reference with an inorganic anion was missing for comparison to clearly identify which effect the structural changes had. Therefore, RoT S2 and S3 (Table 1) could not be scrutinized.

Biodegradation data on cholinium ILs incorporating an inorganic anion, which is not toxic to the microorganisms, is needed for comparison with the available data to make more

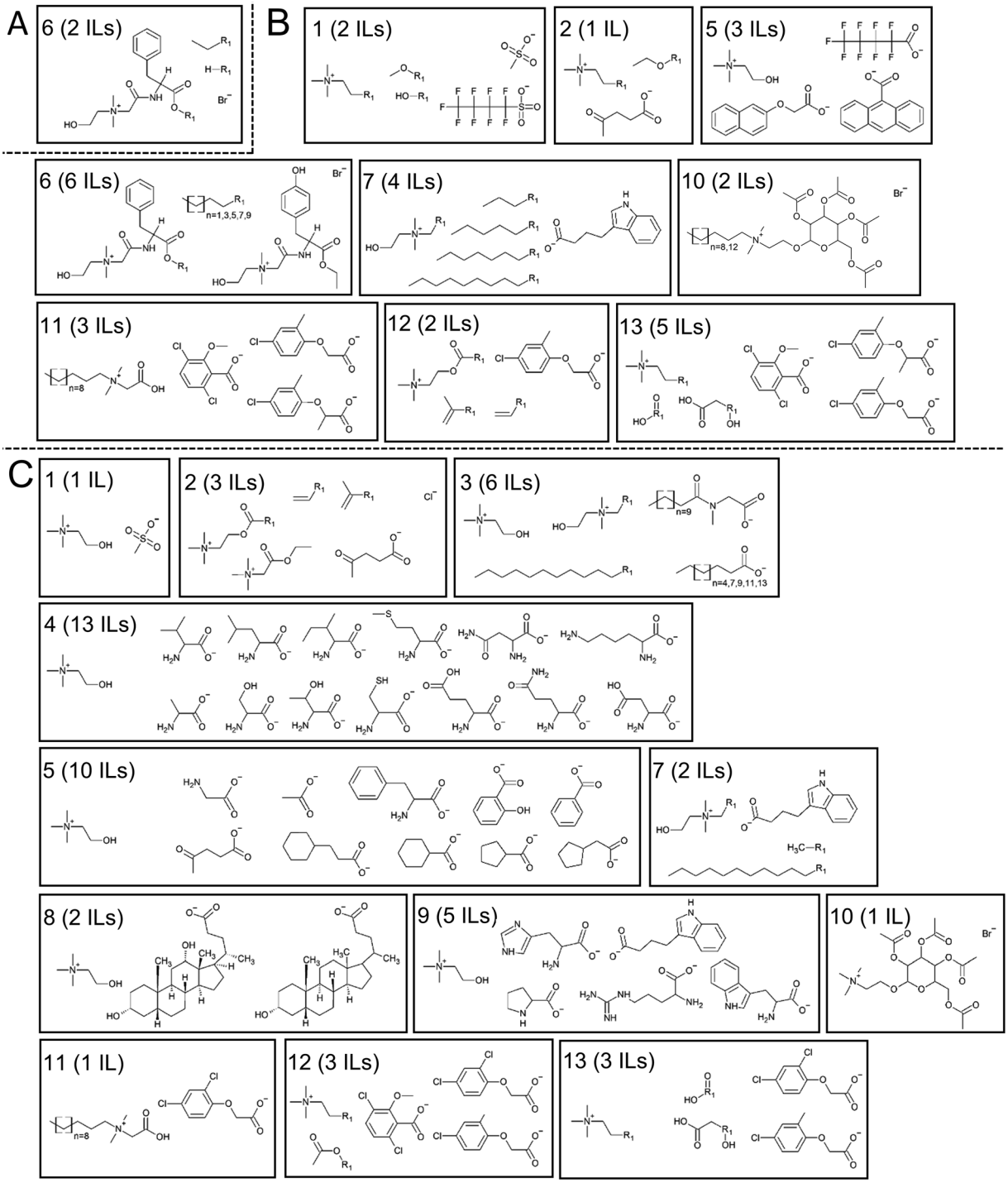


Fig. 7 Cholinium ILs, which were (A) biodegradable by ≤19%, (B) biodegradable by 20–59% and (C) biodegradable by ≥60%, per cluster.

robust statements on the influence of organic anions on biodegradability.

Phosphonium ILs

Clusters 4, 5 and 6 were the only ones that contained amber classified phosphonium ILs (Fig. 1E). All ILs had an octylsulphate or methylsulphate anion in common (clusters 4, 5, and 6, Fig. 8B). Structural analogues regarding the cation contained a bis(trifluoromethylsulphonyl)amide or halogenide anion and were classified red (clusters 1–3, 7, 8, and 10–12, Fig. 8A). Atefi *et al.* identified the octylsulphate anion to promote biodegradability.[108] Furthermore, the results showed that the methylsulphate anion promoted biodegradability, too, which is in line with RoT A1 (Table 1).

Nevertheless, the incorporation of the octylsulphate anion did not necessarily increase the biodegradability as the red

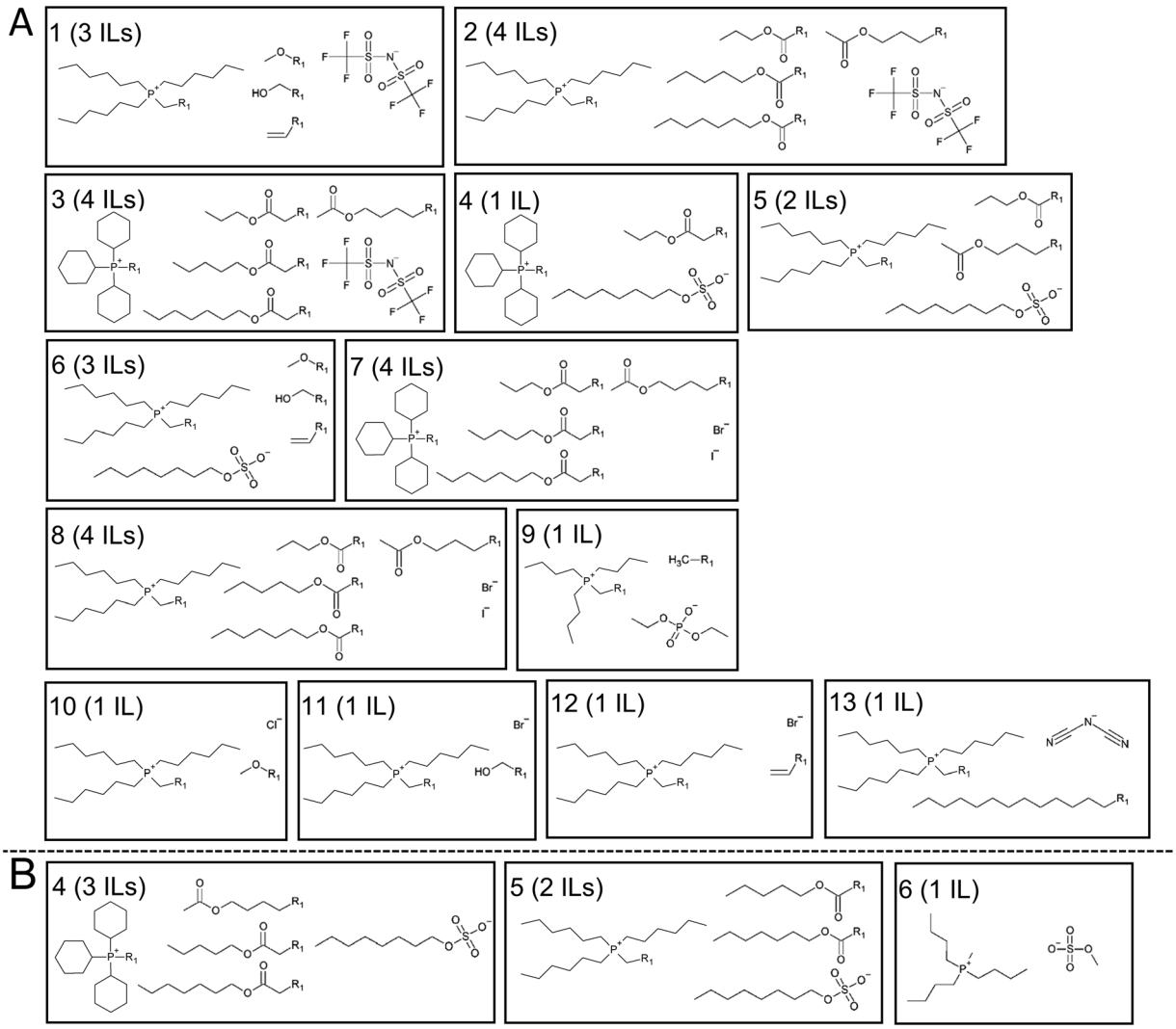


Fig. 8 Phosphonium ILs, which were (A) biodegradable by ≤19% and (B) biodegradable by 20–59% per cluster.

classified ILs in clusters 4, 5 and 6 showed (Fig. 8A). The red and amber classified ILs in clusters 4 and 5 had in common that they all contained an ester group, but differed in the *n*-alkyl chain length and the position of the ester group in this chain (Fig. 8). In cluster 5 an increase in biodegradability could be observed with an increase in the *n*-alkyl chain length (Fig. 8A and B). However, this was not the case in cluster 4. Therefore, the influence of the *n*-alkyl chain was not clear. Furthermore, Atefi *et al.* showed that the alkyl chain length had no influence on the biodegradability of the ILs in clusters 3, 7 and 8.[108] RoT S4 and S9 related to the promoting and hindering effect of alkyl side chain length (Table 1) could not be assessed since data was not available for phosphonium ILs consisting of an inorganic anion and a cation that just incorporates different *n*-alkyl side chains.

In clusters 1, 2 and 3 all ILs contained the bis(trifluoromethylsulphonyl)amide anion and differed in side chains attached to the cation. All were biodegradable ≤19% (Fig. 8A). Comparing the ILs in clusters 2 and 3 (Fig. 8A) to the cation analogues in clusters 4 and 5 (Fig. 8B) a hindering effect on biodegradability by the bis(trifluoromethylsulphonyl)amide anion was shown, which agrees with RoT A6 (Table 1). Furthermore, Atefi *et al.* identified for the ILs in clusters 1 and 2 that the bis(trifluoromethylsulphonyl) amide hindered biodegradability.[108]

Two ILs contained a diethylphosphate (cluster 9) and a dicyanamide anion (cluster 13), respectively, and were not biodegradable (Fig. 8A). Therefore, the anions did not promote biodegradability of these ILs. However, due to the limited data and missing reference compounds it was not possible to assess if the anion hindered biodegradability.

Since clusters 7, 8 and 11 contained non-biodegradable phosphonium cations with an ester or hydroxyl group in the side chain and an inorganic anion, the introduction of an ester or hydroxyl group into the side chains did not increase the biodegradability as shown by Atefi *et al.*[108] Therefore, RoT S1 and S2 were not valid for the tested phosphonium ILs.

Ether groups were included in the cation's side chains in clusters 1, 6 and 10 (Fig. 8A). Three ILs contained the same

cation and differed in the anion. They were classified as red (Fig. 8A). Therefore, the ether group did not have any promoting effect on biodegradability.[108] Hence, the results confirmed RoT S10 (Table 1).

In clusters 1, 6 and 12 three ILs, which contained an allyl group and differed in the anion, were classified red (Fig. 8A). Therefore, the allyl group did not promote biodegradability as Atefi *et al.* showed.[108] Hence, the results were in line with RoT S13 (Table 1).

Pyrrolidinium ILs

The ILs showing a biodegradability ≥60% were allocated to clusters 4, 6, 7 and 10 (Fig. 1F). Having a closer look at these ILs in Fig. 9C some inconsistencies appeared with regard to RoT A6 (Table 1) and the reported biodegradability. In cluster 4 the ILs were structurally characterized by different alkyl side chains in the cation and the anion was bis(trifluoromethylsulphonyl)amide (Fig. 9). Comparing 1-methyl-1-octylpyrrolidinium chloride (cluster 6, Fig. 9C) with 1-methyl-1-octylpyrrolidinium bis(trifluoromethylsulphonyl)amide (cluster 4, Fig. 9C) the biodegradability decreased from 69% to 60%, both measured by OECD 301F.[64,109] This is not a big difference in biodegradability considering the limitations and uncertainties in biodegradation testing related to the used inoculum.[115] Therefore, the biodegradability of 1-methyl-1-octylpyrrolidinium bis(trifluoromethylsulphonyl)amide in cluster 4 contradicts RoT A6 (Table 1). However, the bis(trifluoromethylsulphonyl)amide anion was present in red and amber classified ILs that differed in the side chain attached to the cation in clusters 4 and 5 (Fig. 9A and B). Based on this finding and because the bis(trifluoromethylsulphonyl)amide anion is known to be non-biodegradable[114] the effect on biodegradability was hindering. Eshetu *et al.* concluded for the ILs in cluster 4 that the biodegradability strongly depended on the side chain attached to the cation.[109]

The lauroyl sarcosinate anion had a promoting effect on biodegradability since 1-butyl-1-methylpyrrolidinium lauroyl sarcosinate was classified green (cluster 10, Fig. 9C) and the same cation combined with a bromide anion red (cluster 6, Fig. 9A). Therefore, RoT A3 was true for the lauroyl sarcosinate anion (Table 1).

In the dataset, there was only one IL with a linear alkylsulphate anion (cluster 2, Fig. 9A), which was classified as red. Therefore, this anion did not have any promoting effect on biodegradability as RoT A1 suggested (Table 1). To make a clear statement on the effect of the alkylsulphate anion data on ILs with this anion is needed. Additionally, more data is needed for anions based on amino acids to derive SBRs. In the dataset there was only one IL with such an anion (1-ethyl-1-methyl-pyrrolidinium L-cysteinate), which was classified as amber (cluster 11 in Fig. 9B).

The dicationic pyrrolidinium IL in cluster 1 contained multiple ether groups and was not biodegradable (Fig. 9A). More data is needed for the identification of the influence of dicationic cations since in the dataset there was only one dicationic IL. Regarding monocationic pyrrolidinium ILs with ether groups, the introduction in the alkyl side chain (cluster 5, Fig. 9A) had no positive influence on the biodegradability if

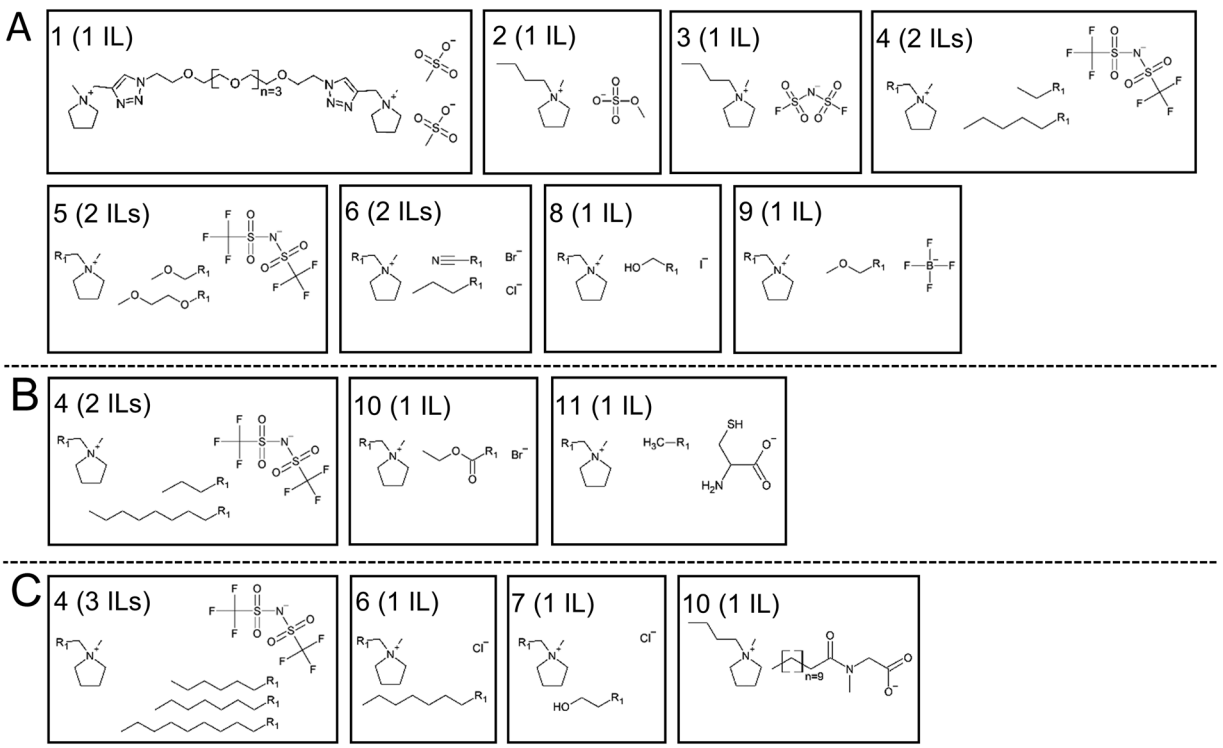


Fig. 9 Pyrrolidinium ILs, which were (A) biodegradable by ≤19%, (B) biodegradable by 20–59% and (C) biodegradable by ≥60%, per cluster.

compared to the corresponding ILs without ether groups (cluster 4, Fig. 9A and B), which were classified red and amber. The finding is in line with RoT S10 (Table 1). Furthermore, the finding by Samorì *et al.* that ether groups did not promote biodegradability is confirmed by comparing clusters 4 and 5.[111]

RoT S4 (Table 1) was proven true for 1-butyl-1-methylpyrrolidinium bromide (cluster 6, Fig. 9A) and 1-methyl-1-octylpyrrolidinium chloride (cluster 6 in Fig. 9C). For these two ILs Neumann *et al.* identified that the IL with the longer alkyl side chains was better biodegradable than the one containing the shorter alkyl residue.[64]

Furthermore, in cluster 4 the *n*-alkyl side chain length increased from propyl to decyl in the IL 1-alkyl-1-methylpyrrolidinium bis(trifluoromethylsulphonyl)amide. In this cluster the biodegradability of the ILs with a hexyl chain and nonyl chain was not in line with RoT S4 since the ILs were not or only slightly biodegradable (cluster 4, Fig. 9A and B), respectively, and the ILs with butyl, heptyl, octyl and decyl side chains were biodegradable (cluster 4, Fig. 9C).

Moreover, conflicting data was available for 1-butyl-1-methylpyrrolidinium bis(trifluoromethylsulphonyl)amide (cluster 4), which showed good biodegradability in the closed-bottle test (OECD 301D),[110] but no biodegradation in manometric respirometry test (OECD 301F).[111] Since the mean value was calculated as described in the methods part, this IL's biodegradability was classified as amber (Fig. 9B). This conflicting data could explain the discrepancy regarding RoT S4 (Table 1) in cluster 4 that there was no promoting effect between the butyl and hexyl side chain in 1-alkyl-1-methylpyrrolidinium bis(trifluoromethylsulphonyl)amide. Nevertheless, the missing promoting effect between the ILs with an octyl and nonyl chain in cluster 4 could not be explained. RoT S9 (Table 1) could not be attested based on the tested structures. Further biodegradation tests on pyrrolidinium ILs incorporating an inorganic anion and a cation, which differ in the *n*-alkyl side chain length are needed to make a statement on this RoT.

