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Benign by Design: Ein Beitrag zur Entwicklung von in der Umwelt biologisch leichter abbaubaren Antibiotika am Beispiel von Fluorchinolonen

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Zusammenfassung

Antibiotika sind aus der Human- und Veterinärmedizin nicht mehr wegzudenken. Ihr hoher Verbrauch führt jedoch zu stetig steigenden Konzentrationen der Wirkstoffe und ihrer Transformationsprodukte in der Umwelt. Antibiotika in der Umwelt haben das Potential Funktionen von Ökosystemen zu stören und tragen zur Entwicklung und Selektion von resistenten Bakterien bei.

Um diese negativen Auswirkungen auf Mensch und Umwelt zu reduzieren, sind vielseitige Lösungen notwendig. Benign by Design (BbD) ist ein wichtiger Baustein dafür. Daher ist es wichtig zu verstehen, inwiefern das BbD Prinzip auf verschiedene Substanzgruppen anwendbar ist und welche Limitierungen zu berücksichtigen sind.

Mit dieser Arbeit soll ein Beitrag zur Entwicklung von in der Umwelt mineralisierbaren Antibiotika entsprechend des Benign by Design Konzeptes geliefert werden. Dies wurde am Beispiel der Fluorchinolonantibiotika durchgeführt, da diese sehr wichtige, aber auch sehr persistente Wirkstoffe sind. Ziel war es, zu verstehen, welche Veränderungen an der Grundstruktur vorgenommen werden können, um Derivate zu erzeugen, die während der Wirkdauer und Lagerung ausreichend stabil bleiben, aber anschließend in der Umwelt möglichst schnell und vollständig mineralisiert werden können.

Im ersten Teil der Arbeit wurden die BbD Ansätze des targeted und non-targeted Re-Designs und *de novo* Designs, sowie die Verwendung von *in silico* Tools zu deren Umsetzung, untersucht. Basierend darauf wurde ein Workflow entwickelt, der eine mögliche Verwendung von computergestützten Methoden innerhalb des BbD Frameworks aufzeigt. In der resultierenden Veröffentlichung wurde dieser Workflow vorgestellt und dessen Chancen und Limitierungen für die Umsetzung des BbD beleuchtet. Die herausgearbeiteten BbD Ansätze bildeten die Grundlage für das folgende targeted und non-targeted Design von Fluorchinolonen.

Der Ansatz des non-targeted Re-Designs wurde für neun verschiedene Substanzen aus der Klasse der Fluorchinolone angewandt. Dafür wurden Transformationsprodukte der Muttersubstanzen mittels Photolyse und Photokatalyse erzeugt. Das resultierende Substanzgemisch wurde hinsichtlich der biologischen Abbaubarkeit und Toxizität untersucht. Dabei konnte gezeigt werden, dass durch die Bestrahlung mit UV-Licht eine Vielzahl an neuen Strukturen entstehen und das Gemisch oft eine gesteigerte biologische Abbaubarkeit im Vergleich zur Muttersubstanz aufweist. Die Zuordnung der gemessenen Abbaueffekte zu einzelnen enthaltenen Strukturen gestaltete sich aufgrund fehlender

Referenzsubstanzen und anwendbarer *in silico* Modelle schwierig. Es konnten daher abschließend keine eindeutig abbaubaren Derivate mit diesem Ansatz identifiziert werden. Trotzdem zeigte sich in den Untersuchungen, dass eine Hydroxylierung der Ausgangssubstanzen sehr wahrscheinlich zu einer verbesserten Abbaubarkeit dieser führt.

Das targeted Re-Design wurde am Beispiel von Fluorchinolon-Glucosamin-Derivaten untersucht. Dabei galt es zu verstehen, inwiefern Glucosamin-Substituenten die biologische Abbaubarkeit beeinflusst. Keine der untersuchten Strukturen konnte in den verwendeten OECD Tests als leicht biologisch abbaubar klassifiziert werden. Es konnte jedoch gezeigt werden, dass durch die Verwendung von acetylierten Glucosamin-Substituenten ein partieller Abbau stattfindet. Diese Erkenntnisse können zukünftig in das gezielte, fragment-basierte Design von grüneren Strukturen einfließen.

Im letzten Teil der Arbeit wurden die Struktur-Bioabbau-Beziehungen von N-heterozyklischen Verbindungen, welche auch die Basis des Fluorchinolon-Grundgerüstes sind, untersucht und leicht biologisch abbaubare Leitstrukturen identifiziert. Dafür wurden 84 verschiedene N-Heterozyklen nach OECD 301 Richtlinien getestet. Basierend darauf wurde zum einen ein lokales 3D-QSAR Modell, insbesondere zur Visualisierung der Effekte der Substituenten im dreidimensionalen Raum, erstellt, als auch Regeln für das Design von umweltfreundlicheren Chinolonen und verwandten Strukturen abgeleitet. Weiterhin wurden abbaubare Strukturen aus der Gruppe der Chinazoline identifiziert, welche vielversprechende Leitstrukturen für das Design von Topoisomerase-Inhibitoren oder anderer Chemikalien darstellen.

Die Diskussion der verschiedenen Ansätze, die Entwicklung eines *in silico* Workflows, sowie die Machbarkeitsstudien am Beispiel von Fluorchinolonen haben demonstriert, dass die Umsetzung von BbD im Wirkstoffdesign möglich ist und wie potentielle Vorgehensweisen aussehen können. Dabei konnte gezeigt werden, dass selbst die als besonders persistent geltenden Fluorchinolonantibiotika das Potential bieten, zu besser abbaubaren Derivaten re-designt zu werden. Es wurde jedoch auch deutlich, dass es keine allgemeingültige Herangehensweise gibt und Methoden den entsprechenden Substanzklassen und Anforderungen angepasst werden müssen. Daher ist es wichtig die vorgestellten BbD Ansätze zukünftig weiter hinsichtlich ihrer Übertragbarkeit auf weitere Substanzklassen zu untersuchen. In diesem Zusammenhang ist es ebenfalls wichtig, experimentelle Bioabbaudaten von weiteren Strukturen zu generieren, um vorhandene Bioabbau-Modelle hinsichtlich ihrer Anwendungsdomäne zu verbessern und für das Design von grünen Wirkstoffen zu nutzen.

Abstract

The high consumption of antibiotics leads to steadily increasing concentrations of the active ingredients and their transformation products in the aquatic environment. Antibiotics in the environment have the potential to disrupt ecosystem functions and contribute to the development and selection of resistant bacteria.

Multiple solutions are needed to reduce such negative impacts on the environment and human health. Benign by Design (BbD) is an important building block in this context. It is therefore crucial to understand how the BbD approaches can be applied to different groups of substances and what limitations need to be considered.

The aim of this work was to contribute to the development of in the environment degradable antibiotics according to the Benign by Design concept on the example of the important but very persistent antibiotic class of fluoroquinolones. The goal was to understand what changes can be made to their structure to generate derivatives that remain as stable as needed during treatment and storage, but can be mineralized fast in the environment later on.