The results on the biodegradability of the ILs in clusters 7 and 8 showed that the effect of hydroxyl groups is not clear as the IL in cluster 7 was biodegradable and the one in cluster 8 was not, even though they were structurally related (Fig. 9A and C).

Further tests are needed for the effect of the introduction of a cyano or ester group into side chains as only for one IL (cluster 6, Fig. 9A and cluster 10, Fig. 9B, respectively) biodegradability data was available.

Structure–biodegradability relationships for cations with less than 12 compounds

Clusters generated by Schrödinger were not used to derive SBRs for ILs containing a morpholinium, DABCO, piperidinium, prolinium, piperazinium and thiazolium cation due to the small number of ILs.

Regarding the twelve morpholinium ILs only the morpholinium cation in combination with the lauroyl sarcosinate anion was biodegradable by ≥60% (Fig. 10C). However, a reference IL was missing in this dataset to explain if the cation was degraded or just the lauroyl sarcosinate anion.

Three morpholinium ILs contained a benzyl or cyano group or phenylalanine moiety in the cation and a halogenide anion. One IL contained a 4-butyl-4-methylmorpholinium cation and a tetrahexafluoroisopropoxyaluminate anion. All four ILs were not biodegradable (Fig. 10A). Compared to 4-ethyl-4-methylmorpholinium bromide, which was slightly biodegradable (Fig. 10B), the benzyl, cyano group and phenylalanine moiety had a hindering effect on biodegradability. Since 4-butyl-4-methylmorpholinium bromide was not biodegradable, the reason that 4-butyl-4-methylmorpholinium tetrahexafluoroisopropoxyaluminate was not biodegradable, could be related to the cation. Nevertheless, the tetrahexafluoroisopropoxyaluminate anion contains only four CH groups, each of which is protected by two trifluoromethyl groups and the Al–O group. Therefore, biodegradation is not likely to take place.

However, the assumptions related to the effect of the benzyl and cyano groups, the phenylalanine moiety and the tetrahexafluoroisopropoxyaluminate anion were only based on one IL per structural fragment or anion respectively and need further investigations that incorporate these structural fragments or anion in known biodegradable morpholinium ILs to identify if they have a hindering effect.

Two morpholinium ILs incorporated a hydroxyl group and a halogenide anion. The IL with a hydroxyethyl group was not biodegradable, whereas the one with the hydroxypropyl was slightly biodegradable (Fig. 10A and B). Further investigations of morpholinium ILs incorporating hydroxyalkyl chains with elongated *n*-alkyl chains (C > 3) are needed to clarify if the increased alkyl chain and therefore the hydroxypropyl compared to the hydroxyethyl group had the promoting effect on biodegradability.

Five 4-alkyl-4-methylmorpholinium bromide ILs differed in the *n*-alkyl chain (C2, C4, C6, C8, C10) (Fig. 10A and B). Only 4-ethyl-4-methylmorpholinium bromide was slightly biodegradable. The ILs with C4, C6, C8, and C10 alkyl chains were not biodegradable. Therefore, the elongated *n*-alkyl chains did not have a promoting effect on biodegradability as Pretti *et al.* concluded.[112] Hence, RoT S4 (Table 1) could not be confirmed.

Regarding the DABCO ILs, data on eight ILs was available. Five DABCO ILs containing *n*-alkyl side chains (C2, C4, C6, C8, C10) in the cation and a bromide anion were slightly biodegradable (Fig. 10B). The highest biodegradability was found for the IL with the ethyl group and the lowest biodegradability for the IL with the hexyl group contradicting RoT S4 (Table 1).

The non-biodegradable DABCO ILs contained *n*-alkyl side chains (C6 or C7) in the cation and a bis(trifluoromethylsulphonyl)amide anion or a hydroxyethyl group in the cation and a dicyanamide anion (Fig. 10A). Since the reference IL, 1-hexyl-1,4-diazabicyclo[2.2.2]octanium bromide, was slightly biodegradable, the bis(trifluoromethylsulphonyl)amide anion had a hindering effect. Based on the limited data, the effect of the elongated *n*-alkyl chains, the hydroxyl group and both organic anions on biodegradability was not clear and needs further investigations.

Seven ILs incorporating a piperidinium cation showed biodegradability between 0% and 100%. 1-(3-Hydroxypropyl)-1-

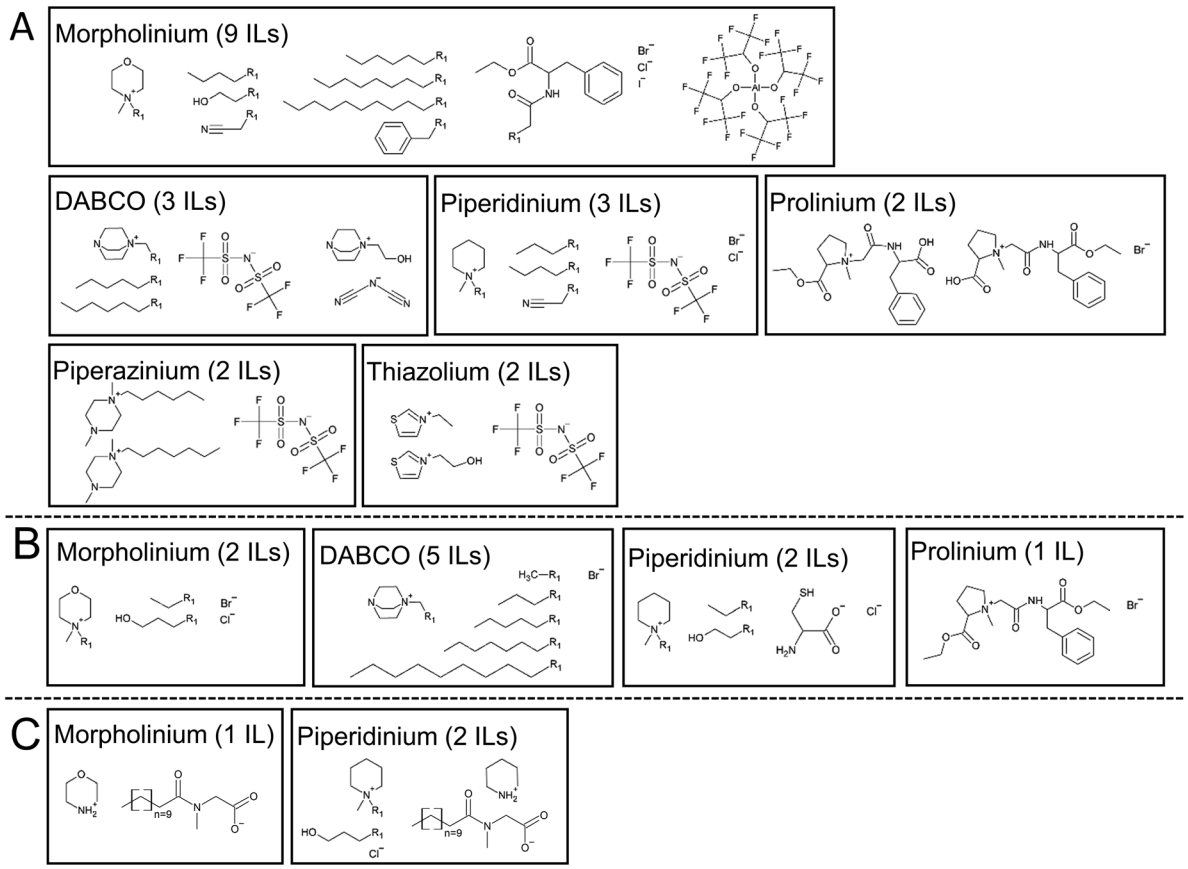


Fig. 10 Morpholinium, DABCO, piperidinium, prolinium, piperazinium and thiazolium ILs, which were (A) biodegradable by ≤19%, (B) biodegradable by 20–59% and (C) biodegradable by ≥60%.

methylpiperidinium chloride and piperidinium lauroyl sarcosinate were biodegradable ≥60% (Fig. 10C). Apparently, the hydroxyl group and the lauroyl sarcosinate anion enhanced the biodegradability of piperidinium ILs. However, 1-(2-hydroxyethyl)-1-methylpiperidinium chloride contained the hydroxyl group too, but was classified amber (Fig. 10B). The decreased biodegradability by the incorporation of a hydroxyethyl could be due to the decreased alkyl chain (RoT S4 Table 1). However, more data are needed to fully understand why the biodegradability decreased that strongly between 1-(3-hydroxypropyl)-1-methylpiperidinium chloride and 1-(2-hydroxyethyl)-1-methylpiperidinium chloride and if the lauroyl sarcosinate anion or the piperidinium cation were relevant for the biodegradability by ≥60%.

The cyano group in 1-(cyanomethyl)-1-methylpiperidinium chloride had no influence on biodegradability since this IL and 1-butyl-1-methylpiperidinium bromide were both not biodegradable (Fig. 10A). However, more data are needed and a reference compound that contains an ethyl and not a butyl side chain to clarify the influence of the cyano group.

Furthermore, the data of piperidinium ILs contained two ILs with an organic anion, which were 1-methyl-1-propylpiperidinium bis(trifluoromethylsulphonyl)amide and 1-ethyl-1-methyl-piperidinium cysteinate. The IL with the bis(trifluoromethylsulphonyl)amide anion was classified as red and the one with the cysteinate anion amber (Fig. 10A and B). More data is needed to clarify the influence of these anions.

The three prolinium ILs contained a bromide anion, a phenylalanine moiety and up to two ethyl groups linked *via* ester groups to the prolinium and phenylalanine moiety. The ILs differed in the presence or absence of the ester linkages to the ethyl groups (Fig. 10A and B). They were classified as red and amber showing that the presence or absence of the ester group had no effect on biodegradability.

The piperazinium ILs 1-hexyl-1,4-dimethylpiperazinium bis(trifluoromethylsulphonyl)amide and 1-heptyl-1,4-dimethylpiperazinium bis(trifluoromethylsulphonyl)amide were not biodegradable and classified red (Fig. 10A).

For thiazolium two ILs, 3-ethylthiazolium bis(trifluoromethylsulphonyl)amide and 3-(2-hydroxyethyl)thiazolium bis(trifluoromethylsulphonyl)amide were not biodegradable showing that the incorporation of the hydroxyethyl group did not increase biodegradability as found by Ford *et al.* (Fig. 10A).[87] Therefore, RoT S1 (Table 1) could not confirmed.

However, based on the limited data for the prolinium, piperazinium and thiazolium cation it was not possible to derive SBRs for the side chains and also for the anion since reference compounds, *e.g.* with an inorganic anion were missing.

The ready biodegradability data of morpholinium, DABCO, piperidinium, prolinium, piperazinium and thiazolium ILs revealed that most of the ILs were not biodegradable by ≥60% indicating that most of them do not seem to be suitable for the development of readily biodegradable ILs. However, more data are needed to clarify this finding.

Insights from the cluster analysis for designing mineralising ILs

The underlying assumption for Benign by Design is that even small changes in the structure of a chemical can have an enormous influence on its property or activity.[116] This effect, called "activity cliff", can be identified by comparing the activity or property of two similar compounds (meaning not the same).[117] The cluster analysis supported the evaluation of a large dataset from an overarching perspective, but also enabled us to get a deeper understanding of the SBRs when having a closer look at the ILs per cluster. Each cluster provided a good starting point to possibly identify the activity cliffs as each cluster consisted of similar compounds. The main issue was to identify the structural fragment, which was responsible for the better biodegradability when comparing two ILs, since often the ILs differed in too many structural fragments and reference compounds were missing. Overall, the cluster analysis facilitated to identify SBRs (Table 4).

The SBRs could be used for structural adjustments of amber and green classified ILs as these are possible candidates for fully mineralising ILs. Table 4 shows that for imidazolium and pyridinium ILs more SBRs were identified than for the remaining 10 cation categories. Due to missing reference compounds and the limited data availability some SBRs were not clear or could not be assessed. Since the aim is to develop safe and sustainable-by-design ILs to prevent environmental pollution and adverse effects on humans and the environment, some of the gaps in SBRs for the development of mineralising ILs should be addressed as a priority.

This study revealed that cholinium ILs are promising compounds for developing mineralising ILs, which is confirmed by RoT C1 (Table 1). The cholinium derivatives showing biodegradability were acetylcholine, betaine and carnitine. Other structural variations at the cholinium cation, as shown before, hindered biodegradability and should be avoided. Testing ILs that combine acetylcholine, betaine and carnitine with a halogenide anion and a biodegradable anion, like alkylsulphates, could give further insights into the influence of ester and carboxyl groups in the cation and close the gap for alkylsulphate anions.

As for the cholinium ILs, these structural fragments and anions should be used in the design of QACs to close gaps and test if these could be candidates for a benign IL.

The data of the pyrrolidinium ILs did not indicate if the cation core structure was biodegradable due to conflicts in data. The disagreement in the assessment of the pyrrolidinium cation was also shown in the literature for RoT C4 (Table 1). Since there were two pyrrolidinium ILs containing a hydroxyl group, but only one of them was biodegradable (clusters 7 and 8, Fig. 9), further tests are needed of ILs that vary in side chains to clarify if the hydroxyl group promotes biodegradability. Furthermore, side chains with ester and carboxyl groups should be incorporated in the pyrrolidinium cation and combined with a halogenide and alkylsulphate anion to close the gaps in SBRs.

In compliance with RoT C7 and C8 (Table 1), the results suggest that imidazolium and phosphonium ILs should not be used for the development of mineralising ILs. The imidazolium core structure was shown to be non-biodegradable by microbial community in activated sludge and metabolites were formed.[65,118] Only in 1-butyl-3-methylimidazolium chloride the imidazolium core was found to be biodegradable.[119] Moreover, the tested phosphonium cations were not biodegradable and slight biodegradability was only reached by an octylsulphate or methylsulphate anion. Therefore, closing the gaps in SBRs for imidazolium and phosphonium ILs is of low priority.

Due to the limited data on morpholinium, DABCO, piperidinium, prolinium, piperazinium and thiazolium ILs nearly all SBRs could not be assessed or clearly identified. Most of the ILs were not biodegradable even though promoting structural fragments according to the RoTs, *e.g.*, medium *n*-alkyl chains, hydroxyl groups, and ester, were incorporated in the ILs. Therefore, the results indicate that these cation core structures should not be of high priority to close gaps in SBRs and to design mineralising ILs.

Further tests according to OECD 302B (Zahn-Wellens test) should be conducted for ILs, which were biodegradable by 20–59%, to get insights on the inherent biodegradability. The Zahn–Wellens test measures the biodegradability under less strict conditions compared to ready biodegradability test methods (*e.g.*, higher bacteria density and diversity). The test could help to identify ILs, which could be candidates for a benign IL and need structural changes according to the here identified SBRs (Table 4).

As mentioned in the Introduction, in the safe and sustainable-by-design assessment further criteria than just environmental biodegradability have to be addressed, which are under development and have to be defined.[25] For an assessment a comprehensive database of high-quality data on biodegradability, toxicity, ecotoxicity and physicochemical properties of ILs is needed, which is not yet available to the best of our knowledge.

Limitations of this study

The dataset on the ready biodegradability of ILs based on Amsel *et al.*[37] was analysed regarding SBRs by *in silico* method clustering. It is a well-known challenge of *in silico* tools and especially of prediction models to process disconnected molecular structures like salts, mixtures and ILs since commonly used fingerprints or structural keys as molecular descriptors are not capable of differentiating between fully connected and disconnected molecules meaning the information that the molecule consists of an anion and cation is lost.[120] Regarding this study, even though MACCS structural keys did not accu-

Table 4 Structure–biodegradability relationships for improved biodegradability of ILs based on the findings of this study. Inorganic anions are not listed as they do not contain any oxidisable carbon. ⬇: hindering biodegradability, ⬆: promoting biodegradability, ⬇⬆: effect not clear, depending on side chain or more data needed, and —: no data

Fragment of IL	Structural fragment	Imid	Pyri	QACs	Chol	Phos	Pyrr	Morph	DABCO	Piperi	Prol	Pipera	Thia
Cation	Mono-cationic	⬇	⬆	⬇⬆	⬆	⬇	⬇⬆	⬇⬆	⬇⬆	⬇⬆	⬇⬆	⬇⬆	⬇⬆
	Polycationic	⬇⬆	⬇	—	—	—	⬇⬆	—	—	—	—	—	—
Side chains of the cation	Amide	⬇	⬇⬆	—	—	—	—	—	—	—	—	—	—
	Amino acid moieties	⬆	⬇	⬆	⬇	—	—	⬇⬆	—	—	—	—	—
	Allyl or vinyl	⬇⬆	⬇⬆	⬇⬆	⬆	⬇	—	—	—	—	—	—	—
	Alkyl chain length ≥C10	⬇	⬇	⬇⬆	⬇	—	—	—	—	—	—	—	—
	Alkyl chain length ≤C9	⬆	⬆ ≤C11	⬇⬆	⬇	⬇⬆	⬇⬆ ≤C10	⬇	⬇⬆	⬇⬆	—	⬇⬆	—
	Carboxyl	⬇⬆	⬆	—	⬇⬆	—	—	—	—	—	⬇⬆	—	—
	Ester	⬆	⬆	—	⬇⬆	⬇	⬇⬆	—	—	—	⬇⬆	—	—
	Ether	⬇⬆	⬇	—	⬇⬆	⬇	⬇	—	—	—	—	—	—
	Hydroxyl	⬇	⬆	⬇⬆	—	⬇	⬇⬆	⬇⬆	⬇⬆	⬇⬆	—	—	⬇⬆
	Cyano	⬇⬆	⬇⬆	—	—	—	⬇⬆	⬇⬆	—	⬇⬆	—	—	—
	Phenyl or benzyl	⬇	⬇⬆	⬇⬆	—	—	—	⬇⬆	—	—	—	—	—
	Increasing the number of side chains	⬇	—	⬇	—	—	—	—	—	—	—	—	—
Anion	Carboxylates, *e.g.*, alkyl carboxylates, cyclopentane or cyclohexane carboxylates, indole-3-butyrate, benzoate, salicylate, desoxycholate or lithocholate, lactate	⬆	—	⬆	⬆	—	—	—	—	—	—	—	—
	(2,4-Dichlorophenoxy)acetate or (4-chloro-2-methylphenoxy)acetate or 3,6-dichloro-2-methoxybenzoate a or 2-(4-chloro-2-methylphenoxy)propionate	—	—	—	⬇⬆	—	—	—	—	—	—	—	—
	Amino acids	⬆	—	⬇	⬆	—	⬇⬆	—	—	⬇⬆	—	—	—
	Polycyclic aromatic carboxylates (*e.g.* naphthoxyacetate and anthracene-9-carboxylate)	—	—	—	⬇	—	—	—	—	—	—	—	—
	Alkylsulphonates	⬇⬆	—	⬇⬆	⬆	—	—	—	—	—	—	—	—
	Alkylsulphates	⬆	⬆	⬇⬆	—	⬇⬆	⬇⬆	—	—	—	—	—	—
	Saccharinate and acesulfamate	⬆	⬇⬆	—	—	—	—	—	—	—	—	—	—
	Perfluoropentanoate	⬇⬆	⬇⬆	—	⬇	—	—	—	—	—	—	—	—
	Perfluorobutanesulphonate	⬇	—	—	⬇	—	—	—	—	—	—	—	—
	Bis(trifluoromethylsulphonyl)amide	⬇⬆	⬇	⬇	—	⬇	⬇	—	⬇⬆	⬇⬆	—	⬇⬆	⬇⬆
	Dicyanamide	⬇⬆	⬇	—	—	⬇⬆	—	—	⬇⬆	—	—	—	—
	Lauroyl sarcosinate	⬆	⬆	—	⬆	—	⬆	⬇⬆	—	⬇⬆	—	—	—

rately represent the anion and cation of ILs, information on the different structural fragments of the examined ILs for calculating the similarity was available. Therefore, MACCS structural keys were appropriate for clustering ILs, which our manual examination of the ILs per cluster confirmed. For future examination of SBRs using software that was designed to process only connected molecular representations an adaptation of MACCS structural keys to ILs is suggested.[41,120]

In this study only ILs were incorporated into the dataset, if they were called ILs in the data source. Nevertheless, QACs, cationic surfactants, like benzalkonium chloride, or anionic surfactants are in use for long and their biodegradability was assessed.[121,122] These were not included in this study if they were not described as ILs in the data source. These compounds, which were not called ILs, but consist also of an anion and cation could give further insights into the design of fully mineralising ILs. A global dataset of ionic compounds available in a database would help in this respect.

Since there was no database on high quality biodegradation data of ILs available, it was necessary to conduct a literature review to obtain the data.[37] In this regard the limitation was that the validity of the used data was restrained since the validation criteria were often not applied.[37] Furthermore, in the literature review mistakes could happen when extracting the structures of the literature data by drawing in order to generate the SMILES codes.[37]

The comparability of the biodegradation data obtained from the literature is also limited since the inoculum used in experimental testing varies between the sources. Regarding the standard test methods by OECD and ISO inoculum from different sources can be used leading to different biological conditions (*e.g.* different microorganism species and density).[115] Furthermore, the biodegradability of a specific IL may vary within a year and at different locations since the microorganism species and density change due to changing environmental conditions (*e.g.* temperature, pH, competing species, nutrients). Therefore, biodegradation tests are only limited reproducible.[115,123] For the development of readily biodegradable ILs it is necessary to take these factors into consideration. In the best case, a benign IL is biodegradable under different environmental conditions.

Regarding the interpretation of the clustering results, based on the used biodegradation data it was not possible to identify if a non-biodegradable IL or even a specific fragment of this IL was toxic to the inoculum. The clustering supports the identification of a change in biodegradability between similar ILs. Further research is needed to connect the changes in biodegradability with ecotoxicity data and to determine, which fragments are not biodegradable due to ecotoxicity.

Conclusion

Previous studies reviewed the experimental data of selected ILs to identify SBRs and to deduce RoTs. However, no study used an *in silico* tool to systematically analyse a large dataset based on the available literature data. Therefore, this study used an *in silico* cluster analysis for the first time to identify SBRs by evaluating a dataset of specifically clustered IL groups. The *in silico* cluster analysis successfully facilitated the SBR identification in the dataset that brought together the ready biodegradability results of 508 ILs of 68 experimental studies. Cluster analysis was required to understand the differences in structures and compare the ILs and their biodegradability. The approach of this study could also be used for datasets of other substance classes to identify SBRs from an overarching perspective.

The overview of SBRs for each cation category serves as a basis for the design of mineralising and non-persistent ILs as a contribution to the overall aim of safe and sustainable ILs. Furthermore, the findings indicate that some knowledge gaps in SBRs need to be closed to enhance the applicability of SBRs in the design of mineralising ILs.

Author contributions

Ann-Kathrin Amsel: conceptualization, methodology, data curation, formal analysis, investigation, visualization, writing – original draft, writing – review & editing, and validation. Oliver Olsson: conceptualization, data curation, methodology, supervision, writing – original draft, writing – review & editing, and validation. Klaus Kümmerer: conceptualization, methodology, funding acquisition, project administration, supervision, resources, writing – original draft, writing – review & editing, and validation.

Conflicts of interest

There are no conflicts to declare.

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Anhang der Publikation 3

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Modelling biodegradability based on OECD 301D data for the design of mineralising ionic liquids†

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Ionic liquids (ILs) are increasingly used, *e.g.* as solvents, electrolytes, active pharmaceutical ingredients and herbicides. If ILs enter the environment due to their use or accidental spills at industry sites, they can pollute the environment. To avoid adverse side effects of persistent ILs in the environment, they should be designed to fully mineralise in the environment after they fulfilled their function during application. (Quantitative) structure–biodegradability relationship models ((Q)SBRs) have been successfully applied in the design of benign chemicals. However, (Q)SBR models have not been widely applied to design mineralising ILs. Therefore, in this study we developed five quantitative structure–biodegradability relationship (QSBR) models based on OECD 301D data from the literature and our own in-house biodegradation experiments. These models can potentially be part of a test battery for designing fully mineralising ILs to increase the overall reliability of the biodegradability assessment and reduce uncertainties. Two datasets were formed and randomly divided into a training set with 233 and 321 compounds and a test set with 26 and 36 compounds, respectively. Both classification and regression models were built using molecular fragments with the aim to predict the classification and continuous biodegradation rate, respectively. The internal and external validations produced a R^2 of 0.620–0.854 for the regression models and accuracy, true positive rate, and true negative rate were between 62 and 100% for the classification models indicating an adequate performance but also a need for improvement. For the models and the test battery presented in this study, further research is needed to demonstrate their applicability.

1. Introduction

Ionic liquids (ILs) are of interest in various application areas because an IL can be tuned to the desired physical and chemical properties by changing its combination of cations and anions.[1] ILs have been examined in application areas such as solvents for cellulose, electrolytes in batteries, solvents for the preparation of perovskite photovoltaics, active pharmaceutical ingredients and herbicides.[2–6] Indeed many quaternary ammonium compounds (QACs) like benzylalkyldimethyl ammonium or alkyltrimethylammonium compounds, which are not called ILs in the literature, have commercial applications, *e.g.* as disinfectants.[7] Actually, they should be included in the group of ILs. ILs can be introduced in the environment through environmentally open applications, at the end-of-life of the mentioned products or accidental spills at industry sites. Some ILs have already been detected in surface water, sediments and wastewater effluents.[8,9] The environmental impact of ILs is of concern because of their (eco)toxicological effects and persistence.[10–14] Many ILs are not biodegradable in the aquatic environment.[15]

There are two categories of ILs: single ILs, which consist of a single, distinct cation and anion, and mixtures of ILs, which contain different cations and anions. For the purpose of this study, the term "ILs" exclusively denote single ILs. In the literature, 16 different cations have been tested for biodegradability ranging from imidazolium, QACs, pyridinium, cholinium, pyrrolidinium, piperidinium, prolinium, piperazinium, phosphonium, morpholinium, quinolinium, 1,4-diazabicyclo(2.2.2) octanium (DABCO), guandinium, sulphonium, thiazolium to triazolium.[15] The tested anions were either organic or inorganic. Organic anions were alkylsulphates, α-amino acids, bis(trifluoromethylsulphonyl)amide, carboxylic acids or dicyanamide. Inorganic anions included halides, tetrafluoroborate or hexafluorophosphate.[15,16] In total, ready biodegradability data are available for 508 ILs in the literature.[15] Of them, 120

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ILs have been identified to be more than 60% biodegradable according to ready biodegradability test methods like the OECD 301 series or ISO 14593.[15] However, just 34 ILs have reached the pass level for ready biodegradability, which is ≥60% biodegradation within 10 days starting from a degradation level of 10% as defined by the OECD guideline for the 301 series.[17–23] Therefore, it is reasonable to consider that IL's design should follow the concept of Benign by Design (BbD).[24] Accordingly, ILs should be designed from scratch for full mineralisation in the environment after their intended usage.[24] This means ensuring that both the anionic and cationic components of ILs undergo complete mineralisation either during wastewater treatment processes or within the natural environment. This aligns with the goal of creating safe and inherently sustainable chemical compounds, as outlined in the "Chemicals Strategy for Sustainability towards a Toxic-Free Environment" by the European Commission.[25]

(Quantitative) structure–biodegradability relationship models ((Q)SBR) can be applied to support the design of readily biodegradable and mineralising ILs. (Q)SBR models help make better informed decisions in the design process prior to the synthesis of chemicals, potentially saving time and resources.[26–31] Most of the models for biodegradability of non-charged chemicals, *e.g.* in EPISuite, Vega, MultiCASE and CATALOGIC, use ready biodegradability data measured according to the MITI test (OECD 301C) and predict either a continuous biodegradation rate or a classification into readily biodegradable or not.[32–36] Furthermore, the software CATALOGIC offers a model based on OECD 301F data which predicts the biodegradation pathway.[34] In fragment-based (Q)SBR models, the modelled relationships between structural fragments and biodegradability, also called alerts, have the advantage that they increase the interpretability of the predictions and help to understand why chemicals are biodegradable or not.[37,38] The relationship can give first insights into which structural adjustments might be needed to design a fully mineralising chemical.[26,27] ILs differ from non-charged organic compounds as they consist of two charged components, the anion and the cation. Both components have their own biodegradability potential if they are of organic nature. One of the components could be biodegradable, while the other may not. This is not represented in the overall biodegradation rate that is measured in tests according to OECD 301 or ISO 14593 and can lead to false conclusions in biodegradability.[39] Inorganic anions or cations lack carbon atoms that could be metabolised by microorganisms and thus do not contribute to the overall biodegradation rate of an IL. Consequently, ILs cannot be treated in modelling approaches similar to uncharged organic compounds and current modelling techniques need to be adapted to accommodate the unique characteristics of ILs.

Barycki *et al.* developed AquaBoxIL to predict the environmental distribution of an IL between water, sediment and organic matter.[40] The models for biodegradability in AquaBoxIL were the first and the only ones reported for ILs until now to the best of our knowledge. They were based on 77 ILs with OECD 310 (CO_2 headspace test) data. The training set included 52 ILs and the test set 25 ILs. 2D and 3D molecular descriptors were used for model building.[40] A classification tree assigns the query IL either to the readily biodegradable or not readily biodegradable class. Depending on the classification result a linear regression quantitative structure–biodegradability relationship (QSBR) model for persistent ILs (training set consisted only of ILs that are biodegradable by ≤60%) or one for readily biodegradable ILs (training set consisted only of ILs that are biodegradable by ≥60%) is applied to predict the percentage of biodegradability for the query IL.[40] AquaBoxIL was mainly built to predict the environmental distribution. Since the applied molecular descriptors are not always easy to interpret regarding structure–biodegradability relationships (SBRs), decisions on specific structural changes in the design for improved biodegradability are not straightforward.[41] Fragment-based models comprising structural alerts are needed for making better-informed decisions in the structural design of ILs.[30,42]

Therefore, this study presents newly developed fragment-based QSBR models based on a newly compiled OECD 301D dataset which comply with the OECD principles for validating (quantitative) structure–activity relationship ((Q)SAR) models.[43,44] We utilised data derived from OECD 301D, recognised as the most rigorous method within the OECD 301 series because readily biodegradable compounds according to this test will be completely biodegradable in surface water. Furthermore, this choice was made to ensure the comprehensive representation of various common ILs, such as imidazolium, pyridinium, QACs, and cholinium ILs, which have been extensively tested through OECD 301D, within our training dataset.[15] In total, five fragment-based QSBR models were developed using MultiCASE's FlexFilters platform[45] with regard to the ease of interpretation and deriving SBRs to support design decisions. Ordinary least squares (OLS) and logistic regression (LR) were used as modelling approaches to support prediction outcomes of continuous biodegradation rate and classification in biodegradable and non-biodegradable ILs, respectively. Additionally, an *in silico* test battery as part of the workflow proposed by Lorenz *et al.* for designing fully mineralising ILs was developed to discuss the possible applications of the models.[30]

2. Materials and methods

2.1 Experimental ready biodegradability data according to OECD 301D

From the Institute of Sustainable Chemistry (INSC) at Leuphana University (Prof. Kümmerer's working group), a dataset based on the in-house OECD 301D biodegradation experiment was provided. The OECD 301D guideline determines the ready biodegradability under aerobic conditions in water.[23] The dataset included 105 ILs (total 116 data points with measured biodegradation rates), 4 organic anions combined with an inorganic cation (6 data points) and 79 non-charged organic compounds (101 data points), which were

structurally related to the ILs. These data include information on whether the test substance is readily biodegradable or not and whether the test was valid. With the help of these data, the biodegradability of the individual compounds was evaluated. The in-house OECD 301D test was described in previous studies.[17,22] The same OECD 301D test protocol and the same inoculum source were used to generate the data. A test compound is considered to be readily biodegradable if it was degraded by ≥60% within a 10-day window starting after 10% degradation was reached.[23]

As per the protocol the biodegradation results were valid if the following conditions are met:

(1) the degradation rates in the duplicates of the test suspension did not differ by >20% after 28 d,

(2) the compound did not inhibit the degradation of the reference compound (sodium acetate) in the toxicity control (sodium acetate must be degraded ≥25% within 14 d based on its share of the total theoretical oxygen demand (ThOD),

(3) the oxygen concentration in the test vessels must not be <0.5 mg L^{-1},

(4) sodium acetate was degraded by ≥60% within 14 d in the positive control,

(5) the oxygen consumption is ≤1.5 mg L^{-1} after 28 d in the blank.

2.2 Compiling the training and test sets

Two datasets, *set_IL* and *set_ILNI*, were compiled to examine the influence of the larger dataset *set_ILNI* on model performance compared to the smaller dataset *set_IL*.[46] Two data sources were used to compile OECD 301D data, (a) data from the INSC in-house biodegradation experiments (section 2.1) and (b) literature data based on OECD 301D as compiled in Amsel *et al.*[15] The following criteria had to be met by the literature data of each IL: (i) tests lasted 28 d, (ii) the allowed concentration of the compound and inoculum of the allowed source was used, and (iii) mineralisation as the ratio between the biochemical oxygen demand (BOD) and the ThOD or chemical oxygen demand (COD) was measured. Sometimes none or just a few validation principles were reported for the data in the literature. Nevertheless, the data were used to expand the dataset. To increase *set_ILNI* the study on benzalkonium chloride of Sütterlin *et al.* was added.[47] The raw data were available and the applied OECD 301D method was similar to the one at INSC.

The literature data and the INSC data were combined. Duplicates were combined into one IL by calculating the mean biodegradation rate. The *set_IL* is just composed of ILs. For the ILs measured at the INSC, stereochemistry was included in the structures. However, the models were not able to consider stereochemistry in their predictions. The *set_ILNI* contained ILs, anions, and non-charged compounds. ILs differing in stereochemistry were considered as duplicates.

Without considering the structures, both *set_IL* and *set_ILNI* were randomly divided into a training and test set. For the test set 10% of the compounds in *set_IL* and *set_ILNI* were used as suggested.[48] The *train_set_IL* contained 233 ILs and the *test_set_IL* 26 ILs. The *set_ILNI* was randomly divided into the *train_set_ILNI* of 321 compounds and the *test_set_ILNI* of 36 compounds. The *train_set_ILNI* contained 73 non-ionic compounds, four anions and 244 ILs. The *test_set_ILNI* contained six non-ionic compounds and 30 ILs.

To characterise the training and test sets, the biodegradability data of the ILs, anions and non-ionic compounds were classified (red: 0–19%, amber: 20–59%, green: ≥60%). The classification is based on the OECD guidelines.[23,49] No or minimal biodegradability equals 0–19% degradation. Inherently biodegradable are compounds that degrade by 20–59% in ready biodegradability tests like OECD 301D.[49] Compounds classified as ≥60% are possibly readily biodegradable. If ≥60% of ThOD was removed within 10 days starting from a degradation level of 10% ThOD, compounds are readily biodegradable.[23]

2.3 Model building

Predictive QSBR models were built using the MultiCASE's FlexFilters platform.[45] The OECD principles for validating (Q)SAR models were followed to increase models' reliability and ensure that the models can be used for REACH registration.[43] The principles are as follows: (1) a defined endpoint, (2) an unambiguous algorithm, (3) a defined domain of applicability, (4) appropriate measures of goodness-of–fit, robustness and predictivity, and (5) a mechanistic interpretation, if possible.[44] Model building was essentially done in three steps: (i) fragmentation of the training compounds, (ii) selection of the most representative fragments (privileged fragments/substructures) that explain the variation of biodegradability of the training set chemicals, and (iii) building a regression model (OLS and LR for continuous regression and classification models, respectively) using these privileged substructures as descriptors. The details of the fragmentation and selection of the most representative fragments are described in the ESI.†

Two types of fragment descriptors were used: (i) fragments based on extended connectivity fingerprint (ECFP) type circular fragments[50] and (ii) element of a special continuous valued fingerprint containing 600 elements developed by Chakravarti.[51] The variable selection using L1 regularisation/Lasso regression was needed to limit the number of unique fragments obtained from the training set chemicals to prevent overfitting.[37,52–54] In the variable selection those structural fragments were picked that were relevant to the biodegradability potential of the training chemicals.

For the model IL_FP_cont 600 elements of the fingerprints were considered as descriptors. However, after the variable selection step using L1 regularisation/Lasso regression, only 61 elements were found to be relevant to biodegradability potential (Table S2†). For the models IL_Al_cont, IL_Al_class, ILNI_Al_cont and ILNI_Al_class 70, 29, 130 and 60 fragments, respectively, were found to be relevant (Tables S3, S4, S5 and S6†). These fragments are also called alerts.

Several modelling approaches are available, *e.g.* multiple linear regression, partial least squares, artificial neural network, random forest, support vector machine and many

more, which all have their strengths and weaknesses.[55] In this study, simple and well-known OLS and LR modelling in conjugation with fragment descriptors were used (second OECD principle for validating (Q)SAR models). Both approaches, OLS and LR, were chosen since they differ in the prediction outcome (continuous biodegradability rate and classification, respectively). Furthermore, they are easy to interpret due to the linear relationship between descriptors and the target property. On this basis, five models were developed to compare the different modelling approaches and training sets. Models using alerts as descriptors, OLS or LR, were built for both training sets, *train_set_IL* and *train_set_ILNI*. Additionally, OLS and elements of fingerprint as descriptors were used for a model based on *train_set_IL* (Table 1). The constructed regression models were then used for ready biodegradability prediction of new ILs. The continuous regression models' endpoint was ready biodegradability potential based on OECD 301D (ranging between 0 and 100%) (IL_FP_cont, IL_Al_cont, ILNI_Al_cont, Table 1). The classification models (IL_Al_class, ILNI_Al_class, Table 1) produced a probability value (ranging between 0.0 and 1.0), which can be separated in two classes (readily biodegradable and not readily biodegradable based on OECD 301D) by applying a threshold (usually 0.5) (first OECD principle for validating (Q)SAR models).

All models have in common that they were built using rigorously identified fragment-based activity privileged substructures, providing easy interpretability and a mechanistic interpretation to enable better-informed decisions in the design of readily biodegradable ILs. These fragments are annotated with the quantitative relationship with biodegradability (regression coefficients). While predicting a test chemical, these fragments are identified in the test chemical and therefore the mechanistic explanations for the predictions can be constructed (fifth OECD principle for validating (Q)SAR models). Hence, a non-biodegradable fragment could be replaced by a better biodegradable one to increase the biodegradability of the whole IL. Both approaches, ordinary and logistic regression were chosen since they have an advantage over the other at different steps in the design process. A classification is appropriate for design decisions at the beginning of the process as they can be used to separate ILs into biodegradable and non-biodegradable ones. The classification provides first insights into which ILs should be focused on to develop readily biodegradable ILs. In contrast, after the first classification step continuous biodegradability rates help to answer questions like which IL of the biodegradable ones is the best biodegradable IL or which IL is the best candidate for further structural adjustments when all ILs are not biodegradable.