Firstly, the BbD approaches of targeted and non-targeted re-design and *de novo* design were identified and investigated. Furthermore, a workflow was developed, which describes a possible use of computer-aided methods within those approaches. In the resulting paper this workflow was presented and the chances and limitations of its implementation within the BbD framework were discussed. The presented BbD approaches and the workflow were the starting point for the following targeted and non-targeted design approaches.

The non-targeted re-design approach was applied to nine different fluoroquinolones (FQ). Therefore, transformation products of the parent compounds were generated by photolysis and photocatalysis. The resulting mixtures of transformation products were investigated with regard to their biodegradability and toxicity. It was shown that irradiation with UV light leads to the formation of a large number of new structures and that the mixture of these often exhibits increased biodegradability compared to the parent compounds. Since only the mixtures were investigated, it was not possible to assign biodegradation effects to individual structures. Due to the lack of reference substances and applicable *in silico* models, no potentially degradable derivatives could be identified using this approach. However, it could be verified, that the hydroxylation of the FQ structures via photolysis or photocatalysis possibly leads to improved biodegradability.

Targeted re-design was investigated using the example of fluoroquinolone-glucosamine

derivatives. The aim was to understand how glucosamine substituents affect the biodegradability of the structures. None of the investigated structures could be classified as readily biodegradable in the OECD tests. However, it could be shown that the acetylated glucosamine substituents led to a partial degradation. These findings could be further used in the targeted fragment-based design of greener structures.

In the last part, the structure-biodegradation relationships of N-heterocyclic compounds, which are the basis of FQs, were investigated and readily biodegradable lead scaffolds were identified. Therefore, 84 different N-heterocycles were evaluated according to the OECD 301 guideline. Based on the results, a local 3D-QSAR model was developed and rules for the design of more environmentally friendly quinolones and related structures were derived. Furthermore, degradable structures from the group of quinazolines were identified, which might be promising lead scaffolds for the design of topoisomerase inhibitors and other chemicals.

The herein presented BbD approaches and *in silico* workflow, as well as the case studies for fluoroquinolones have demonstrated that the implementation of BbD in the drug design process is possible and how such workflows might look like. It could be shown that even fluoroquinolone antibiotics, which are considered particularly persistent, have the potential for a design towards more degradable derivatives. However, it also became clear, that there is no one-fits-all approach and that methods have to be adapted to the respective substance classes and use cases. Therefore, it is important to further investigate the BbD approaches presented here to determine their transferability to other substance classes. In this regard, it is also necessary to generate more consistent biodegradation data of a variety of compounds to improve existing biodegradation models regarding their applicability domain, so that they can be used for the design of greener compounds.

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Abkürzungsverzeichnis

BbD Benign by Design

CBT Closed Bottle Test

DOC Gelöster organischer Kohlenstoff (engl. dissolved organic carbon)

DMSO Dimethylsulfoxid

FQ Fluorchinolone

HPLC Hochleistungsflüssigkeitschromatographie

LBT Leuchtbakterientest

LC-MS Gekoppelte Flüssigchromatographie und Massenspektrometrie

MEC Measured Environmental Concentration

MRT Manometrische Respirationstest

MS Massenspektrometrie

PNEC Predicted No-Effect Concentration

SDG Sustainable Development Goals

TP Transformationsprodukte

WHO World Health Organization

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1 Einleitung

1.1 Antibiotika in der Umwelt

Antibiotika sind seit ihrer Entdeckung im Jahr 1928 aus der modernen Medizin nicht mehr wegzudenken. Sie ermöglichen nicht nur die Behandlung von Infektionskrankheiten, sondern auch viele fortgeschrittene medizinische Eingriffe (Davies und Davies, 2010). In den letzten Jahrzehnten ist der Verbrauch von Antibiotika daher weltweit enorm gestiegen. In Deutschland gehören sie zu den umsatzstärksten Wirkstoffgruppen mit einem Umsatz von 920 Mio. Euro im Jahr 2014 allein im humanmedizinischen Sektor (GERMAP, 2015). Dies entspricht 17,4 definierte Tagesdosen pro 1000 gesetzlich Versicherten. Verlässliche Zahlen für einen weltweiten Verbrauch sind schwierig zu finden, man schätzt den globalen Verbrauch auf durchschnittlich 14,3 definierte Tagesdosen pro 1000 Personen mit großen räumlichen Unterschieden (Browne et al., 2021).

Im Laufe der Zeit wurden viele Wirkstoffe, wie auch Antibiotika, so optimiert, dass sie eine möglichst hohe chemische Stabilität aufweisen. Das Ziel dabei ist, eine möglichst unkomplizierte Lagerung und Anwendung zu gewährleisten und die Entstehung von ungewollten Abbauprodukten zu vermeiden. Eine hohe chemische Stabilität geht jedoch auch mit einer hohen Persistenz in der Umwelt einher (Leder et al., 2015). Mit dem hohen Verbrauch von Antibiotika ist daher auch der Nachweis stetig steigender Konzentrationen der Wirkstoffe im Abwasser und der Umwelt zu verzeichnen (Chow et al., 2021; Kümmerer, 2009a,b).

Die Anwesenheit von Spurenschadstoffen im Wasser ist eine der größten Herausforderungen für ein nachhaltiges Wassermanagement (Kümmerer et al., 2019; Rogowska et al., 2020). Die Elimination der Substanzen in konventionellen Kläranlagen ist oft unvollständig oder findet gar nicht statt. Durch Methoden der erweiterten Abwasserbehandlung entstehen oft sogar Transformationsprodukte, welche dann in die Umwelt gelangen. Sowohl ihre Strukturen als auch ihr Verhalten in der Umwelt sind größtenteils

unbekannt und oft kaum bzw. gar nicht erforscht (Fatta-Kassinos et al., 2011; Michael et al., 2014; Zwiener, 2007). Teilweise sind die entstandenen Transformationsprodukte sogar toxischer und damit noch schädlicher für die Umwelt als die Muttersubstanzen selbst (Casellas et al., 2013). Zudem stellen die Methoden der erweiterten Abwasserbehandlung keine wirklich nachhaltige Lösung des Problems dar, da die verwendeten Methoden oft einen hohen Energieverbrauch, sowie eine geringe Effizienz und Effektivität haben. Hinzu kommt, dass weltweit bis zu 80% der Abwässer überhaupt keiner Form der Abwasserbehandlung unterzogen werden, sodass die Wirkstoffe somit direkt in die aquatische Umwelt gelangen (UN, 2017).

Antibiotika hemmen per Definition das Wachstum von Bakterien und haben damit ein großes Potential Ökosysteme und deren Funktionen zu stören, sobald sie sich in der Umwelt anreichern (Polianciuc et al., 2020). Außerdem tragen Antibiotika in der Umwelt und in Kläranlagen nachweislich zur Resistenzbildung bei Bakterien bei (Gullberg et al., 2011; Tello et al., 2012). Lange wurde angenommen, dass Antibiotika-Konzentrationen unterhalb der minimalen Hemmkonzentration keine nennenswerte Auswirkung auf Mikroorganismen in der Umwelt haben. Inzwischen ist jedoch bekannt, dass bereits weitaus geringere Konzentrationen das Wachstum von Bakterien beeinträchtigten und insbesondere mutierten, und somit resistenten, Bakterien einen Wachstumsvorteil ermöglichen. Diese Konzentrationen, auch „minimal selective concentration“ genannt, können in einem Bereich von 1/10 bis zu 1/230 der minimalen Hemmkonzentration liegen (Gullberg et al., 2011). Für Ciprofloxacin, einen Vertreter der Fluorchinolone, entspricht das 2,5 $\frac{\text{ng}}{\text{mL}}$ bis 100 $\frac{\text{pg}}{\text{mL}}$. Dabei handelt es sich um Konzentrationen, die in der Umwelt bereits vor Jahren gemessen wurden (Chow et al., 2021; Kümmerer, 2009a,b; Umweltbundesamt, 2011).

Resistente pathogene Mikroorganismen stellen ein immer größeres Gesundheitsrisiko für die Menschheit dar. Man geht bereits jetzt von weltweit bis zu 700.000 Todesfällen pro Jahr aufgrund von resistenten Krankheitserregern aus (Jasovský et al., 2016). Laut World Health Organization (WHO) sind sie eine der größten Bedrohungen für die globale Gesundheit, Nahrungssicherheit und Entwicklung (WHO, 2015). Viele Infektionskrankheiten werden erneut zu einer Bedrohung, da die verwendeten Antibiotika wirkungslos werden. All das ist bereits eine reelle Gefahr, während auf der anderen Seite weltweit noch immer nicht alle Menschen ausreichend Zugang zu Antibiotika haben (Browne et al., 2021; Mendelson et al., 2016). Unter anderem die WHO betonte daher die Dringlichkeit, eine weitere Resistenzbildung einzugrenzen und veröffentlichte dafür einen Akti-

onsplan (WHO, 2015). Auch zur Umsetzung der Sustainable Development Goals (SDG) ist eine umfangreiche Lösung aus verschiedenen Blickwinkeln dringend nötig (Jasovský et al., 2016). Dies betrifft besonders das SDG 3 (Gesundheit und Wohlergehen). Aber auch andere Bereiche wie die der SDGs 1 (Keine Armut), 2 (Kein Hunger), 6 (Sauberes Wasser), 8 (Gute Arbeit und Wirtschaftswachstum), sowie 12 (Nachhaltiger Konsum und Produktion) sind stark von resistenten Krankheitserregern betroffen (Jasovský et al., 2016).

1.2 Fluorchinolonantibiotika

Eine besonders wichtige Klasse der Antibiotika stellen die Chinolon- bzw. Fluorchinolonantibiotika dar. Seit der Entdeckung des ersten Chinolonantibiotikums Nalidixinsäure in den 60er Jahren folgten weitere Generationen, welche vor allem auch fluorierte Verbindungen beinhalten. Das Fluoratom an Position 6 des Chinolongrundgerüsts wurde vor allem deshalb eingeführt, weil dadurch eine vielfache Steigerung der antibakteriellen Aktivität erreicht werden konnte. Fluorchinolone (FQ) sind synthetische Breitbandantibiotika. Durch ihre Interaktion mit der DNA-Gyrase und Topoisomerase IV inhibieren sie die DNA Synthese von Bakterien und führen dadurch zu Bakteriostase oder Zelltod (Aldred et al., 2014). FQ werden sowohl in der Human- als auch Veterinärmedizin eingesetzt, insbesondere in der Humanmedizin oft auch als Reserveantibiotika. Das heißt, FQ kommen oft erst dann zum Einsatz, wenn andere Antibiotikaklassen nicht mehr wirksam sind. Unter anderem sind sie deshalb von der WHO als „critically important“ eingestuft (WHO, 2012). Bezogen auf ihren Verbrauch gehören Fluorchinolone mit 17% globalem Marktanteil zu der drittgrößten Gruppe der Antibiotika (van Doorslaer et al., 2014).

Fluorchinolone sind in der Umwelt nicht abbaubar (Alexy et al., 2004) und können, wie viele andere Arzneistoffe, in der Kläranlage nicht, oder nur unvollständig entfernt werden (Kümmerer, 2009a,b; van Doorslaer et al., 2014). Durch ihren hydrophoben Charakter werden sie in den allermeisten Fällen durch Sorption am Klärschlamm um bis zu 50-60% eliminiert. Durch Verfahren der erweiterten Abwasserbehandlung entstehen eine Vielzahl von Transformationsprodukten. Für einen prominenten Vertreter der FQ, Ciprofloxacin, konnten bis zu 38 mögliche Transformationsprodukte identifiziert werden (Haddad und Kümmerer, 2014). Auch andere Vertreter der Fluorchinolone wurden hinsichtlich ihrer Transformationsprodukte (TP) untersucht, sodass zahlreiche Publikationen mit Struk-

turvorschlägen existieren (Liu et al., 2012; Sirtori et al., 2011; Zhang et al., 2019). Bei der Untersuchung der Eigenschaften dieser TP konnte außerdem gezeigt werden, dass diese in der Regel nicht biologisch abbaubar sind und teilweise noch immer toxische Wirkungen auf Umweltbakterien haben (Oliveira et al., 2015; Sirtori et al., 2011; Sturini et al., 2015; Vargas et al., 2008; Vasquez et al., 2013).

Im Jahr 2011 veröffentlichte das Umweltbundesamt eine Zusammenfassung der Monitoringdaten zu Umweltkonzentrationen von Arzneimitteln (Umweltbundesamt, 2011). Dafür wurde die gemessene Umweltkonzentration (Measured Environmental Concentration (MEC)) der Predicted No-Effect Concentration (PNEC), also der Konzentration bei der man davon ausgeht, dass keine negativen Effekte zu erwarten sind, gegenübergestellt. Bei 28 der untersuchten Wirkstoffen war die gemessene Umweltkonzentration größer als die PNEC, weshalb diese Substanzen als gefährlich für die Umwelt eingestuft wurden. Darunter waren Ciprofloxacin sowie weitere 14 Antibiotika. Auch global wurde das MEC/PNEC Verhältnis von Ciprofloxacin untersucht und war in 30% der getesteten Umweltproben aus 47 verschiedenen Ländern in einem kritischen Bereich (Booth et al., 2020).