2.4 Model validation

The validation of QSBRs was divided into internal and external validation as proposed by OECD to assess the goodness-of-fit and the predictivity, respectively (details are described in the ESI†).[44] The typical performance measures accuracy, sensitivity (true positive rate, TPR), specificity (true negative rate, TNR) and area under the curve (AUC) were evaluated for classification models (Table S7†), since they help to understand the model's performance in predicting both classes, biodegradable and non-biodegradable ILs.[44,48] For the OLS models the commonly used squared correlation coefficient R^2 was evaluated which ranges from 0.0 to 1.0 (Table S7†).[44,48]

For the development of new (Q)SBR models, it is important to define the domain of applicability (AD) of the models to prevent potentially unreliable results for query chemicals with very different chemistry. The AD is a "theoretical region in chemical space" and depends on the chemicals in the training set and the descriptors used to model the endpoint.[56] In general, the AD informs about to which chemical structures the models can be applied.[57] Clustering was performed using the "*R*" package *rtsne* to visualise and study the chemical space defined by the ILs.[58] The two-dimensional (2D) *t*-distributed stochastic neighbour embedding (*t*-SNE) methodology was applied.[59] The 600-element continuous-valued fingerprints of the ILs (section 2.3) were used for clustering.

2.5 Developing an *in silico* test battery for designing fully mineralising ILs

The ECHA recommends applying all available independent and valid models for one endpoint to increase the overall reliability of the prediction.[43] Independent models means that the models differ in descriptors, structural alerts or training sets.[43] Therefore, an *in silico* test battery was developed to structure the application of the newly developed models in the

Table 1 Set-up for the five biodegradability models of ILs. Logistic regression (LR) and ordinary least squares (OLS)

	Model 1 IL_FP_cont	Model 2 IL_Al_cont	Model 3 IL_Al_class	Model 4 ILNI_Al_cont	Model 5 ILNI_Al_class
Training set number of chemicals	233 Ionic liquids			321 Ionic liquids and non-ionic compounds	
Test set number of chemicals	26 Ionic liquids			36 Ionic liquids and non-ionic compounds	
Number of descriptors/alerts	61	70	29	130	60
Techniques	Fingerprints, OLS	Alerts, OLS	Alerts, LR,	Alerts, OLS	Alerts, LR,
Prediction outcome	Continuous rate in % of the ThOD	Continuous rate in % of the ThOD	Classification in biodegradable or not	Continuous rate in % of the ThOD	Classification in biodegradable or not

design process of mineralising ILs. As outlined in the workflow for the benign design of newly or redesigned chemicals using *in silico* tools in a study by Lorenz *et al.* an *in silico* test battery supports the identification of the most promising molecules regarding improved environmental biodegradability.[30] The workflow by Lorenz *et al.* started with a pool of molecules that were generated by one of the BbD approaches, which are the targeted or non-targeted *de novo* and targeted or non-targeted redesign.[30] This pool was the starting point for the development of the *in silico* test battery in this study, which aims to limit the pool of molecules to the most promising ones regarding environmental biodegradability by combining different models for this property and guide their application.

3. Results and discussion

3.1 Training and test sets used for modelling

Applying the criteria defined in section 2.2 to the literature data, 25 studies out of 31 containing OECD 301D data were appropriate for the dataset. From the literature in total 231 data points were collected for 201 ILs (dataset, Table 2). 7 of 25 studies contained data measured in the in-house OECD 301D biodegradation experiment at INSC. These 7 studies reported 77 data points for 75 ILs. For *set_IL* 192 ILs from the literature were combined with 75 ILs measured in the in-house OECD 301D biodegradation experiment at INSC (Table 2). After removing the duplicates, *set_IL* contained 259 ILs that differed in the organic cation and the side chains attached to it and were combined with organic or inorganic anions. For *set_ILNI* 196 ILs from the literature were used after removing the stereoisomers from the dataset. These data were combined with 90 ILs, four organic anions combined with an inorganic cation, 79 non-ionic compounds from the INSC in-house OECD 301D data leading to a total number of 357 compounds (Table 2).

The ILs in *set_IL* and *set_ILNI* were the only ones for which OECD 301D data were available that complied with the criteria defined for the literature data in section 2.2. Using OECD 301D data ensured the inclusion of many common ILs, like imidazolium, pyridinium, QACs and cholinium ILs.[15] More than 50% of the compounds in *set_IL* and *set_ILNI* were measured in the same laboratory at the INSC using the same OECD 301D test protocol and the same inoculum source. Data from the INSC were used for 139 of 259 ILs in *set_IL* and for 240 of 357 compounds in *set_ILNI*. The *set_IL* and *set_ILNI* were randomly divided into a training set of 233 and 321 compounds and a test set with 26 and 36 compounds, respectively.

The number of compounds per compound category and biodegradability in the *train_set_IL* and the *test_set_IL* are shown in Fig. 1. The prevalent cations in the *train_set_IL* were imidazolium (75 ILs), pyridinium (61 ILs), QACs (40 ILs) and cholinium (43 ILs) (Fig. 1A). Just a few morpholinium (3 ILs), pyrrolidinium (3 ILs), piperidinium (2 ILs), prolinium (5 ILs) and phosphonium (1IL) ILs were in *train_set_IL* (Fig. 1A). Most of the ILs in the *test_set_IL* were imidazolium (10 ILs), pyridinium (7 ILs), QACs (3 ILs) and cholinium (4 ILs). Additionally, one prolinium IL and one phosphonium IL were included in the *test_set_IL* (Fig. 1B).

In each category, most of the compounds in *train_set_IL* and *test_set_IL* were not equally distributed over the biodegradability classes. Just for QACs in the *test_set_IL* there is an equal number of compounds per class. Since *set_IL* was randomly divided into a training set and a test set, the distribution of ILs over biodegradability classes and compound category was not influenced. Without considering the compound category, the compounds were not equally distributed over the biodegradability classes as well. In the *train_set_IL*, 87, 99 and 47 ILs can be assigned to the biodegradability classes 0–19%, 20–59% and ≥60%, respectively. In the *train_set_IL*, the number of biodegradable ILs is less than that for non-biodegradable and slightly biodegradable ILs. In the *test_set_IL* seven ILs were biodegradable by 0–19%, 15 ILs by 20–59% and four ILs by ≥60% showing a higher number for slightly biodegradable ILs than for biodegradable and non-biodegradable ILs.

Compared to *set_IL*, *set_ILNI* comprised additional compounds to examine the influence of the larger dataset *set_ILNI*

Table 2 Data used for *set_IL* and *set_ILNI*

		Dataset	set_IL	set_ILNI
Literature data	ILs (data points)	201 (231) of them measured at INSC: 75 (77)	192 (222) of them measured at INSC: 67 (69)	196 (231) of them measured at INSC: 70 (77)
	Anions (data points)	0	0	0
	Non-charged compounds (data points)	0	0	0
INSC in-house OECD 301D data	ILs (data points)	105 (116)	75 (79)	90 (116)
	Anions (data points)	4 (6)	0	4 (6)
	Non-charged compounds (data points)	79 (101)	0	79 (101)
Number after removing duplicates	ILs	294	259	274
	Anions	4	0	4
	Non-charged compounds	79	0	79
Total number of compounds		377	259	357
Characteristics		Stereoisomers, ILs, anions, non-charged compounds	Stereoisomers, just ILs	No stereoisomers, ILs, anions, non-charged compounds

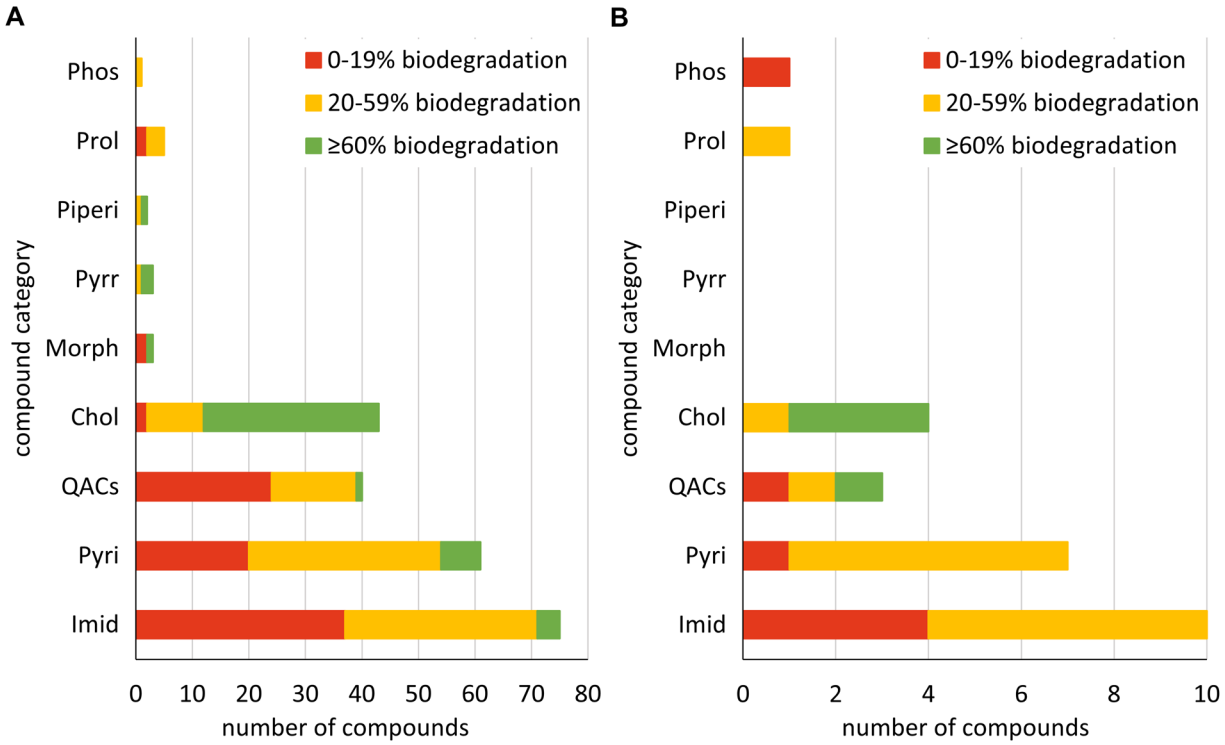

Fig. 1 Characterisation of the *train_set_IL* and *test_set_IL*. Classification of biodegradation data of ILs in (A) the training set and (B) the test set. The number of compounds for each compound category relates to the different combinations of side chains attached to the cation core structure and the anions. The biodegradation classification refers to the whole IL including side chains and anions. Imidazolium (Imid), pyridinium (Pyri), quaternary ammonium compounds (QACs), cholinium (Chol), morpholinium (Morph), pyrrolidinium (Pyrr), piperidinium (Piperi), prolinium (Prol), and phosphonium (Phos).

on model performance. Therefore, one piperazinium and one thiazolium IL, as well as four anions (organic anion in combination with inorganic cation) and 79 non-ionic compounds that are structurally related to the ILs were used for *set_ILNI*. Similar to *train_set_IL* and *test_set_IL* most of the ILs belong to the categories of imidazolium (79 ILs), pyridinium (69 ILs), QACs (37 ILs) and cholinium ILs (41 ILs) (Fig. 2). Additionally, the *test_set_ILNI* contained one prolinium and one thiazolium IL. The non-ionic compounds were not divided into different categories to show the ratio between ILs and non-ionic compounds in Fig. 2. Regarding the biodegradability of the compounds in both subsets, in each category, the compounds in *train_set_ILNI* and *test_set_ILNI* were not equally distributed over the biodegradability classes. Without considering the categories, the compounds are nearly equally distributed over the biodegradability classes. In the *train_set_ILNI* of a total of 321 compounds, 117 compounds were biodegradable by 0–19%, 105 by 20–59% and 99 by ≥60%. In the *test_set_ILNI* of a total of 36 compounds 12 compounds were biodegradable by 0–19%, 11 by 20–59% and 13 by ≥60%.

Fig. 1 and 2 show that the number of ILs in the presented datasets is not equally distributed among the compound categories. It was shown that more valid biodegradation data based on OECD 301D is needed for morpholinium, pyrrolidinium, piperidinium, prolinium, phosphonium, piperazinium, thiazolium, guandinium, DABCO, quinolinium, sulphonium and triazolium ILs.[15]

3.2 Biodegradability models for ILs and validation results

The five models were developed with respect to the OECD principles for validating (Q)SAR models (Table S8†). The endpoint was biodegradability according to OECD 301D, and the models predict a continuous value as the percentage of ThOD (models IL_FP_cont, IL_Al_cont, ILNI_Al_cont) or a classification in biodegradable or non-biodegradable ILs (models IL_Al_class, ILNI_Al_class).

The results of internal and external validation (section 2.4) are summarised in Table 3. The model IL_AL_class had better accuracy in the training (98%) and test set (96%) compared to model ILNI_Al_class (92% in the training set and 81% in the test set) meaning it classified the ILs more correctly into biodegradable and non-biodegradable. Furthermore, the model IL_AL_class showed better sensitivity and specificity in both the training set (91% and 100% respectively) and test set (75% and 100% respectively). Therefore, IL_AL_class also assigned the ILs more correctly to single classes, biodegradable and non-biodegradable, than ILNI_Al_class. Both models have in common that they had a better specificity than sensitivity meaning they were better in predicting non-biodegradable compounds correctly. The AUC value of the test set was larger

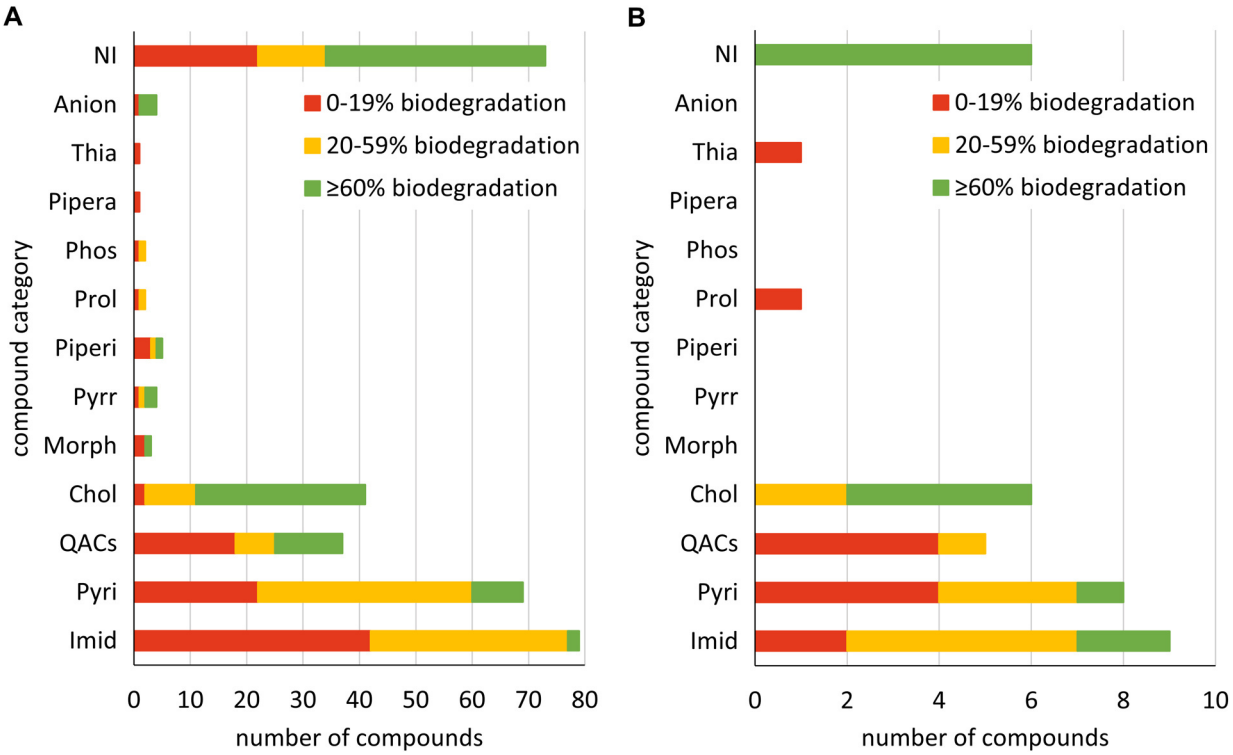

Fig. 2 Characterisation of the *train_set_ILNI* and *test_set_ILNI*. Classification of biodegradation data of compounds in (A) the training set and (B) the test set. The number of compounds for each compound category relates to the different combinations of side chains attached to the cation core structure and the anions. The biodegradation classification refers to the whole IL including side chains and anions. Imidazolium (Imid), pyridinium (Pyri), quaternary ammonium compounds (QACs), cholinium (Chol), morpholinium (Morph), pyrrolidinium (Pyrr), piperidinium (Piperi), prolinium (Prol), phosphonium (Phos), piperazinium (Pipera), thiazolium (Thia), and non-ionic (NI).

Table 3 Results for internal and external validation. Area under the curve (AUC), true negative rate (TNR), and true positive rate (TPR)

		Model 1 IL_FP_cont	Model 2 IL_Al_cont	Model 3 IL_Al_class	Model 4 ILNI_Al_cont	Model 5 ILNI_Al_class
Internal validation	Accuracy	—	—	98%	—	92%
	TPR	—	—	91%	—	80%
	TNR	—	—	100%	—	96%
	AUC	—	—	0.99	—	0.97
	R^2	0.814	0.843	—	0.788	—
External validation	Accuracy	—	—	96%	—	81%
	TPR	—	—	75%	—	62%
	TNR	—	—	100%	—	91%
	AUC	—	—	0.82	—	0.90
	R^2	0.854	0.687	—	0.620	—

for the model ILNI_Al_class (0.90) than for the model IL_AL_class (0.82) (Table 3). In contrast, the AUC in the training set for IL_AL_class was 0.99 and therefore larger than that for ILNI_Al_class (AUC of 0.97 in the training set). Since the AUC is higher than 0.5 the models are able to discriminate between biodegradable and non-biodegradable ILs.

Of all OLS models, IL_Al_cont had the best goodness-of-fit (R^2 of 0.843 for the training set). The model ILNI_Al_cont had the worst goodness-of-fit (R^2 of 0.788 for the training set) and the worst predictivity (R^2 of 0.620 for the test set) (Table 3). The model IL_FP_cont showed a R^2 of 0.854 for the test set. Compared to model IL_Al_cont (R^2 of 0.687 for the test set) and ILNI_Al_cont (R^2 of 0.620 for the test set) model IL_FP_cont had therefore the best predictivity of continuous values for biodegradability. The plots for comparison of the predicted *vs.* experimental biodegradation rates for the continuous regression models are visualised in Fig. S1–S6.† The validation results for the models ILNI_Al_cont and ILNI_Al_class were worse than those for the models IL_Al_class and IL_Al_cont. The results showed that the *train_set_ILNI* compared to *train_set_IL* did not increase the performance. Overfitting of the models is not very likely. On

the one hand, the results indicate that the performance metrics of the internal and external validation are not very different. On the other hand, the number of descriptors is lower than the training data points and was limited to the relevant ones for the biodegradability potential using L1 regularisation/Lasso regression.

In order to compare the performance of the newly developed models with models for biodegradability from the literature for charged and non-charged compounds the performance measures and the training sets have to be considered. To assess whether a model's algorithm is better than the other, the same training and test set and performance measures have to be used.[60] Since the used training sets in this study were not used in other studies, it is not possible to assess whether the models from the literature are better or worse performing. The classification model in the AquaBoxIL showed an accuracy, sensitivity and specificity for the test set of 96%, 94% and 100%, respectively.[40] R^2 was 0.726 for the model for persistent ILs and 0.881 for the model for readily biodegradable ILs.[40] The accuracy, sensitivity, specificity and R^2 related to the test set of the classification model and the two linear regression models are larger than those of the models presented here in this study. However, the training set of the model in Barycki *et al.*[40] contained 52 ILs and is therefore smaller than the training sets used in this study (233 and 321 ILs). The smaller the number of ILs, the less skewed the distribution of readily biodegradable and not readily biodegradable ILs, and the structural similarity of biodegradable and non-biodegradable ILs could have positively influenced the performance.[61] This cannot be proven since the experimental biodegradation data used for AquaBoxIL and information on the structures in the training and test set were missing.[40]

The performance of previous models for non-charged compounds was between 69 and 92% for accuracy, sensitivity, and specificity and between 0.7 and 0.9 for R^2.[32–35,42,62–64] The performance is similar to the newly developed models IL_FP_cont, IL_Al_class and ILNI_Al_class in this study. However, Table 3 shows that models IL_Al_cont and ILNI_Al_cont are not in the range of 0.7–0.9 for R^2 of models described in the literature. Nevertheless, the combination of fragment-based descriptors with OLS and LR for model building resulted in adequate models for predicting the biodegradability of ILs. To improve the models' performance, an increase of the size of the datasets while covering a wider variety of structural classes might be considered.

3.3 Clustering of the training sets

Fig. 1A and 2A provide first insights into the AD since they show which cations were included in the training sets. The models can only be applied to ILs incorporating these cations. However, for every new query IL a check is needed whether it falls within the AD or not since the side chains attached to the cation and the anion have an influence on this, too. Examples for anions, cations and side chains attached to the cation are visualised for *train_set_IL* in Fig. 3 and for *train_set_ILNI* in Fig. 4. The tSNE coordinates are available in Tables S9 and S10† for every compound. In the *t*-SNE plots in Fig. 3 and 4 compounds with different structural fragments are located away from each other, while similar compounds are located close to each other. The 600-element continuous-valued fingerprints were able to separate the compounds regarding their structural fragments and their biodegradability as clusters of the same colour show (Fig. 3 and 4). Imidazolium, pyridinium ILs, and QACs are mainly non-biodegradable as can been seen from the structural fragments shown for each cluster. Most of the biodegradable ILs belong to the group of cholinium ILs. Some α-amino acids, which are just available in *train_set_ILNI* and highlighted as non-ionic compounds in Fig. 4B, are biodegradable as well.

ILs were not available for every combination of cations, anions and side chains. Predictions for ILs that are structurally not related to the training set compounds would be based on extrapolations and could be possibly unreliable.[37] Therefore, the model's performance (Table 3) is only provided within the AD.

Most of the ILs available in the training sets are imidazolium, pyridinium, QACs and cholinium ILs. Only a few morpholinium, pyrrolidinium, piperidinium, prolinium, phosphonium, piperazinium and thiazolium ILs were included. Therefore, the AD is broader for imidazolium, pyridinium, QACs and cholinium ILs than for the under-represented ILs. The models cannot predict the biodegradability of ILs that are not represented in the training sets and therefore not structurally related to the training set compounds, *e.g.* DABCO, guandinium, quinolinium, sulphonium and triazolium, and of mixtures of ILs. Therefore, there is a need for more experimental data based on OECD 301D for morpholinium, pyrrolidinium, piperidinium, prolinium, phosphonium, piperazinium, thiazolium, guandinium, DABCO, quinolinium, sulphonium and triazolium ILs to enlarge the AD.

The ADs of the models for biodegradability prediction in the AquaBoxIL were visualised in a William's and Insubria plot.[40] Both plots identify response outliers and chemicals that are outside the AD due to their structure. The plots differ in their applicability. The William's plot can be used for chemicals for which experimental data are available, while the Insubria plot is used for chemicals without experimental data.[48,65] However, it was not mentioned, which structural features the training and test set contained. Therefore, it is not possible to compare the AD of AquaBoxIL and the models presented in this study.

3.4 Application of models for designing environmentally mineralising ILs

The five models were developed to support the design of biodegradable ILs. Since they differ in the training set or descriptors or structural alerts, they can be considered as independent from each other. Therefore, according to ECHA[43] all five models should be applied to increase the reliability of the overall biodegradability assessment of ILs. Hence, this section explores how these models could be applied in an *in silico* test battery (Fig. 5) that is part of the workflow for the benign

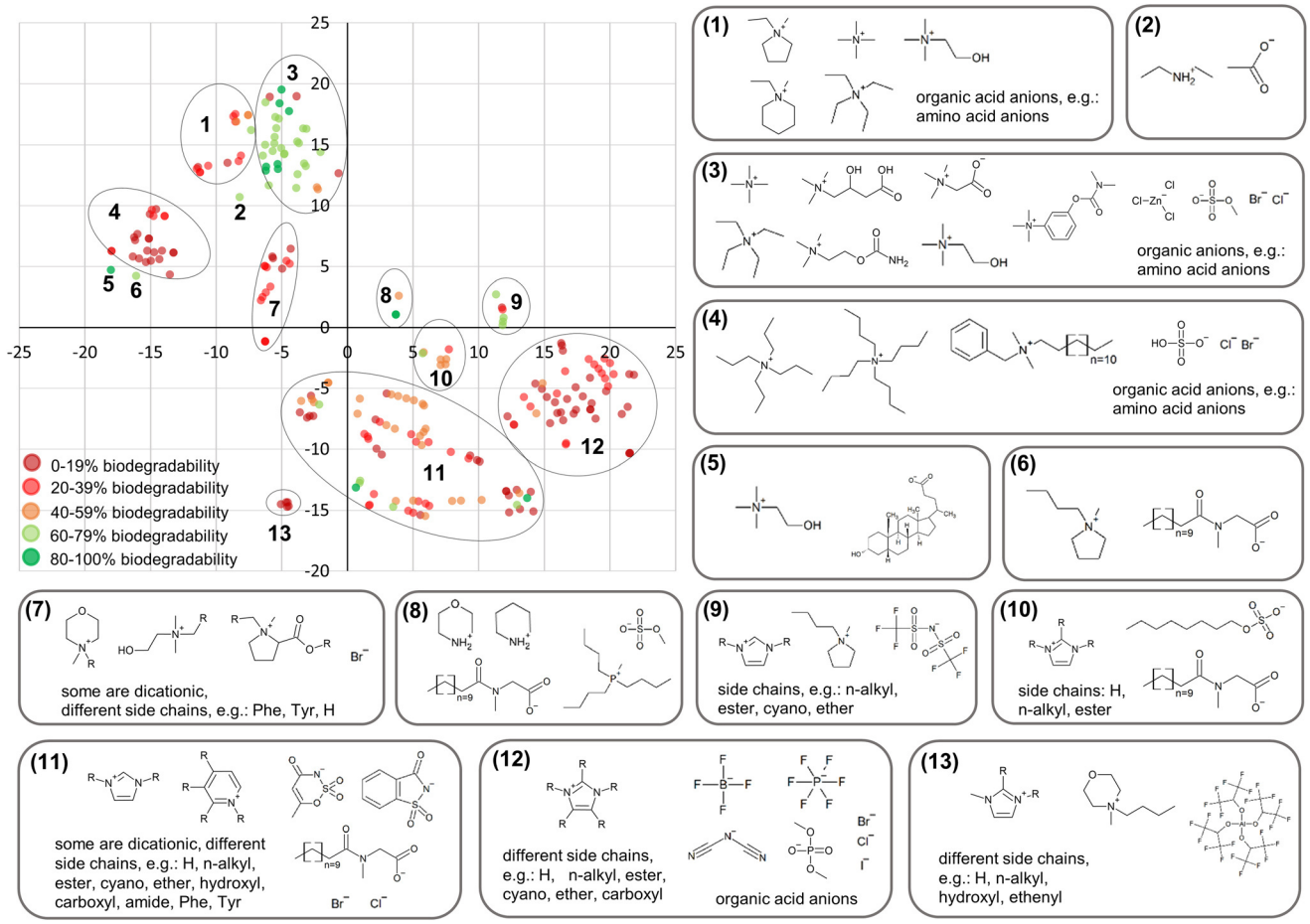

Fig. 3 Structural fragments of compounds in *train_set_IL* and *t*-SNE plot highlighted for the biodegradability. Phenylalanine (Phe) and tyrosine (Tyr).

design of chemicals presented in Lorenz *et al.*[30] The test battery starts with a pool of ILs either in the *de novo* or redesign of chemicals (Fig. 5). Just statistical QSBR models are included since these are the only ones available for ILs and the endpoint ready biodegradability according to OECD 301D.

The advantage of biodegradability alerts was demonstrated in the rational redesign of atenolol and metoprolol.[26,27] Accordingly, Fig. 5 proposes to apply IL_Al_class and ILNI_Al_class at first to gain insights from two independent models and two different sets of alerts (one for each model). The models facilitate to separate the pool of new or redesigned ILs into biodegradable and non-biodegradable ILs (Fig. 5). The model ILNI_Al_class performs not as well as IL_Al_class, but contains more alerts compared to IL_Al_class (60 compared to 29). Therefore, ILNI_Al_class helps to understand why some ILs are biodegradable and others are not. In this step three different outcomes are possible: 1. all ILs in the pool of newly developed or redesigned ILs are biodegradable, 2. some are biodegradable and some are not, and 3. no IL is biodegradable.

However, after the classification, a continuous biodegradation rate is needed to decide which IL in the class of biodegradable ILs (outcomes 1 and 2) or non-biodegradable ILs (outcome 3) is the best candidate to change structural fragments and to design a biodegradable IL. The approach of first using a classification model and then a model predicting a continuous value was demonstrated to be useful for prioritising chemicals in chemical safety assessment regarding their carcinogenicity.[66] Therefore, this study suggests the combination of a classification and a continuous biodegradation rate in Fig. 5 to support the prioritisation. In this respect, both models IL_Al_cont and ILNI_Al_cont are suitable since they generate a continuous biodegradation rate and their performance is adequate (Table 3). If in outcomes 1 and 2 the predicted biodegradation rate is ≤60% structural adjustments are needed and the workflow would start from the beginning with the pool of molecules. The models' alerts and the identified SBRs in Amsel *et al.* could support to identify which structural changes are needed.[16] In particular, the 130 alerts in model ILNI_Al_cont, the model with the most alerts, give insights into SBRs and could help to design a fully mineralising IL.

The model IL_FP_cont should be applied to confirm the predictions of IL_Al_cont and ILNI_Al_cont since it generates continuous rates with the best performance in the test set

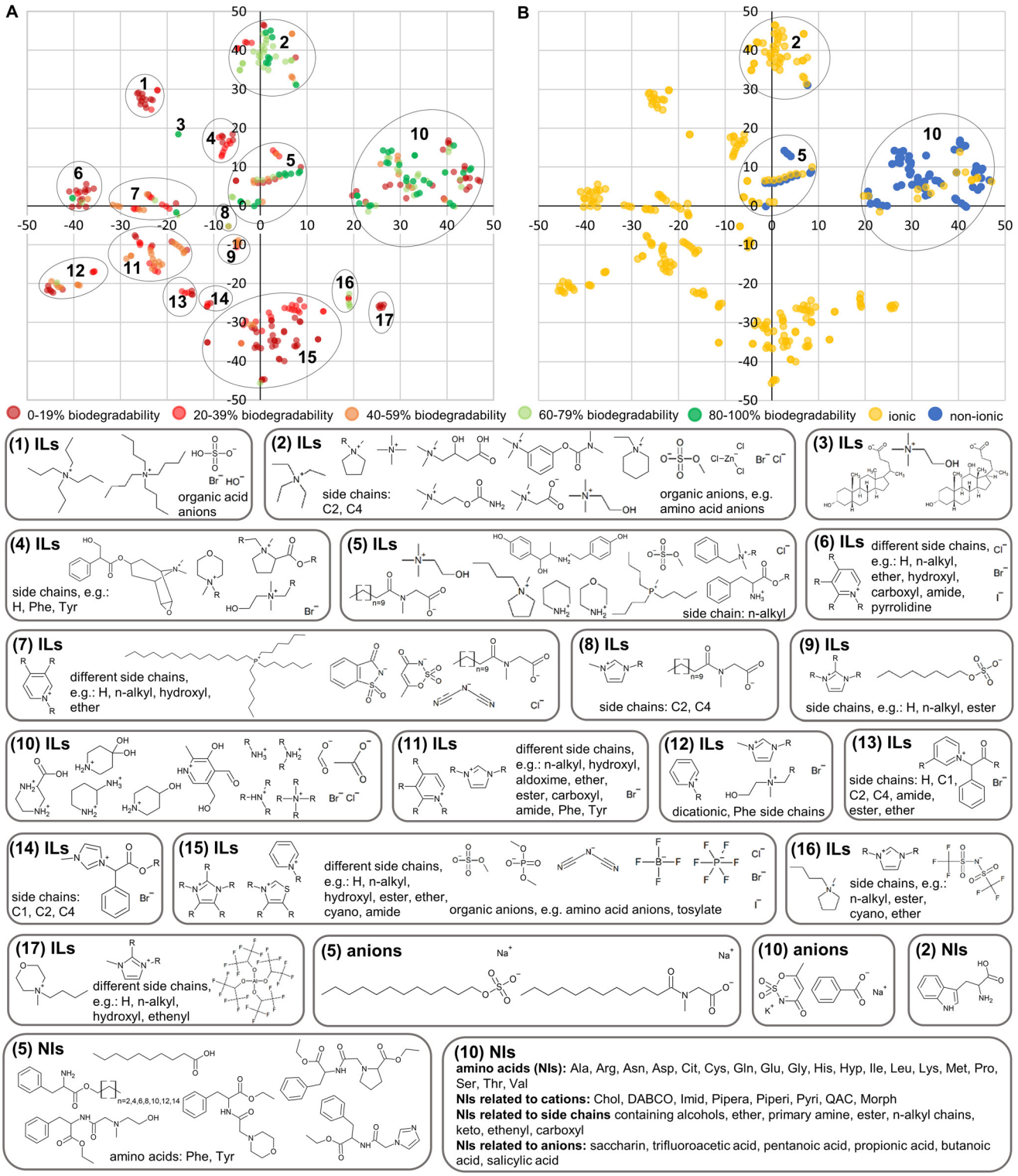

Fig. 4 Structural fragments for the ILs, anions and non-ionic compounds in *train_set_ILNI* for each cluster in the *t*-SNE plot, which is highlighted for (A) biodegradability and (B) non-ionic and ionic compounds. Alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cholinium (Chol), citrulline (Cit), cysteine (Cys), 1,4-diazabicyclo[2.2.2]octanium (DABCO), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), hydroxyproline (Hyp), imidazolium (Imid), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), morpholinium (Morph), phenylalanine (Phe), piperazinium (Pipera), piperidinium (Piperi), proline (Pro), pyridinium (Pyri), quaternary ammonium compounds (QACs), serine (Ser), threonine (Thr), tyrosine (Tyr), and valine (Val).

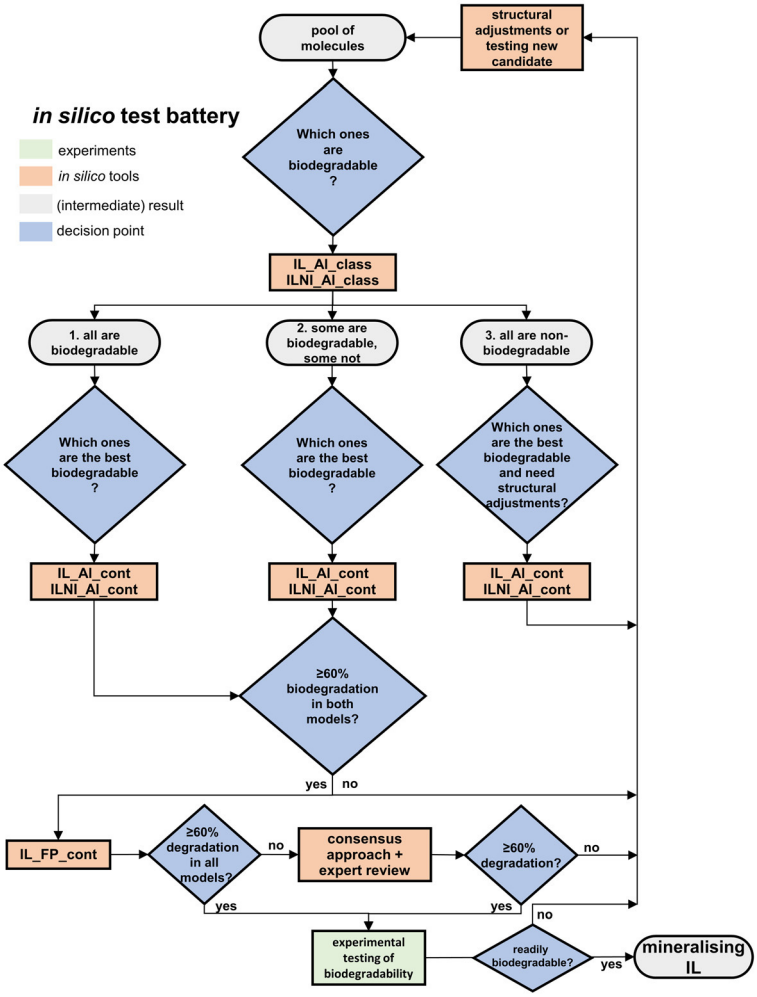

Fig. 5 Possible applications of models in an *in silico* test battery for designing fully mineralising ILs.

compared to IL_Al_cont and ILNI_Al_cont (Table 3). If all three models indicate that the IL is biodegradable by ≥60%, its ready biodegradability should be tested in a laboratory experiment (Fig. 5). The pass level for ready biodegradability in OECD 301D is ≥60% removal of ThOD within 10 days starting from a degradation level of 10%.[23] If the three models do not agree with their outcome, a consensus approach or an expert review as proposed by the workflow for the benign design of chemicals in Lorenz *et al.* might be helpful to increase the confidence of the assessment and reduce uncertainties.[30] If the outcome indicates that the IL is not biodegradable, structural changes can be made to possibly increase the biodegradability. The redesigned IL would be included in the pool of molecules and its biodegradability be predicted. If the consensus approach or expert review confirms a biodegradation rate of ≥60%, the IL should be tested in the laboratory for ready biodegradability. A mineralising IL was designed if it is readily biodegradable in experimental testing. Non-biodegradable ILs that differ in structural fragments with training set ILs might be tested in the laboratory as well. The new data of biodegradable and non-biodegradable ILs could be included in the training sets and possibly improve the models' performance.