Die typischen Eintragsquellen in die Umwelt sind kommunale Abwässer, Krankenhausabwässer, industrielle Abwässer aus der Herstellung und das Ausbringen von Klärschlamm oder Gülle auf Agrarflächen (Millanao et al., 2021). Da FQ hoch aktive Antibiotika sind, ist zu erwarten, dass sie auch ökotoxikologische Effekte in der Umwelt haben. Tatsächlich gehören FQ zu den Substanzen, die die größte Wirkung auf aquatische Umweltbakterien in *in vitro* Studien gezeigt haben (Felis et al., 2020). Dazu gehören sowohl akute Effekte, die direkt die mikrobielle Gemeinschaft stören, als auch Langzeiteffekte, wie die Selektion von resistenten Mikroorganismen. Eine Korrelation zwischen dem Verbrauch von FQ in der Viehzucht und der Entwicklung von klinischen resistenten Bakterienstämmen konnte bereits belegt werden (Kenyon, 2021).

Die negativen Auswirkungen auf Mensch und Umwelt durch Antibiotika im Abwasser und der aquatischen Umwelt können durch erweiterte Abwasserbehandlungen und andere „end-of-pipe“ Methoden offensichtlich nicht umfassend gelöst werden. Im Sinne einer grünen Chemie und Pharmazie ist es daher wichtig über ganzheitliche Lösungen, über den gesamten Lebenszyklus eines Moleküls hinweg, nachzudenken.

1.3 Grüne Pharmazie und Benign by Design

Die zwölf Prinzipien der „Green Chemistry“ beschreiben, wie Chemikalien und Prozesse zu gestalten sind, um die Erzeugung von für den Menschen oder die Umwelt gefährlichen Substanzen zu reduzieren und im besten Fall sogar vollständig zu verhindern. Im Rahmen der bisher diskutierten Problematik von Mikroschadstoffen in der aquatischen Umwelt ist insbesondere das zehnte der Prinzipien wichtig: „Design for Degradation“ (Anastas und Warner, 2000). Chemische Verbindungen sollen demnach so entwickelt werden, dass sie nach ihrer Verwendung bzw. der Erfüllung ihrer Funktion in ungefährliche Abbauprodukte abgebaut werden können und nicht persistent in der Umwelt sind. Im Bezug auf pharmazeutische Wirkstoffe bedeutet dies, bei ihrer Entwicklung nicht nur die pharmakologische Aktivität und die für die Anwendung notwendigen Eigenschaften zu optimieren, sondern auch das Schicksal der Substanz nach der Anwendung von Beginn an zu berücksichtigen. Ein Ziel der grünen Pharmazie ist deshalb, das Verhalten und die mögliche Wirkung auf die Umwelt der Wirkstoffe als Faktor in die Entwicklung zu integrieren, d.h. die Umweltgefährdung bei gleichbleibender oder sogar verbesserter Wirkung zu minimieren (Jordan und Gathergood, 2013; Kümmerer, 2007; Leder et al., 2015).

Darauf baut auch das Benign by Design (BbD) Konzept auf (Kümmerer, 2007, 2016). Dies kann sowohl durch das Design von komplett neuen Molekülen (*de novo* Design) mit den gewünschten Eigenschaften umgesetzt werden, als auch durch die Veränderung bereits bekannter und verwendeter Wirkstoffstrukturen (Re-Design). Denn oft können bereits kleine strukturelle Veränderungen eine große Auswirkung auf bestimmte Eigenschaften wie Löslichkeit, Aktivität oder Bioabbaubarkeit haben (Leder et al., 2015; Rastogi et al., 2014a,b, 2015).

Leder et al. (2015) veröffentlichten ein erstes Framework zum *de novo* und Re-Design von umweltfreundlicheren Substanzen. Am Beispiel von verschiedenen β-Blockern konnte erfolgreich gezeigt werden, wie das Re-Design bekannter Strukturen hin zu besser abbaubaren Derivaten umgesetzt werden kann. Damit wurde zum ersten mal ein experimentelles Framework für das sogenannte „non-targeted re-design“ von Wirkstoffen veröffentlicht (Rastogi et al., 2014a,b, 2015). Das Prinzip des „targeted re-designs“ konnte am Beispiel von Ciprofloxacin exemplarisch umgesetzt werden. Dabei wurde die Struktur von Ciprofloxacin so modifiziert, dass zumindest teilweise eine Mineralisierung und Inaktivierung der verbleibenden Struktur erreicht werden konnte. Dafür wurde der Cyclopropyl-

Rest des Ciprofloxacin durch ein Hemiaminal ersetzt. Dieser Hemiaminal-Linker kann bei niedrigen pH-Werten hydrolysiert werden, was in einem mineralisierbaren Hemiaminal-Rest und der verbleibenden Grundstruktur resultiert (Leder et al., 2021).

Das Ziel von BbD spiegelt sich in verschiedenen regulatorischen Strategien der EU wieder, wie der „Strategy for a non-toxic environment“ oder dem „Strategic Approach to Pharmaceuticals in den Environment“. Weiterhin veröffentlichte die Europäische Kommission 2020 im Rahmen des Green Deals die „Chemicals Strategy for Sustainability“ (EC, 2020). Das Konzept von „Safe and sustainable-by-design“, das im Rahmen dieser Strategie vorgestellt wurde, unterstreicht sowohl die Dringlichkeit, Chemikalien so zu entwickeln, dass sie sicher für Menschen und Umwelt sind und als auch die Wichtigkeit, im Rahmen dessen neue Methoden und Frameworks zu entwickeln.

Bisher werden Umweltaspekte erst sehr spät in der Wirkstoffentwicklung berücksichtigt. Für ein grüneres und nachhaltigeres Wirkstoffdesign sollten Endpunkte, wie zum Beispiel die leichte biologische Abbaubarkeit, bereits in den frühen Prozess als wichtige Kriterien implementiert werden (Puhlmann et al., 2021). Die Implementierung von Benign by Design in den regulären Prozess der Wirkstoffentwicklung könnte durch die Entwicklung und Publikation von Workflows, Strategien und Best Practice Beispielen stimuliert werden.

2 Aufbau und Ziel der Arbeit

Um die dargestellten negativen Auswirkungen auf Mensch und Umwelt durch nicht abbaubare Antibiotika in der Umwelt einzudämmen, war das übergeordnete Ziel dieser Arbeit einen Beitrag zur Entwicklung von umweltfreundlicheren Antibiotika zu liefern. Dabei sollten bereits bekannte Strategien untersucht, adaptiert und weiterentwickelt werden, um deren Potential und Übertragbarkeit auf andere Stoffgruppen zu untersuchen.

Das konkrete Ziel der Arbeit war, zu verstehen, welche Änderungen an den Molekülstrukturen der Fluorchinolonantibiotika vorgenommen werden können, um eine pharmakologisch aktiv Substanz zu entwickeln, welche während der Lagerung und Wirkdauer im Körper stabil ist, aber anschließend im Körper und/oder der Umwelt in ein nicht mehr aktives Derivat abgebaut, oder im besten Fall vollständig mineralisiert wird. Dadurch kann der Selektionsdruck in der Umwelt reduziert, und die Entwicklung von resistenten Mikroorganismen verlangsamt werden.

Tabelle 1: Liste der Arbeitspakete (AP) und den entsprechenden Publikationen

	AP	**Titel der Veröffentlichung**
Publikation 1	Diskussions-Papier	**Lorenz, S.**, Amsel, A.K., Puhlmann, N., Reich, M., Olsson, O., Kümmerer, K. (2021): Toward Application and Implementation of in Silico Tools and Workflows within Benign by Design Approaches. ACS Sustainable Chemsitry and Engineering. 9, 37, 12461-12475
Publikation 2	Targeted Re-Design	**Lorenz, S.**, Suaifan, G., Kümmer, K. (2022): Designing benign molecules: The influence of O-acetylated glucosamine-substituents on the environmental biodegradability of fluoroquinolones. Chemosphere. 309 (2), 136724
Publikation 3	Struktur-Bioabbau-Beziehungen	Suk, M., **Lorenz, S.**, Kümmerer, K. (2023): Identification of environmentally biodegradable scaffolds for the benign design of quinolones and related substances. Sustainable Chemistry and Pharmacy. 31, 100947

Die folgenden vier Arbeitsschwerpunkte wurden im Laufe der Disseration näher untersucht:

(1) Welche Herangehensweisen existieren im Rahmen des Benign by Design? Wie können die verschiedenen Ansätze kategorisiert werden? Wie können computergestützte Methoden (*in silico*) bei dem Design von grüneren Chemikalien und Arzneimitteln helfen und wie könnte ein entsprechender Workflow aussehen? (**Diskussions-Papier**)

(2) Kann das Framework des non-targeted Re-Designs, wie am Beispiel von β-Blockern (Leder et al., 2015) gezeigt, auf die Stoffgruppe der Fluorchinolone übertragen werden? (**Non-targeted Re-Design**)

(3) Können gezielte strukturelle Veränderungen, zum Beispiel durch das Hinzufügen von Zuckersubstituenten, die biologische Abbaubarkeit von Fluorchinolonen verbessern? (**Targeted Re-Design**)

(4) Welche Struktur-Bioabbau-Beziehungen und biologisch abbaubare Leitstrukturen können bei der Untersuchung von diversen N-heterozyklischen Verbindungen identifiziert werden? Wie können diese in das grüne Design von Fluorchinolonen einfließen? (**Struktur-Bioabbau-Beziehungen**)

Zu jedem dieser Arbeitsschwerpunkte wurden eigene wissenschaftliche Studien durchgeführt. Zu drei der genannten Arbeitsschwerpunkte wurden Artikel in internationalen peer-reviewed Fachzeitschriften publiziert (Tabelle 1). Auf diese Weise sollten aktuelle Lösungsansätze und Tools für das Design von umweltfreundlicheren Antibiotika aufgezeigt werden und zu einem wissenschaftlichen Diskurs anregen. Die dabei gewonnenen experimentellen Ergebnisse und konzeptionellen Vorschläge sollen dazu beitragen zukünftige Strategien und Gesetzgebungen umzusetzen, sowie weitere Forschung und Anwendung im Bereich des Wirkstoffdesigns zu stimulieren.

Die experimentellen Ergenisse des 2. Arbeitsschwerpunktes wurden nicht in einer Fachzeitschrift veröffentlicht. Das methodische Vorgehen sowie die entsprechenden Ergebnisse werden daher in diesem Rahmenpapier ausführlicher dargestellt als die der übrigen Arbeitsschwerpunkte.

3 Benign by Design Ansätze und die Verwendung von *in silico* Tools

3.1 Problemstellung

Die „Chemicals Strategy for Sustainability towards a Toxic-Free Environment“ der Europäischen Kommission beinhaltet verschiedene Aspekte, deren Umsetzung für „sustainable-by-design“ Chemikalien und eine „zero chemical pollution“ wichtig sind (EC, 2020). Diese Aktionsfelder umfassen unter anderem innovative neue Tools für die Risikoabschätzung sowie das Design von nachhaltigen und sicheren Chemikalien. Benign by Design (BbD) kann dafür einen wertvollen Beitrag liefern. Um Chemikalien und Arzneimittel in Zukunft so zu entwickeln, dass ihr Verbleib und ihre Abbaubarkeit in der Umwelt schon beim Design berücksichtigt werden, ist es daher wichtig verschiedene Benign by Design Ansätze zu verstehen und systematisch anwenden zu können.

Sogenannte *in silico* Tools spielen im Rahmen von BbD eine große Rolle. Im Vergleich zu *in vitro* und *in vivo* Tests sind sie günstiger, schneller und erfordern keine Tierversuche. Ihre Verwendung wird daher schon in verschiedensten Bereichen des Risikoassessments von Behörden empfohlen (ECHA, 2008). Es existieren bereits verschiedene Workflows, um das Risiko oder die Toxizität von Chemikalien mit Hilfe von Modellen zu bewerten (Escher und Fenner, 2011). Inwiefern solche Tools in einen Workflow für das Benign by Design implementiert werden können, wurde jedoch noch nicht tiefgreifend beleuchtet.

Ziel der Arbeiten zu diesem Arbeitsschwerpunkt war es daher, zunächst die bisher verfolgten Ansätze des BbD aus der Literatur zu identifizieren. Dafür wurden Veröffentlichungen gesichtet, in denen experimentell oder konzeptionell das BbD Konzept zum (Re)-Design von in der Umwelt besser abbaubaren Substanzen verwendet wurde. Weiterhin wurden verschiedene *in silico* Tools und deren Anwendungsbereiche und Limitie-

rungen untersucht. Basierend darauf wurde ein Workflow entwickelt, der die Verwendung von computergestützten Tools und Methoden innerhalb des BbD Frameworks aufzeigt. Es wurden außerdem Hilfestellungen herausgearbeitet, die dabei unterstützen können, den Workflow für spezifische Problemstellungen anzupassen.

3.2 Benign by Design Ansätze

Das Design und Re-Design von Chemikalien, wie z.B. Wirkstoffen oder Pestiziden, beruht zum einen auf der Annahme, dass kleinere strukturelle Anpassungen im Molekül zu Veränderungen der Eigenschaften und Aktivitäten führen können, als auch dass ähnliche Strukturen vergleichbare Eigenschaften aufweisen (Struktur-Eigenschafts-Beziehungen). Dieser Ansatz kann ebenso auf die biologische Abbaubarkeit von Molekülen angewendet werden. Kleine Veränderungen in der Struktur, wie z.B. das Hinzufügen einer Hydroxylgruppe, kann bereits zu signifikanten Änderungen in der biologischen Abbaubarkeit führen (Boethling et al., 2007; Rastogi et al., 2014a,b, 2015). Die Struktur-Eigenschaftsbeziehungen wiederum liegen den *in silico* Modellen zur Vorhersage der Abbaubarkeit zu Grunde.