3.5 Evaluation of the developed models for biodegradability of ILs

Since around 80% of the wastewater is not treated worldwide, ILs can be introduced into the environment *via* wastewater or leakages.[67] The endpoint ready biodegradability according to OECD 301D was chosen since it is the most stringent method of the OECD 301 series and many of the common ILs were tested according to this test method.[15] Therefore, this study developed models for the endpoint ready biodegradability according to OECD 301D of ILs using literature data and INSC in-house OECD 301D data addressing this topic for the first time.

Both datasets, *set_IL* and *set_ILNI* are unique since more than 50% of the compounds were measured in the same laboratory at the INSC using the same OECD 301D test protocol, validation criteria and similar inoculum leading to increased data quality compared to the literature data for which different inoculum sources, concentrations and microorganism diversities were used and not all validation criteria were reported.

For every individual model, the training set defined the AD as it determined the representative fragment descriptors and the alerts (section 3.3). The models might not cover important SBRs that are relevant for biodegradability predictions of a query compound. Hence, the models are not able to make reliable predictions for a query IL that differs in too many fragments from training set compounds.[37,68] Therefore, just within the model's AD reliable predictions can be made according to the model's performance, and extrapolations in predictions are avoided.

As the validation results showed, the QSBR models successfully predicted the biodegradability of common ILs, like imidazolium, pyridinium, QACs and cholinium ILs (section 3.2). The models can be applied in a test battery to design environmentally readily mineralising ILs. Uncertainties regarding the biodegradability of a newly designed IL are addressed after the *in silico* design process by testing the biodegradability in the laboratory. Hence, QSBR models are versatile tools for planning of experiments and selecting the most promising candidates.

4. Conclusion

Previous biodegradation models for ILs focused on modelling the environmental distribution of ILs between water, sediment and organic matter. These models used OECD 310 (CO_2 headspace test) literature data of 77 ILs. In our study, we used 294 ILs' biodegradability data (OECD 301D, ready biodegradability) for five fragment-based QSBR models using the MultiCASE's FlexFilters platform. Well-known and easily interpretable modelling approaches were applied, OLS and LR, to build models with two different outcomes, a continuous biodegradation rate and a classification model, respectively. The models successfully predicted the biodegradability of common ILs, like imidazolium, pyridinium, QACs and cholinium ILs. Additionally, the models were developed in agreement with the OECD principles for the validation to increase their reliability and their acceptance for regulatory purposes. Thus, this application showed that OECD principles can be implemented in biodegradation prediction models of ILs, even for the most stringent method of the OECD 301 series, OECD 301D. The internal and external validation results were adequate to predict the biodegradability of ILs. The *train_set_ILNI* did not increase the model's performance compared to *train_set_IL* even though it contained more ILs. Furthermore, the reasonably good prediction performance suggests an application of the models in a test battery for the design of environmentally mineralising ILs to increase the overall reliability of the assessment of newly developed or redesigned ILs. The test battery supports the candidate selection for synthesis and testing while saving time. In the test battery different models for environmental biodegradability according to OECD 301D were applied. The *in silico* test battery as part of the workflow for the benign design of newly developed or redesigned chemicals using *in silico* tools was successfully demonstrated. The test battery will help practitioners to understand when which model could be applied in the assessment of biodegradability to limit the pool of newly developed or redesigned ILs to the mineralising ones. However, best practice examples are needed to demonstrate the applicability of the models and the test battery and the ease of interpretation of the alerts. Better performance could be possibly achieved by increasing the size of the datasets while covering a wider variety of structural classes. Biodegradability is a central endpoint for a benign IL regarding its end-of-life. Bioaccumulation and (eco)toxicity have to be examined as well for which (Q)SAR models should be developed if not yet available to support the design of benign ILs.

Author contributions

Ann-Kathrin Amsel: conceptualisation, methodology, data curation, formal analysis, investigation, visualisation, writing – original draft, writing – review & editing, and validation; Suman Chakravarti: conceptualisation, methodology, software, data curation, formal analysis, investigation, supervision, resources, visualisation, writing – original draft, writing – review & editing, and validation; Oliver Olsson: conceptualisation, data curation, investigation, visualisation, writing – original draft, writing – review & editing, and validation; Klaus Kümmerer: conceptualisation, methodology, investigation, supervision, resources, writing – original draft, writing – review & editing, and validation.

Conflicts of interest

There are no conflicts of interest to declare.

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Anhang der Publikation 4

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Design and ready biodegradability of monocationic and dicationic L-phenylalanine-based ionic liquids

In Bearbeitung

Design and ready biodegradability of monocationic and dicationic L-phenylalanine-based ionic liquids

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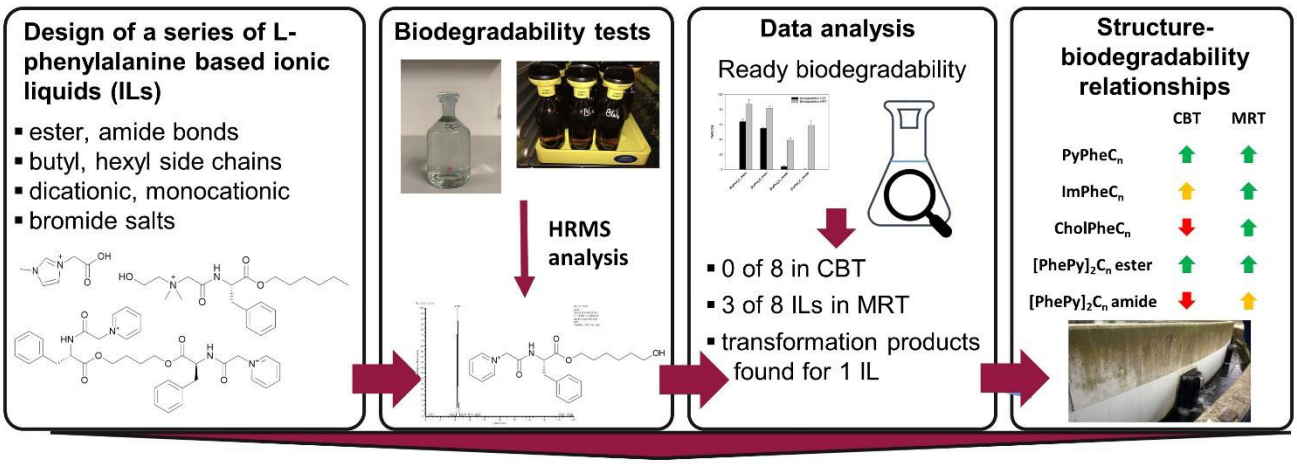
Graphical abstract



Abstract: Many ionic liquids (ILs) are ecotoxic, cytotoxic and persistent in the environment. To prevent environmental pollution and detrimental effects on humans and the environment, ILs should be non-(eco)toxic, non-bioaccumulative and biodegradable in the environment while preserving functional performance, which is in line with the EU´s Chemical Strategy for Sustainability. Therefore, a series of mono- and dicationic L-phenylalanine-based ionic liquids (Phe ILs) were designed and ready biodegradability investigated by a modified closed bottle test (CBT, OECD 301D) and by the manometric respiratory test (MRT, OECD 301F). Among the monocationic Phe ILs different cationic headgroups containing a hexyl residue connected to the L-phenylalanine moiety were studied. Pyridinium structures were selected for dicationic ILs, which differ in the alkyl spacer (C_n n = 4 and 6) and linkage (ester or amide bond). For the monocationic Phe ILs biodegradation increased in the following order ImPheC$_6$ < CholPheC$_6$ < PyPheC$_6$ in the MRT, while only PyPheC$_6$ could be classified as readily biodegradable (≥ 60% biodegradation). CholPheC$_6$ was fully mineralized in the MRT via a biphasic degradation process, in which the second phase needed a crucial adaption to the substrate after the alkyl chain was mineralized. The degradation product of ImPheC$_6$, ImAc, was persistent and therefore, prevented ImPheC$_6$ from complete mineralization. Biodegradation of the dicationic ILs was affected by the linkage, showing complete

mineralization of the ester structures in the MRT, whereas the amide counterparts were only partially biodegradable. No effect of the alkyl spacer on biodegradability was found for the esters while biodegradation in the amide series increased with a hexyl chain in comparison to the butyl residue. Under surface water conditions, the ester series exhibited biodegradation, but not ready biodegradation, and the amide series remained persistent. Overall, the results demonstrated the potential of biobased ILs for the development of greener and more sustainable ILs.

Keywords: Ready biodegradability; Benign by design; Ionic liquids; OECD 301; Structure-biodegradability-relationships; Safe and sustainable by design

Synopsis: Ionic liquids could be important chemicals towards greener synthesis, yet ecotoxicity and environmental persistence contradict such objective. This study highlights the use of biodegradable structures for environmental design of ionic liquids.

1. Introduction

Ionic liquids (ILs) have received growing interest due to their catalytical properties, very low vapor pressure, non-flammability, high thermal stability, and broad range of solvating properties.[1–4] Moreover, since many of their physical properties can be specifically tuned for a targeted application by variation of the cation and anion, ILs offer a wide range of potential industrial applications.[1,3–7] However, many ILs have been shown to be ecotoxic, persistent, cytotoxic, can form highly toxic degradation products, and also inhibit human acetylcholinesterase.[8–16] To reduce their possible detrimental impact on humans and the environment, safe and sustainable-by-design ILs are recommended, which consider incorporating an environmentally benign design concept and are part of the EU´s Chemicals Strategy for Sustainability – Towards a Toxic-Free Environment.[17] Essential and highly desirable properties of ILs are therefore no (eco)toxicity, no bioaccumulation, stability

during application and biodegradability to prevent environmental pollution while preserving functional performance.[18]

An approach to developing greener ILs is based on the utilization of renewable resources, nature-inspired and naturally occurring molecules, e.g. amino acids.[19–23] However, the use of naturally occurring or inspired molecules does not ensure high biodegradability or low ecotoxicity, as demonstrated by a series of monocationic Phe ILs (**Figure 1**).[19–22,24] While many of these ILs exhibited higher biodegradation with an increasing alkyl residue, only the pyridinium series ($PyPheC_n$) showed ultimate biodegradation in the closed bottle test (CBT, OECD 301D).[19,22] Furthermore, ecotoxicity of ILs increased with longer alkyl chains independently of the cationic structures resulting in complete inhibition of biodegradation in some cases.[20,21,24] This increased ecotoxicity is also known for other commercially used ILs and may therefore interfere with an environmentally benign design.[25,26] Similar correlations are reported for cytotoxicity and lipophilicity of ILs in general.[11,27] To reduce cytotoxicity of such structures the introduction of a dicationic element is considered.[12,28–31] A dicationic structure can be formed either by a bola-type structure, incorporating two hydrophilic groups and a spacer molecule, or by a gemini-structure with two hydrophilic and hydrophobic groups linked via a spacer. Applying such polycationic structure in imidazolium ILs showed lower ecotoxicity in comparison to the corresponding monocationic ILs, but none of the investigated ILs were biodegradable.[30]

In order to promote biodegradation, ester and amide bonds are suggested by the literature as environmentally degradable bonds.[32] Hence, this study proposes the utilization of hydrolysable ester and amide bonds, in hydrophobic L-phenylalanine-based dicationic ILs to overcome biological persistence in the environment. While both esters and amide can be hydrolyzed enzymatically by esterases or amidases, abiotic cleavage at environmental pH occurs only for esters, whereas amides are generally stable towards hydrolysis under environmental conditions.[33] In addition, esters enable the targeted elimination and detoxification of ILs with long alkyl chain after their intended

application, following the 'soft' drug design approach.[34] The aim of soft drug design is the design of inherently safer pharmaceuticals by integration of metabolization into the drug design e.g. remifentanil.[35] However, this approach is not limited to pharmaceuticals, as demonstrated by many more environmentally benign surfactants and antimicrobial surfactants, which rely on the utilization of degradable bonds embedded in the spacer substructure.[36,37] Therefore, designing hydrophobic dicationic ILs based on L-phenylalanine and targeted destabilization via ester bonds is of particular interest for possibly designing mineralizing ILs. Hence, the aim of this study is the evaluation of ready biodegradability of four new gemini-ILs ($[PyPhe]_2C_4$ ester, $[PyPhe]_2C_6$ ester, $[PyPhe]_2C_4$ amide, $[PyPhe]_2C_6$ amide), incorporating degradable bonds, and the investigation of the influence of the alkyl spacer and the ester and amide linkage on biodegradability (**Figure 1**). To investigate ready biodegradability a modified CBT and the manometric respiratory test (MRT, OECD 301F) were chosen which simulate biodegradation at surface water and sewage treatment plant conditions, respectively. Additionally, biodegradability of previously synthesized monocationic ILs ($PyPheC_6$, $CholPheC_6$ and $ImPheC_6$, **Figure 1**) and the identified stable degradation product 1-(carboxymethyl)-3-methyl-1H-imidazol-3-ium cation (ImAc) were evaluated in the MRT, i.e. at the higher α- and β-diversity of microorganisms compared to the CBT. To study only the biodegradability of the cations, bromide was used as non-toxic and carbon free anion. Both biodegradation assays were accompanied by high-resolution mass spectrometry (HRMS) to monitor primary elimination and to identify degradation products.

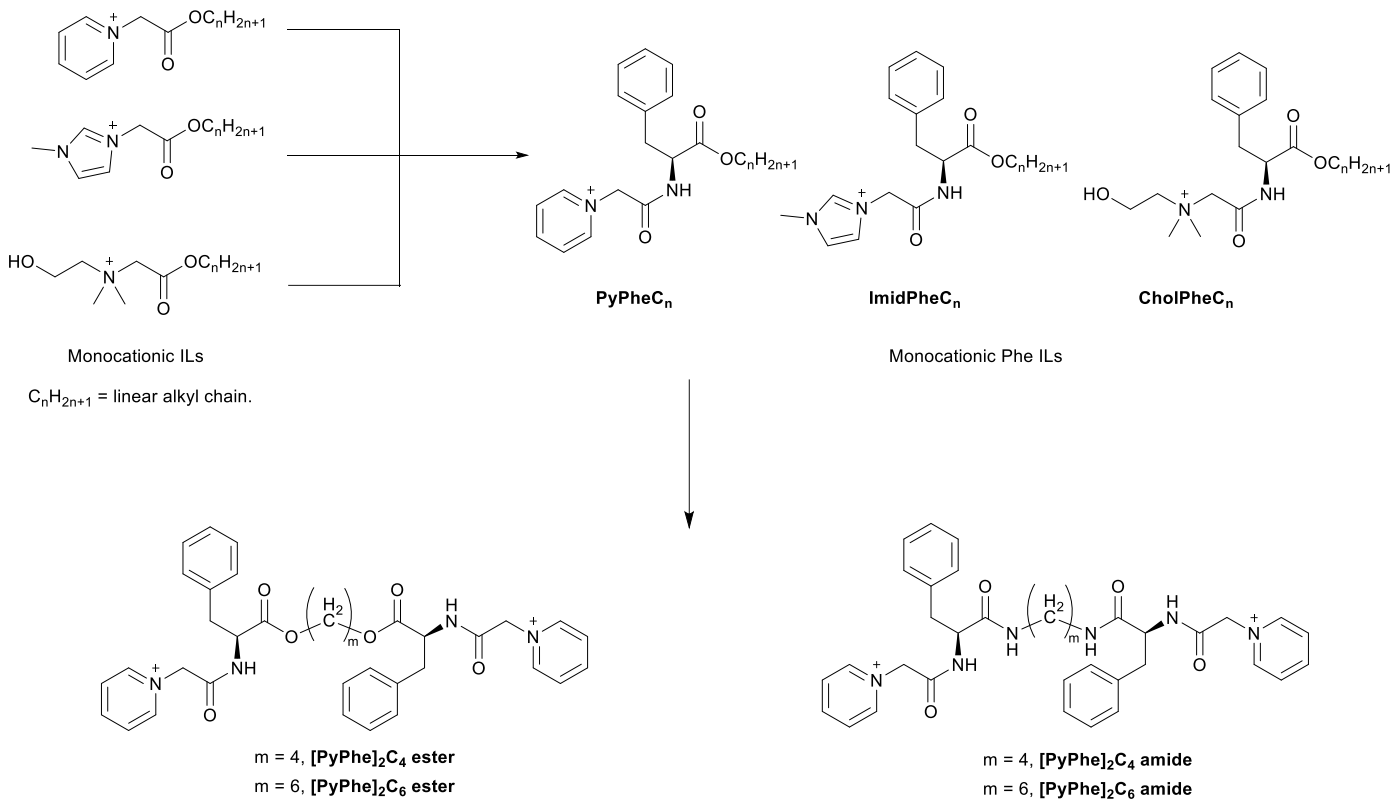


Figure 1: Structural design of monocationic and dicationic ILs by incorporation of a L-phenylalanine-based moiety and ester and amide bonds (all ILs bromide salts).

2. Materials and methods

2.1. Evaluation of ready biodegradability under aerobic conditions at surface water conditions - CBT

To simulate biodegradation at surface water levels an optode-based CBT (Fibox3 system, Regensburg, Germany) was employed using 2 drops L^{-1} of secondary effluent for the inoculum derived from a municipal sewage treatment plant (Abwasser, Grün & Lüneburg Services GmbH, Lüneburg, Germany, 325,000 eq. inhabitants).[38] For the assay a series of blanc, quality control, toxicity control to exclude false-negative results and test vessels were prepared. As reference compound for both quality and toxicity control sodium acetate was used as suggested by the OECD.[39] The test concentration of each test compound and the reference compound corresponded to a theoretical oxygen demand of 5 mg O_2 L^{-1} without nitrification ($ThOD_{NH3}$). All CBT bottles were incubated for 28 days at 20 ± 1°C in the dark and the oxygen consumption was determined every day. Samples for the HRMS analysis were taken at day 0 and day 28 without any additional filtration and stored at -20°C until analysis. Validity of the results was demonstrated by applying five criteria defined in the OECD guideline.[39]

2.2. Evaluation of ready biodegradability under aerobic conditions at sewage treatment plant conditions - MRT

Ready biodegradability was further investigated by the MRT using the OxiTop system (OC110-System, WTW GmbH, Weilheim, Germany) and NaOH pellets to fixate released CO_2 in accordance to OECD guideline 301F.[39] 30 mg SS L^{-1} of activated sludge were applied as inoculum source, derived from a municipal sewage treatment plant (Abwasser, Grün & Lüneburg Services GmbH, Lüneburg, Germany, 325,000 eq. inhabitants). By utilization of activated sludge as a source of microorganisms with a high diversity, the MRT simulates biodegradation in sewage treatment plants

142 rather than surface water. Prior use, the sludge was washed three times with tap water to remove the
143 DOC content of the inoculum and to reduce blanc oxygen consumption. Similarly to the CBT, the
144 MRT consisted of a series of blanc, quality control, test vessels and toxicity control to monitor
145 general toxic effects on the microorganisms. Additionally, the MRT uses a sterile control containing
146 320 mg L^{-1} NaN_3 to investigate abiotic degradation of the ILs. The test concentrations of the ILs
147 corresponded to a theoretical oxygen demand of 30 mg O_2 L^{-1} with ($ThOD_{NO3}$) and without
148 ($ThOD_{NH3}$) incorporation of nitrification. All vessels were incubated for 28 days at 20 ± 1°C with
149 constant stirring in a climate cabinet in the dark. Samples for HRMS analysis were taken at day 0
150 and day 28, filtered through a 0.45 µm PES filter (Macherey-Nagel, Düren, Germany) and stored at -
151 20°C. The results were considered to be valid if five validity criteria defined in OECD were met.[39]
152
153 **2.3. Identification of degradation products by HRMS analysis**
154 Primary elimination and identification of possible degradation products was done by an UHPLC
155 (Ultimate 3000, Thermo Scientific, Dreieich, Germany) coupled to an LTQ-Orbitrap-XL (Thermo
156 Scientific, Dreieich, Germany) equipped with a HESI source (Thermo Scientific, Dreieich,
157 Germany). HRMS-spectra were acquired in positive mode at 30,000 FWHM (m/z 400) and 35 NCE
158 (**Table S1**). Chromatographic separation was carried out on a CN-Hypersil Gold column (150 x 2.1
159 mm, 1.9 µm, Thermo Fisher Scientific, Germany) in gradient mode. The eluents consisted of 0.1%
160 formic acid (A) and acetonitrile (B). The Gradient started at 1% A and hold for 1.5 min, then
161 increased to 90% A after 10 min and was hold for 6 min. After that the aqueous phase decreased
162 back to 99% within 0.5 min and was hold for 3.5 min. The column temperature and the flow rate
163 were set to 20°C and 0.3 mL min^{-1} respectively. The injection volume was 10 µL for MRT and CBT
164 samples.
165

3. Results and discussion

3.1. Biodegradation of monocationic L-phenylalanine ILs in the MRT

The results of the MRT showed that all monocationic Phe ILs exhibited biodegradation and achieved higher biodegradation levels compared to the CBT (**Table 1**, **Figure 2a-c**). All compounds showed no general toxic effects to the inoculum even at the higher MRT´s concentrations, since in the toxicity control degradation exceeded 25% $ThOD_{NO3}$ within 14 days, which is in accordance to CBT results and previously reported ecotoxicity assays.[19–21] Thus, general toxic effect of the ILs on the inoculum hindering biodegradability can be excluded. However, only $PyPheC_6$ classified as readily biodegradable (80 ± 13.2% $ThOD_{NO3}$) according to the OECD guideline 301F. Moreover, within the monocationic Phe ILs series only $PyPheC_6$ showed significant nitrification.