Vier Ansätze für das Design von neuen Molekülen im Kontext des BbD wurden in der Literatur identifiziert (Tabelle 2):

(i) non-targeted *de novo* Design

(ii) targeted *de novo* Design

(iii) non-targeted Re-Design

(iv) targeted Re-Design

Alle vorgestellten Ansätze beruhen darauf, einen Pool an neuen Molekülen mit potentiell verbesserten Umwelteigenschaften zu erzeugen. Diese Moleküle werden entweder durch *in silico* Tools oder mit Hilfe von experimentellen Methoden erzeugt. Anschließend müssen die vielversprechendsten Strukturen identifiziert und ihre Eigenschaften näher untersucht werden.

Sowohl für das *de novo* als auch das Re-Design können targeted oder non-targeted Ansätze verfolgt werden. Der Unterschied dieser Methoden besteht darin, dass bei einem

Tabelle 2: Identifizierte BbD Ansätze, um einen Pool von Molekülen mit potentiell verbesserten Umwelteigenschaften zu erzeugen (**Publikation 1**)

	Non-targeted	**Targeted**
De novo	Scannen großer Mengen von Strukturen aus Datenbanken bezüglich ihrer Funktion, Bioabbaubarkeit und (Öko)Toxizität	Kombination molekularer Fragmente, welche bekannt dafür sind eine bestimmte Eigenschaft zu erzeugen
Re-Design	Non-targeted Synthese oder *in silico* Erzeugung von TPs, sowie anschließendes Screening dieser für Funktion, Bioabbaubarkeit und (Öko)Toxizität	Gezielte Integration von strukturellen Fragmenten in bekannte Strukturen, welche potentiell die Funktion und Bioabbaubarkeit verbessern und die (Öko)Toxizität verringern

targeted Ansatz strukturelle Fragmente, welche dafür bekannt sind bestimmte Eigenschaften zu beeinflussen, genutzt werden um Moleküle gezielt anzupassen oder neu zu designen. Dies beruht in der Regel auf einem großen Erfahrungsschatz und Expertenwissen. Der non-targeted Ansatz hingegen zeichnet sich durch eine nicht zielgerichtete Erzeugung von neuen Molekülen aus. Diese so erzeugten Moleküle mit unbekannten Eigenschaften können dann näher untersucht werden. Dieser Ansatz ist oft schneller und vor allem ist dafür weniger spezifisches Fachwissen über Struktur-Eigenschaftsbeziehungen nötig.

3.3 Workflow für die Verwendung von *in silico* Tools im Kontext von BbD

Nachdem ein oder mehrere Moleküle unter Verwendung der vorgestellten BbD Ansätze entworfen wurden, kann der im **Publikation 1** vorgestellte Workflow für die Bewertung und Optimierung dieser Strukturen herangezogen werden (**Publikation 1**, Abbildung 2). Dabei soll bewertet werden, ob die vorgeschlagenen Strukturen die vorgesehenen Eigenschaften besitzen (z.B. biologische Abbaubarkeit und keine Ökotoxizität), während sie

trotzdem ihre ursprüngliche oder gewünschte Funktion erfüllen.

Die für die Anwendung des Workflows nötigen Schritte und Überlegungen umfassen:

- die Repräsentation des Moleküls in einem maschinenlesbaren Format,
- die Definition der relevanten Endpunkte,
- die Auswahl der passenden Modelle und Methoden,
- die Evaluation und Interpretation der Ergebnisse,
- sowie das anschließende Datenmanagement.

Die dafür wichtigen Überlegungen wurden ausführlich in **Publikation 1** dargestellt. Weiterhin wurde diskutiert, welche Grenzwerte für die Beurteilung einer grüneren Chemikalie herangezogen werden sollten. Dies umfasst vor allem die Überlegung, dass eine Substanz nur so stabil sein sollte wie für die Lagerung und Anwendung nötig, jedoch nicht darüber hinaus.

Wird der Workflow für das non-targeted (Re)Design verwendet, kann dieser als eine Art Filter fungieren. Dabei werden die nicht zielgerichtet erzeugten Moleküle, die die gewünschten Kriterien nicht erfüllen, nach und nach aussortiert. Übrig bleibt eine verkürzte Auswahl an vielversprechenden Molekülen, welche anschließend durch Synthese und experimentelle Versuche weiter untersucht werden können. Da durch die *in silico* Methoden die Liste der Moleküle aber bereits stark verkürzt wurde, spart man bei der Untersuchung im Labor Zeit und Ressourcen.

Beim targeted Design von neuen Molekülen kann der Workflow als eine Art Schleife angewendet werden. Ein Molekül wird anhand der gewählten Methoden und Endpunkte bewertet. Werden die Kriterien nicht erfüllt, können Modifikationen an dem Molekül durchgeführt und dieses anschließend neu bewertet werden, bis die gewünschten Eigenschaften eingestellt wurden.

Um Bioabbaubarkeit als einen Endpunkt in die Entwicklung von Chemikalien zu integrieren, sind verfügbare und gute Modelle eine Voraussetzung. Dies bezieht sich zum einen auf die Performance der Modelle, als auch deren Anwendungsdomäne. Bei der Betrachtung der bisher zur Verfügung stehenden Modelle zur Vorhersage der Bioabbaubarkeit wurden jedoch einige Limitierungen deutlich.

Modelle zur Vorhersage der Bioabbaubarkeit performen oft schlechter als Modelle für andere Endpunkte, die sich z.B. auf physikochemische Eigenschaften konzentrieren. Dies

ist unter anderem darauf zurückzuführen, dass für den Bioabbau eine Vielzahl an Prozessen in der Umwelt eine Rolle spielen, wie die Aufnahme in die Zellen, der Transport der Substanzen innerhalb der Zelle, sowie die Anwesenheit, Aktivität und Spezifität von Enzymen. Weiterhin beinhalten Bioabbau-Tests, wie alle biologischen Tests, eine gewisse Unsicherheit. Mit Ausnahme von *single-strain* Tests, sind die vorhandenen Enzyme und Mikroorganismen in den meisten Fällen nicht bekannt, die Tests sind also oft eine „Black Box“. Da die Zusammensetzung der Enzyme und Bakterien je nach Ort und Jahreszeit unterschiedlich sein kann, können auch die Testergebnisse schwanken. Diese Schwankungen führen bereits in den Trainingsdaten zu Unsicherheiten, die sich natürlich auch auf die damit trainierten Modelle auswirken.

Experimentelle Bioabbaudaten für ein Modell sollten daher im Optimalfall nach einer spezifischen Test-Guideline mit der gleichen Inokulum Quelle erzeugt werden. Solche Datensätze sind jedoch sehr rar und beinhalten nur eine geringe Auswahl an Chemikalien, was die Entwicklung von generalisierten Modellen sehr schwierig macht. Um das Angebot der Bioabbau-Modelle zu verbessern, sind experimentelle Daten aus vergleichbaren Tests für eine Vielzahl an Strukturen nötig.