$CholPheC_6$ (74 ± 5.2% $ThOD_{NH3}$) and $ImPheC_6$ (64 ± 1.4% $ThOD_{NH3}$) on the other hand were ultimately biodegradable in the MRT, yet not readily biodegradable as the criteria of the 10-days window was not met. While no enhancement compared to the reported CBT results was observed in terms of biodegradation level for $ImPheC_6$ in the MRT, the biodegradability of $CholPheC_6$ increased significantly compared to its previous biodegradation results in the CBT.[19] In the previous CBT, the alkyl residues of the ester were degraded via a monophasic process to yield CholPhe.[19] However, under the higher diversity of microorganisms in the MRT a biphasic process was identified (**Figure 2b**). During the first phase, a degradation level of 34 ± 4.0% $ThOD_{NH3}$ was observed on day 18, corresponding to the mineralization of the hexyl residue and the formation of CholPhe, the stable degradation product of the $CholPhe_n$ series in CBT. Thereafter, an additional degradation phase was initiated, which resulted in ultimate biodegradation, meaning ≥60% ThOD was degraded without passing the required 10-day window for being classified readily biodegradable.[40,41] Due to the structural similarity of the Phe ILs and the previously identified biodegradation pathway of the pyridinium and imidazolium ILs, the degradation is proposed to proceed via an amidase catalyzed step to L-phenylalanine and *N*-(carboxymethyl)-2-hydroxy-*N*,*N*-dimethylethanaminium (CholAc),

191 which were both completely mineralized by the inoculum in the second biodegradation phase
192 (**Figure 3**).[19] Since this second stage only occurred after 18 days in the MRT, an essential adaptation
193 of the microorganisms is needed to fully degrade the cholinium IL, which did not occur in the CBT
194 even after test prolongation to 42 days.[19] The duration of the lag-phase was not influenced by the
195 presence of sodium acetate as readily biodegradable co-substrate. However, once this adaptation is
196 complete, CholPhe undergoes rapid mineralization. The lower biodegradability of the cholinium IL
197 in the CBT compared to $PyPheC_6$ and $ImPheC_6$ is contradictory to the biodegradability trend
198 observed for the ILs.[9,42] An explanation could be that this trend is true for the pure cholinium cation,
199 but not for the Phe derivative.[43–47]
200 The results of the MRT were supported by the HRMS analysis showing no degradation products for
201 $PyPheC_6$ and $CholPheC_6$, whereas the ultimately biodegradable $ImPheC_6$ was degraded to ImAc.
202 ImAc was also proposed by Stolte et al. as a stable biodegradation product of [OMIM][Cl].[48] ImAc
203 was persistent to biodegradation in both, CBT (-3 ± 0.3% $ThOD_{NH3}$) and MRT (-10 ± 1.2%
204 $ThOD_{NH3}$), as well as to abiotic degradation in the sterile control, thereby rendering the imidazolium
205 ILs not attractive as biodegradable scaffold (**Figure 2d**). The biological persistence of ImAc might
206 imply that 3-methylimidazolium-based ILs are generally not able to undergo complete mineralization
207 in the environment or sewage treatment plants. In the sterile control, all monocationic Phe ILs
208 showed between 97 to 100% primary elimination after hydrolysis to the corresponding acids (**Table**
209 **1**). All in all, biodegradation results of monocationic ILs showed that $CholPheC_6$ is another fully
210 mineralizing IL and should be prioritized to design new derivatives.

213 **Table 1:** Biodegradability of the investigated substances in CBT and MRT (n = 2). The sterile
214 control was not prepared for the CBT. n.d. = not determined, SC = Sterile control; TS = Test
215 substance.

Substance	Sample	CBT		MRT		Result
		[% $ThOD_{NH3}$] mineralization	[%] primary elimination	[% $ThOD_{NO3}$] mineralization	[%] primary elimination	
$PyPheC_6$	TS	46 ± 4.2 [19]	100	80 ± 13.2	100	Partially biodegradable[b] in CBT, readily biodegradable[c] in MRT
	SC	n.d.	n.d.	4	97	Sensitive to hydrolysis
$CholPheC_6$	TS	30 ± 1.0 [19]	100	74 ± 5.2[a]	100	Partially biodegradable in CBT, ultimately biodegradable[d] in MRT
	SC	n.d.	n.d.	19[a]	100	Sensitive to hydrolysis
$ImPheC_6$	TS	51 ± 1.6 [19]	100	64 ± 1.4[a]	100	Partially biodegradable in CBT, ultimately biodegradable in MRT
	SC	n.d.	n.d.	8[a]	96	Sensitive to hydrolysis
ImAc	TS	-3 ± 0.3	n.d.	-10 ± 1.8[a]	0	Biologically persistent[e] in CBT and MRT
	SC	n.d.	n.d.	4[a]	0	Resistant to abiotic degradation
$[PyPhe]_2C_4$ ester	TS	64 ± 3.8	100	87 ± 6.6	100	Ultimately biodegradable in CBT, readily biodegradable in MRT
	SC	n.d.	n.d.	0	98	Sensitive to hydrolysis
$[PyPhe]_2C_6$ ester	TS	55 ± 1.3	100	82 ± 2.8	100	Partially biodegradable in CBT, readily biodegradable in MRT
	SC	n.d.	n.d.	0	97	Sensitive to hydrolysis
$[PyPhe]_2C_4$ amide	TS	5 ± 3.6	0	39 ± 2.8	61	Biologically persistent in CBT, partially biodegradable in MRT
	SC	n.d.	n.d.	0	0	Stable to hydrolysis
$[PyPhe]_2C_6$ amide	TS	4 ± 2.0	0	59 ± 6.9	100	Biologically persistent in CBT, partially biodegradable in MRT
	SC	n.d.	n.d.	0	0	Stable to hydrolysis

216 [a] Calculation based on $\%ThOD_{NH3}$

217 [b] Partially biodegradable: 10% ThOD ≤ biodegradability ≤ 60% ThOD after test end

218 [c] Readily biodegradable: ≥ 60% ThOD within the required 10-day window [39]

219 [d] Ultimately biodegradable: ≥ 60% ThOD without the required 10-day window [40,41]

220 [e] Biological persistent: ≤ 10% ThOD

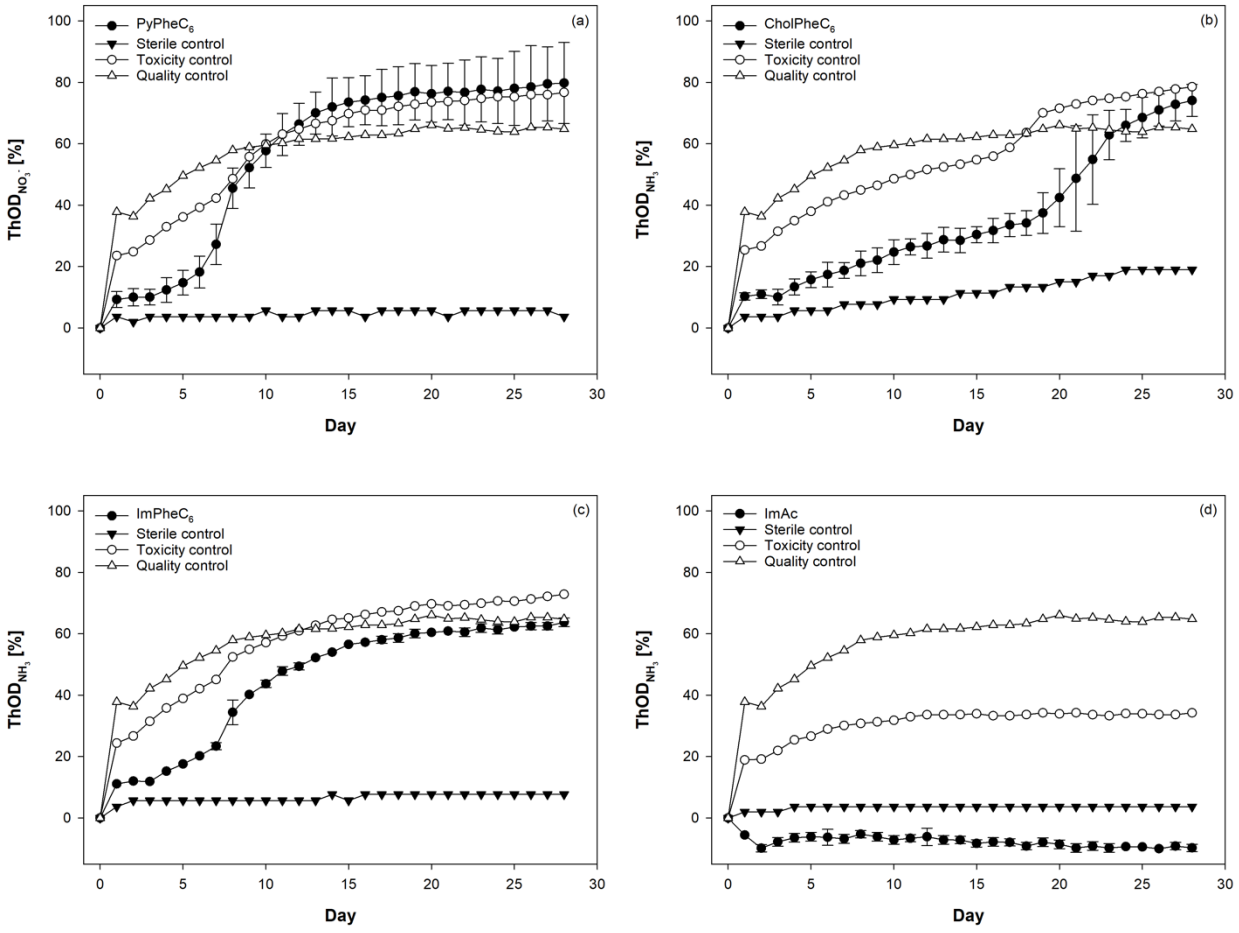


Figure 2: Biodegradability of (a) $PyPheC_6$, (b) $CholPheC_6$, (c) $ImPheC_6$ and (d) ImAc in the MRT (n = 2).

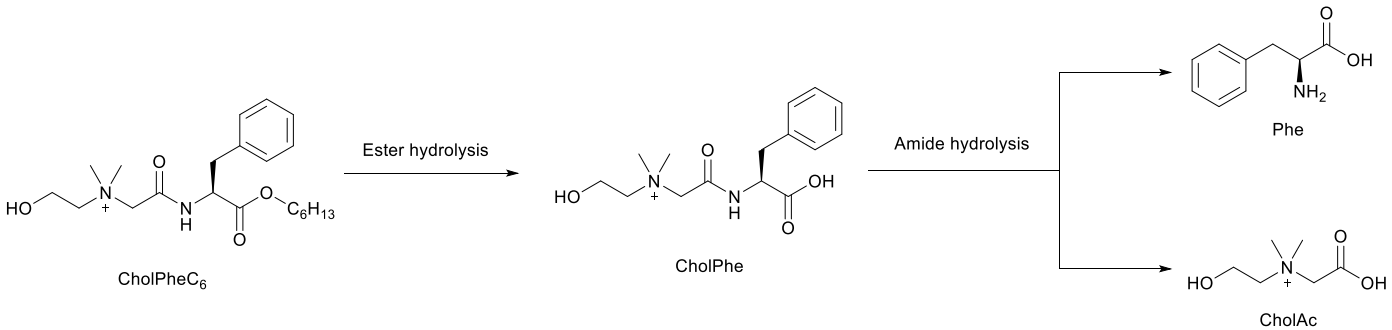


Figure 3: Proposed degradation pathway of $CholPheC_6$ in the MRT.

3.2. Biodegradation of dicationic L-phenylalanine esters

While both gemini esters, the C_4-ester and C_6-ester, exhibited biodegradation in the CBT, the C_4-ester demonstrated slightly better degradation (64 ± 3.8% $ThOD_{NH3}$) compared to the C_6-ester. But since the 10-day window requirement was not met, $[PyPhe]_2C_4$ ester was only classified as ultimately biodegradable and not readily biodegradable (**Figure 4a**). The C_6 ester showed 55 ± 1.3% $ThOD_{NH3}$, which was also close to being classified as ultimately biodegradable. Nevertheless, $[PyPhe]_2C_6$ ester could only be considered as partially biodegradable (**Figure 4b**). Although, it is expected that $[PyPhe]_2C_6$ ester might likely be at least ultimately biodegradable in biodegradation assays, which are less stringent compared to the CBT since they use a higher microorganisms' diversity and density e.g., OECD 301F. The high biodegradability of the ILs already at CBT conditions implies no bioaccumulation or persistence in the aquatic environment. Moreover, none of the compounds showed toxic effects according to the toxicity control. In comparison to $PyPheC_n$ ILs (n = 4 and 6), which were biodegradable by 49 ± 0.3% $ThOD_{NH3}$ and 46 ± 4.2 $ThOD_{NH3}$, respectively, the gemini structures performed only slightly better indicating neither negative nor positive influence of the gemini element on biodegradability or toxicity in the CBT.[19] Regarding the biodegradation kinetics no effect of the length of the alkyl spacer was observed between both gemini structures. The HRMS analysis showed the formation of $PyPheC_nOH$ and PyPhe as degradation products (**Figure 6**). All in all, the results indicate that both dicationic pyridinium esters could be viable structures for new greener ILs.

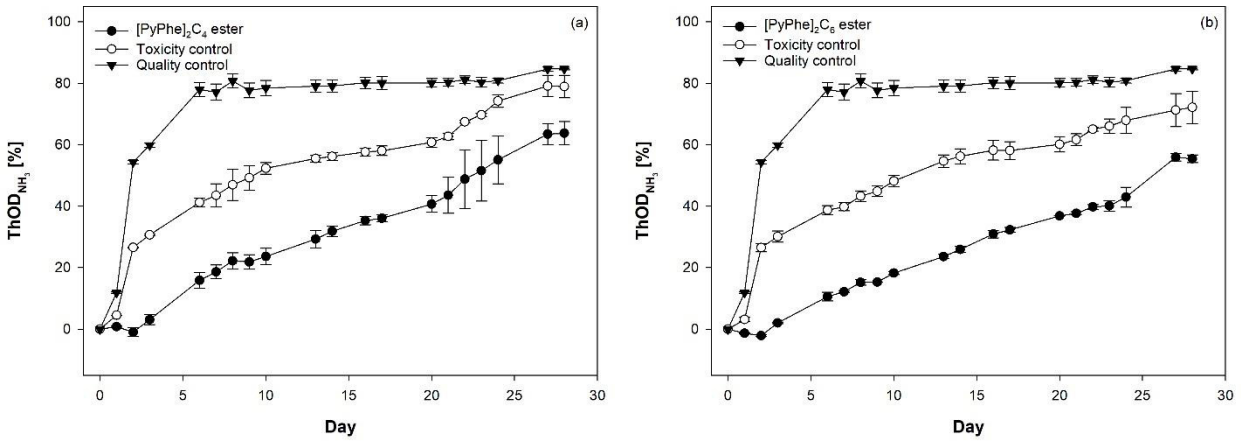


Figure 4: Biodegradability of (a) $[PyPhe]_2C_4$ ester and (b) $[PyPhe]_2C_6$ ester in the CBT (n = 2).

Since the gemini esters did not pass the ready biodegradability criteria in the CBT, a MRT was performed as additional biodegradation assay. In contrast to the CBT, both esters were readily biodegradable in accordance to OECD guideline 301F reaching 87 ± 6.6% $ThOD_{NO3}$ and 82 ± 2.8% $ThOD_{NO3}$, respectively, thereby demonstrating no influence of the alkyl spacer on biodegradability as already suggested by the CBT results (**Figure 5a-b**). In addition, no effect of the gemini-form was observed on either biodegradation level or kinetics in comparison to $PyPheC_6$ in the MRT (**Figure 3a, 5b**). No general toxic effects towards the activated sludge were observed, which was indicated by the absence of a lag-phase in the toxicity controls. The rapid and complete biodegradation of the esters was confirmed by accompanied HRMS analysis showing complete elimination after 28 days and no degradation products. Complete primary degradation of the esters to the corresponding $PyPheC_mOH$ and PyPhe was also obtained in the sterile controls via abiotic hydrolysis (**Table 1**). Based on these findings, the biodegradation pathway is expected to be in accordance to the $PyPheC_n$ series in the CBT forming $PyPheC_nOH$ and PyPhe as first transformation products, which undergo further degradation to L-phenylalanine and PyAc (**Figure 6**).