Im Rahmen der Dissertation wurde daher auch diese Forschungslücke aufgegriffen und die Datengrundlage für ein lokales Bioabbau-Model generiert (**Publikation 3**). Die diskutierten Ansätze des targeted und non-targeted Re-Designs sollen zudem am Beispiel der Fluorchinolonantibiotika im Folgenden näher betrachtet werden.

4 Non-targeted Re-Design von Fluorchinolonen

4.1 Problemstellung

Die Untersuchung verschiedener Strukturvariationen ist zum Beispiel ein in der Wirkstoffentwicklung übliches Vorgehen (Puhlmann et al., 2021). Bereits kleine Veränderungen an der Struktur von Molekülen können zu signifikanten Änderungen in deren Umweltverhalten führen (Boethling et al., 2007; Leder et al., 2015), ohne dass ihre für die Anwendung wichtigen Eigenschaften und Aktivitäten verloren gehen müssen. Dabei werden meist die für die Aktivität verantwortlichen Fragmente des Moleküls (Leitstruktur) beibehalten, andere Substituenten aber verändert (Leder et al., 2015). Für Substituenten, welche potentiell die biologische Abbaubarkeit eines Moleküls beeinflussen, sind bereits einige Zusammenhänge bekannt (Boethling et al., 2007; Kümmerer, 2010).

Gezielte Synthesen verschiedener Variation können allerdings sehr zeitaufwendig und teuer sein. Es bietet sich daher an, entweder mit Hilfe von *in silico* Tools verschiedene Strukturen zu erzeugen und daraus eine Vorauswahl zu treffen (**Publikation 1**) oder eine sogenannte non-targeted Synthese durchzuführen (Leder et al., 2015; Rastogi et al., 2014a,b, 2015). Dabei können zum Beispiel Methoden der erweiterten Abwasserbehandlung (wie Ozonierung oder Photolyse) verwendet werden, um eine Vielzahl an Transformationsprodukten zu erzeugen, welche in den meisten Fällen weiterhin die Leitstruktur der Muttersubstanz enthalten.

Rastogi et al. (2014a,b, 2015) zeigten am Beispiel verschiedener β-Blocker, wie eine non-targeted Synthese mit anschließender Identifikation von in der Umwelt abbaubaren Strukturen aussehen kann. Dabei wurden aus bekannten Ausgangssubstanzen mittels Photolyse Transformationsprodukte erzeugt und anschließend die Reaktionslösung einem Bioabbautest unterzogen, sowie die Strukturen mittels LC-MS aufgeklärt. All

jene Transformationsprodukte, welche eine biologische Abbaubarkeit zeigten und die gewünschte Leitstruktur weiterhin enthielten, wurden anschließend mittels verschiedener *in silico* Methoden näher untersucht. Wenn möglich, wurden vielversprechende Strukturen auch als Einzelsubstanzen getestet und ihre Bioabbaubarkeit und Aktivität in *in vitro* Tests verifiziert. Rastogi et al. konnten damit zeigen, dass im Fall der untersuchten β-Blocker bereits die Addition einer Hydroxlygruppe zu einer verbesserten Mineralisierung in der Umwelt führt, während die Wirkungsweise dadurch sehr wahrscheinlich nicht beeinträchtigt wird. Damit wurde außerdem ein Arbeitsablauf entwickelt, der es ermöglicht eine Vielzahl an Strukturen mit geringen strukturellen Veränderungen schnell und kostengünstig hinsichtlich ihrer Bioabbaubarkeit zu untersuchen.

Um festzustellen, inwiefern sich dieses gezeigte Vorgehen auch auf andere Substanzgruppen übertragen lässt, wurden im Rahmen der Dissertation verschiedene Fluorchinolone entsprechend diesem Vorgehen untersucht. Dafür wurden mittels Photolyse TP erzeugt und anschließend hinsichtlich ihrer biologischen Abbaubarkeit und Aktivität bzw. Toxizität untersucht.

4.2 Methoden

Das Konzept der non-targeted Synthese wurde auf neun verschiedene Substanzen angewendet (Abbildung 1). Dazu gehörten insgesamt sechs Antibiotika aus der Gruppe der (Fluor)Chinolone (Oxolinsäure, Nalidixinsäure, Lomefloxacin, Marbofloxacin, Moxifloxacin, Prulifloxacin) sowie drei Grundstrukturen aus der Gruppe der Chinolone (CG01, CG02, CG03). Die Strukturen wurden ausgewählt, um innerhalb der Substanzklasse einen möglichsten weiten Strukturraum abzudecken und Erkenntnisse über den Einfluss verschiedener funktioneller Gruppen oder Atome an unterschiedlichen Position zu untersuchen.

Die nicht zielgerichtete Erzeugung von neuen Derivaten wurde mittels UV-Photolyse und UV-Photokatalyse erreicht. Dafür wurden Lösungen der Muttersubstanzen (c = 20 $\frac{\text{mg}}{\text{L}}$ und c=70 $\frac{\text{mg}}{\text{L}}$ bis 90 $\frac{\text{mg}}{\text{L}}$) einer Quecksilber-Mitteldrucklampe (TQ150, UV Consulting Peschl, Mainz) für maximal 64 min in einem 1 L Batch-Photoreaktor ausgesetzt. Im Falle der Photokatalyse wurden zudem 500 $\frac{\text{mg}}{\text{L}}$ Titandioxid (Degussa P25) hinzugefügt. Die Probenentnahme erfolgte jeweils nach 0, 2, 4, 8, 6, 32 und 64 Minuten. Die Proben der Photokatalyse wurden vor der Analyse für 5 min bei 400 rpm zentrifugiert.

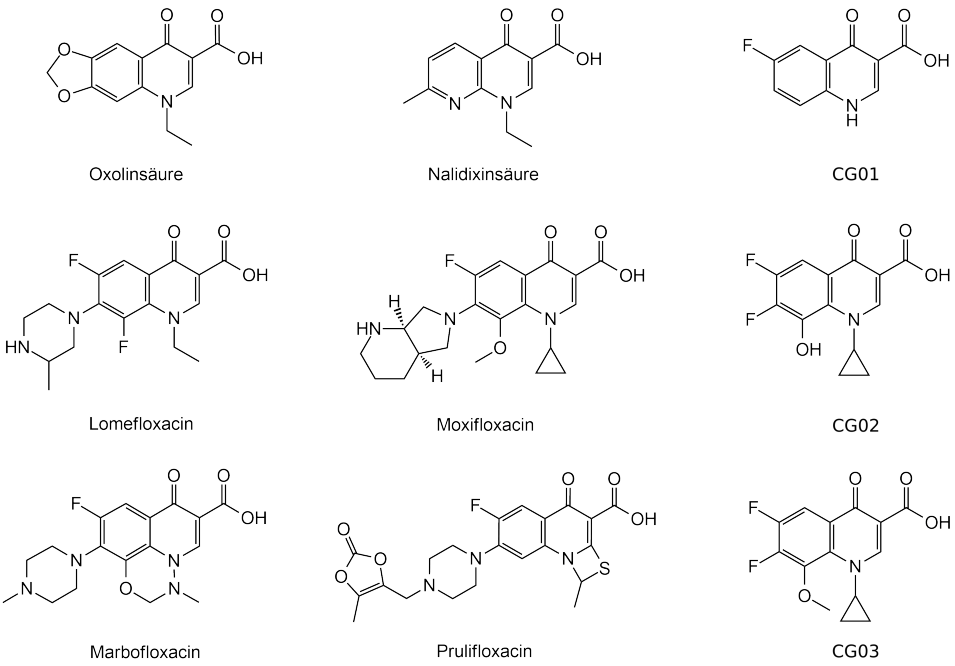


Abbildung 1: Strukturen der (Fluor)Chinolone, die im Rahmen des non-targeted Ansatzes untersucht wurden