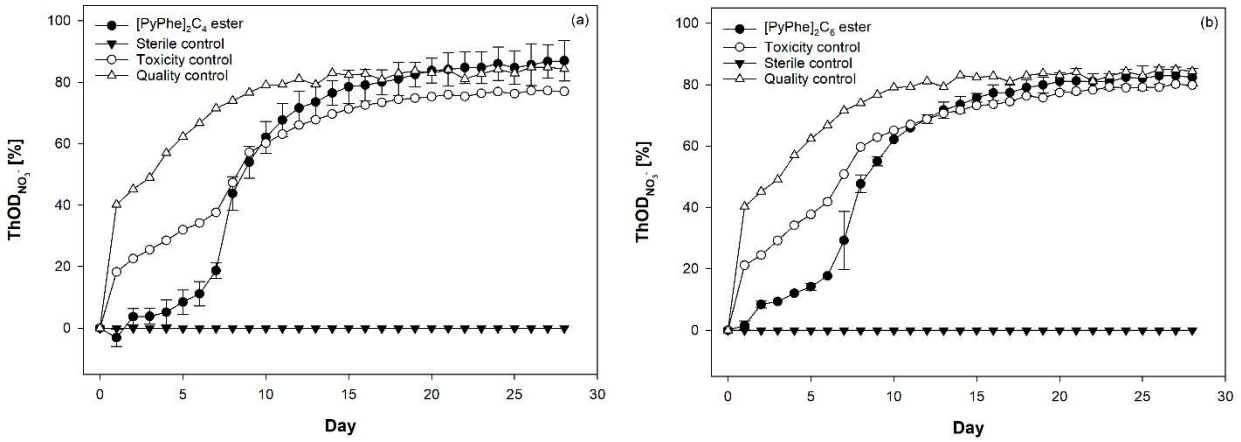


Figure 5: Biodegradability of (a) $[PyPhe]_2C_4$ ester and (b) $[PyPhe]_2C_6$ ester in the MRT (n = 2).

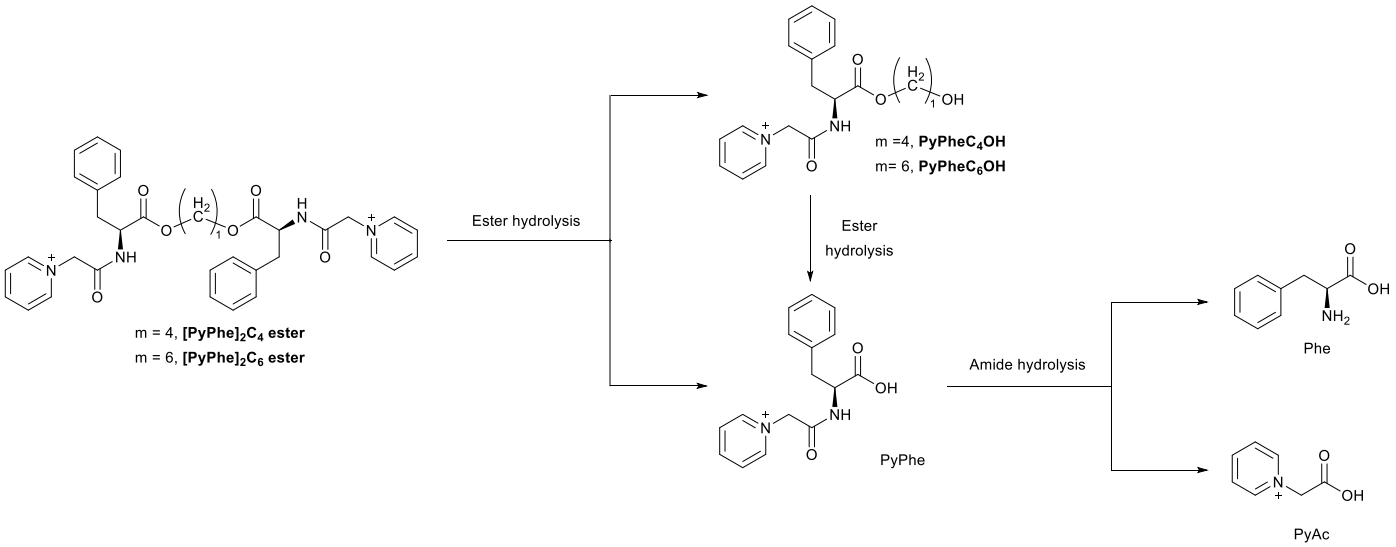
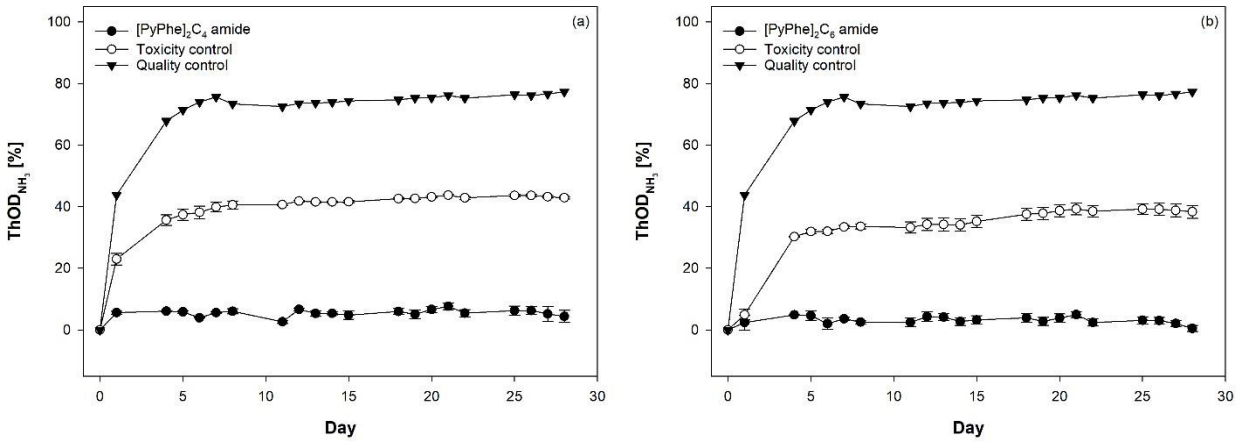


Figure 6: Proposed degradation pathway of $[PyPhe]_2C_m$ ester in the MRT (m = 4 and 6).

3.3. Biodegradation of dicationic L-phenylalanine amides

The dicationic L-phenylalanine amide spacer analogues of the esters were found to show no biodegradation under CBT conditions and were thereby classified as not readily biodegradable (**Figure 7a-b**). This is in accordance to our previous results, which showed no degradation of a series of 3-methylimidazolium amide-derivatives, while the corresponding esters showed mineralization of the alkyl alcohols.[49] The results therefore showed that amides are not always biodegradable and have to be used carefully when designing environmentally biodegradable ILs.[32] Furthermore, neither

276 [PyPhe]$_2$C$_m$ amide displayed toxic effects on the inoculum excluding false-negative results due to
277 general antimicrobial effects. HRMS investigations confirmed biological persistence by showing no
278 primary elimination.



279

280 **Figure 7:** Biodegradability of (a) [PyPhe]$_2$C$_4$ amide and (b) [PyPhe]$_2$C$_6$ amide in the CBT (n = 2).

281

282 In contrast to the CBT, the [PyPhe]$_2$C$_m$ amide series compounds were partially biodegradable at
283 MRT conditions (**Figure 8a-b)**. While the amide analogues were biodegradable but not readily at
284 MRT conditions, their final biodegradation levels were significant lower in comparison to the ester
285 analogues in the MRT (**Figure 5a-b, 8a-b**). These differences between the CBT and MRT can be
286 attributed to the higher α- and β-diversity of microorganisms in the activated sludge, which enhances
287 biodegradability. Therefore, in contrast to the CBT results, the MRT results help to identify
288 structure-biodegradability relationships and describe trends for the [PyPhe]$_2$C$_m$ amide series. Like
289 the ester analogues, the length of the alkyl spacer had only minor influence on the biodegradation
290 level and kinetics of the [PyPhe]$_2$C$_m$ amides. Yet, the final biodegradation level of [PyPhe]$_2$C$_6$ amide
291 (59 ± 6.9% ThOD$_{NO3}$) was higher compared to the C$_4$ analogue (39 ± 2.8% ThOD$_{NO3}$) and was close
292 to the 60% mark for ultimate biodegradability. Furthermore, the amide structures exhibit a lag-phase
293 of 9 and 13 days for [PyPhe]$_2$C$_4$ amide and [PyPhe]$_2$C$_6$ amide, respectively as well as slow kinetics
294 while the lag-phase of [PyPhe]$_2$C$_6$ amide increased slightly indicating necessary adaptation of the

microorganism in the biodegradation process. However, even after adaption, the degradation kinetics were rather slow in comparison to $[PyPhe]_2C_m$ ester. Accompanied HRMS analysis showed no degradation products. Like their ester analogues, no IL displayed any general toxic effects. Hydrolytic stability of $[PyPhe]_2C_m$ amide was higher compared to the esters indicated by no primary elimination in the sterile control for the amides whereas the esters showed complete elimination after 28 days. Since the difference between both gemini-forms is the spacer utilizing either ester or amide bonds, the enzymatic hydrolysis of these elements is assumed to be the rate limiting step.

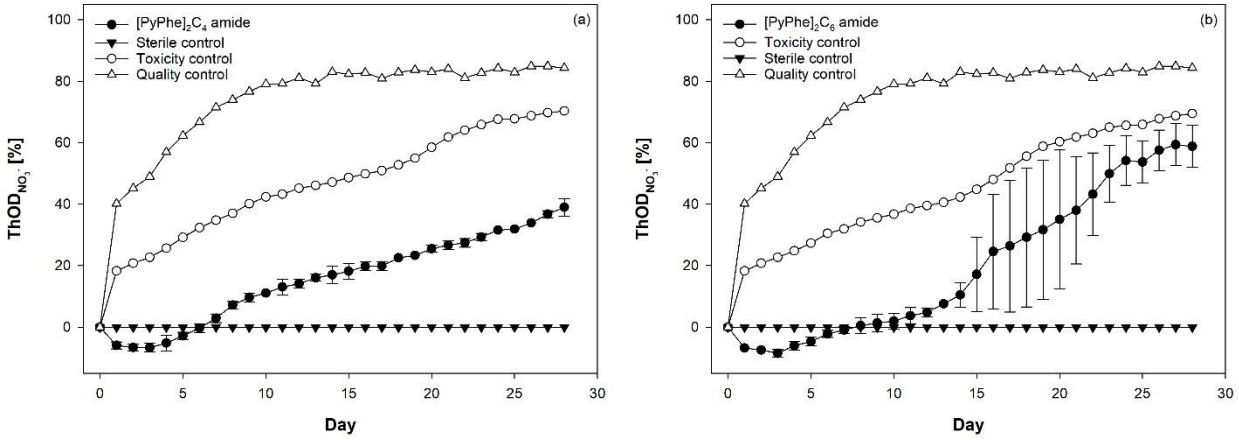


Figure 8: Biodegradability of (a) $[PyPhe]_2C_4$ amide and (b) $[PyPhe]_2C_6$ amide in the MRT (n = 2).

4. Conclusion

Ready biodegradability of previously investigated Phe ILs and their gemini-pyridinium analogues were assessed at CBT and MRT conditions. The MRT results of $CholPheC_6$ and $ImPheC_6$ contradicted our previously reported CBT results by showing an increased biodegradability for $CholPheC_6$, which was ultimately biodegradable in the MRT. Moreover, the imidazolium salt showed the least biodegradation of all monocationic ILs in the MRT, while it was the second most biodegradable IL in the previously reported CBT study. However, only $PyPheC_6$ was readily biodegradable. Even though mineralization of $CholPheC_6$ required a crucial adaptation of 18 days of the microorganisms in the MRT, $ImPheC_6$, which was partially biodegradable at CBT conditions, is

not preferred over the cholinium IL in designing greener ILs as the persistent degradation product ImAc is formed. Although all gemini ILs were biodegradable under MRT conditions, it was obvious that the amide structures were generally more resistant towards biotic and abiotic degradation. Furthermore, only the ester series could be classified as readily biodegradable, whereas the amide structures were only partially biodegradable. Therefore, ester-based ILs are preferred especially in combination with longer alkyl chains as the esters are also degraded abiotically. The ester-based gemini-form had no effect on the fate in the biodegradation assay in comparison to the non-gemini IL. Based on our results, we suggest using the gemini ILs to reduce possible toxic effects of long alkyl chains in ILs while maintaining ready biodegradability. However, amide linkage should be used carefully as it may reduce biodegradability, especially under surface water conditions. Finally, both the monovalent $PyPheC_6$ and $CholPheC_6$ as well as the divalent ester series can be referred as greener ILs. Future research should be directed towards optimizing the pyridinium element using renewable resources to obtain even greener ILs.

Conflicts of interest

There are no conflicts of interest to declare.

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Anhang der Publikation 5

Suk, Morten; Amsel, Ann-Kathrin; Karpichev, Yevgen;
Gathergood, Nicholas; Kümmerer, Klaus (2024).

Design and ready biodegradability of monocationic and dicationic L-phenylalanine-based ionic liquids

In Bearbeitung

Supplementary Information

Design and ready biodegradability of monocationic and dicationic L-phenylalanine-based ionic liquids

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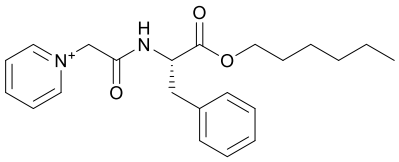
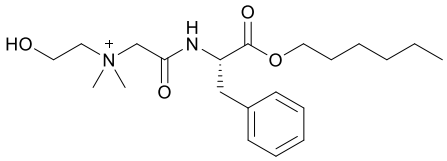
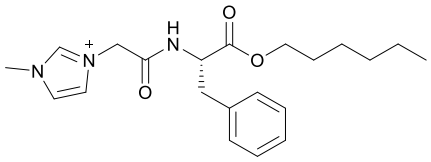
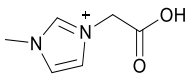
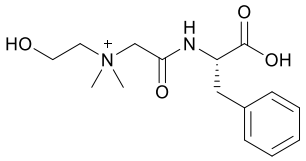
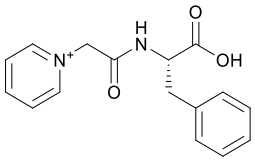
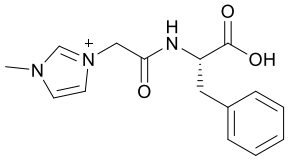
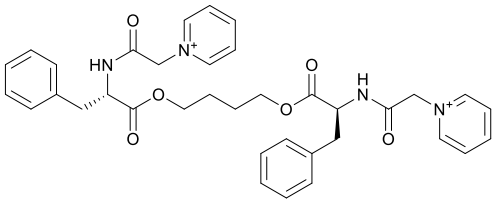
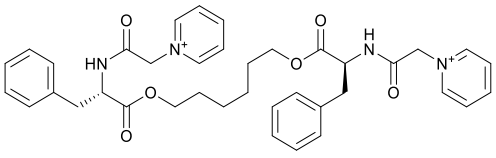
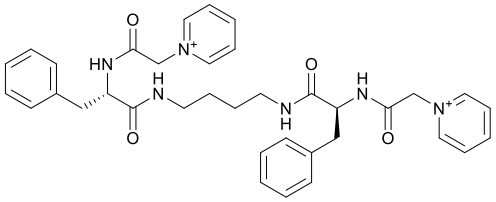
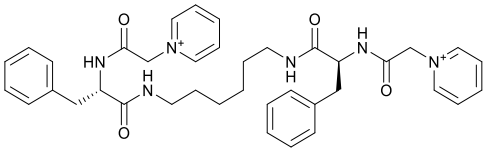
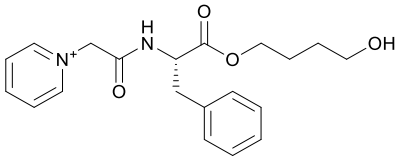
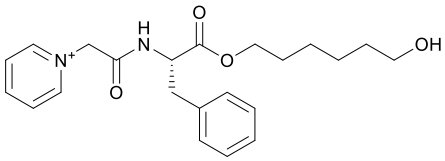
Table S1: HRMS^n analysis of the investigated ILs and their proposed degradation products (Δ m/z $\leq$ 5 ppm).

Substance	Structure of cation	Molecular formula	RT [min]	MS^1	MS^2	Molecular formula
PyPheC$_6$		$C_{22}H_{29}N_2O_3^+$	4.39	369.2180	239.1182 285.1236 132.0809 206.0814	$C_{15}H_{15}ON_2^+$ $C_{16}H_{17}O_3N_2^+$ $C_9H_{10}N^+$ $C_{11}H_{12}O_3N^+$
CholPheC$_6$		$C_{21}H_{35}N_2O_4^+$	4.38	379.2596	249.1598 295.1652 132.0808 335.2329	$C_{14}H_{21}O_2N_2^+$ $C_{15}H_{23}O_4N_2^+$ $C_9H_{10}N^+$ $C_{19}H_{31}O_3N_2^+$
ImPheC$_6$		$C_{21}H_{30}N_3O_3^+$	4.38	372.2293	288.1347 242.1292	$C_{15}H_{18}O_3N_3^+$ $C_{14}H_{16}ON_3^+$
ImAc		$C_6H_9N_2O_2^+$	4.11	141.0661	95.0605 123.0554 96.0682	$C_5H_7N_2^+$ $C_6H_7ON_2^+$ $C_5H_8N_2^+$
CholPhe		$C_{15}H_{23}N_2O_4^+$	4.06	295.1659	249.1560 251.1392	$C_{14}H_{21}O_2N_2^+$ $C_{13}H_{19}O_3N_2^+$
PyPhe		$C_{16}H_{17}N_2O_3^+$	4.05	285.1241	239.1181	$C_{15}H_{15}ON_2^+$
ImPhe		$C_{15}H_{18}N_3O_3^+$	4.05	288.1350	242.1290 141.0660	$C_{14}H_{18}ON_3^+$ $C_6H_9O_2N_2^+$
[PyPhe]$_2$C$_4$ ester		$C_{36}H_{40}N_4O_6^{2+}$	4.66	312.1470	239.1177 357.1806 285.1232 339.1701	$C_{15}H_{15}ON_2^+$ $C_{20}H_{25}O_4N_2^+$ $C_{16}H_{17}O_3N_2^+$ $C_{20}H_{23}O_3N_2^+$
[PyPhe]$_2$C$_6$ ester		$C_{38}H_{44}N_4O_6^{2+}$	4.65	326.1634	239.1180 367.2018 385.2122 285.1235	$C_{15}H_{15}ON_2^+$ $C_{22}H_{27}O_3N_2^+$ $C_{22}H_{29}O_4N_2^+$ $C_{16}H_{17}O_3N_2^+$
[PyPhe]$_2$C$_4$ amide		$C_{36}H_{42}N_6O_4^{2+}$	4.64	311.1637	239.1180 355.2130 271.6420 120.0446	$C_{15}H_{15}ON_2^+$ $C_{20}H_{27}O_2N_4^+$ $C_{31}H_{37}O_4N_5^{2+}$ $C_7H_6ON^+$

$[PyPhe]_2C_6$ amide		$C_{38}H_{46}N_6O_4^{2+}$	4.69	325.1791	239.1175 383.2435 285.6570 120.0442	$C_{15}H_{15}ON_2^+$ $C_{22}H_{31}O_2N_4^+$ $C_{33}H_{41}O_4N_5^{2+}$ $C_7H_6ON^+$
$PyPheC_4OH$		$C_{20}H_{25}N_2O_4^+$	4.25	357.1822	285.1243 239.1187 132.0812 206.0818	$C_{16}H_{17}O_3N_2^+$ $C_{15}H_{16}ON_2^+$ $C_9H_{10}N^+$ $C_{11}H_{12}O_3N^+$
$PyPheC_6OH$		$C_{22}H_{29}N_2O_4^+$	4.29	385.2141	239.1187 285.1244 132.0812 206.0819	$C_{15}H_{16}ON_2^+$ $C_{16}H_{17}O_3N_2^+$ $C_9H_{10}N^+$ $C_{11}H_{12}O_3N^+$

22