Die Konzentration an gelöstem organischen Kohlenstoff (dissolved organic carbon, DOC) in den Photolyse-Proben wurde mittels Total Organic Carbon-Analysator (TOC 5000 Shimadzu GmbH, Duisburg) gemessen. Anhand dessen konnte der Grad der Mineralisierung während der Photolyse bestimmt werden. Die Primärelimination der Muttersubstanzen wurde durch Messungen mittels Hochleistungsflüssigkeitschromatographie (HPLC) verfolgt. Dafür wurde eine Dionex Ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham) mit Umkehrphasen-Säule (RP18 EC 125x4 mm Nucleodur 100-5) verwendet. Das Fließmittel wurde graduell aus 0,1 % Ameisensäure in Wasser und 100 % Acetonitril zusammengesetzt. Die entstandenen Transformationsprodukte wurden mit einem amaZon SL Ionenfallen-Massenspektrometer (Bruker Daltonic, Bremen) und einer hochauflösenden LTQ Orbitrap XL (Thermo Fisher Scientific,Waltham) analysiert. Die Parameter der Ionenfallen wurden durch vorherige Optimierung mit der Muttersubstanz eingestellt und es wurde im positiven Ionisierungsmodus gemessen.

Das durch die Photo(kata)lyse erzeugte Gemisch der Transformationsprodukte wurde anschließend in biologische Abbautests überführt. Dafür wurden zwei Tests aus der OECD 301 Guideline verwendet – der Closed Bottle Test (301 D) und der Manometrische

Respirationstest (301 F). Beide Tests untersuchen die leichte biologische Abbaubarkeit der Testsubstanzen, unterscheiden sich jedoch in der verwendeten Bakteriendichte und -diversität sowie der eingesetzten Substanzkonzentration. Für den Testansatz wird das Substanzgemisch, mineralisches Medium und das Inokulum (Kläranlagenablauf oder Belebtschlamm, Abwasser Grün & Lüneburg Services GmbH, Lüneburg, 325.000 Einwohner) in eine luftdicht verschlossene Testflasche überführt und bei konstanter Temperatur über 28 Tage im Dunkeln gelagert. Zur Validierung des Tests wurden außerdem Proben für Blindwert, Qualitätskontrolle und Toxizitätskontrolle mitgeführt. Beim CBT wird anhand einer faseroptischen Sauerstoffbestimmung indirekt auf den biologischen Abbau geschlossen. Der Manometrische Respirationstest (MRT) nutzt Oxitop-Köpfe (Xylem Water Solutions Deutschland GmbH, Langenhagen) um den Sauerbedarf zu messen. Der prozentuale Abbau wird anschließend anhand Gleichung 4.1 bestimmt. Weitere Informationen zu den biologischen Abbautests können der OECD Richtlinie entnommen werden (OECD, 1992).

$$\text{Abbau } [\%] = \frac{BSB}{ThOD} \cdot 100 \tag{4.1}$$

BSB = gemessener biologischer Sauerstoffbedarf in mg O_2 pro mg Testsubstanz

ThOD = theoretischer Sauerstoffbedarf in mg O_2 pro mg Testsubstanz

Um den Abbau der einzelnen TP in dem Reaktionsgemisch zu analysieren, wurden an Tag 0 und Tag 28 Proben aus den Ansätzen der Bioabbautests entnommen und mittels LC-MS analysiert. Die Peakflächen der einzelnen Signale von beiden Zeitpunkten wurden anschließend verglichen. Somit konnten Aussagen über die Entwicklung der einzelnen TP während des CBT/MRT getroffen werden.

Um einen ersten Eindruck bezüglich der Aktivität der TP im Vergleich zur Muttersubstanz zu erhalten, wurde das Substanzgemisch verschiedener Bestrahlungszeiten aus den Photo(kata)lyse-Versuchen in einem kinetischen Leuchtbakterientest (LBT) untersucht (Menz et al., 2013). Bei diesem Test können drei verschiedene Endpunkte gemessen werden: die akute Leuchthemmung, die chronische Leuchthemmung und die chronische Wachstumshemmung. Für die Muttersubstanzen konnten diese Endpunkte für verschiedene Konzentrationen gemessen werden. Dadurch konnte mit Hilfe einer logistischen Regressionskurve eine Dosis-Wirkungsbeziehung bestimmt werden (Abbildung A1). Anhand dieser bekannten Dosis-Wirkungsbeziehung und der gemessenen Konzentration

der Muttersubstanz in dem Substanzgemisch nach der Bestrahlung, konnte die erwartete theoretische Hemmung der Muttersubstanz berechnet werden. Ist die gemessene Hemmung größer als die theoretisch berechnete Hemmung, kann dies ein Hinweis auf potentiell toxische bzw. aktive Transformationsprodukte sein.

4.3 Ergebnisse und Diskussion

Für alle neun untersuchten Strukturen wurde die Muttersubstanz durch UV-Strahlung vollständig eliminiert. In den meisten Fällen blieb der DOC bei vollständiger Primärelimination jedoch konstant (Abbildung 2). Dies deutet auf die Entstehung von photostabilen TP hin. Durch die LC-MS Untersuchungen konnte die Bildung neuer Strukturen bestätigt werden. Die non-targeted Synthese von neuen Derivaten mit struktureller Ähnlichkeit zur Muttersubstanz war daher erfolgreich.

Für die weitere Untersuchung der biologischen Abbaubarkeit wurde eine definierte Bestrahlungszeit für jede Substanz individuell ausgewählt. Die gewählte Bestrahlungszeit richtete sich nach der Kinetik der detektierten TP und der Primärelimination der Muttersubstanz.

Bei allen sechs untersuchten (Fluor)Chinolonen konnte mindestens eine leichte Steigerung der biologischen Abbaubarkeit nach Bestrahlung im CBT oder MRT festgestellt werden (vgl. Tabellen 3 & 4). Dies ist ein erster Hinweis darauf, dass womöglich vollständig oder teilweise biologisch abbaubare TP entstanden sind. Alle Muttersubstanzen (unbestrahlte Proben) waren im CBT oder MRT nicht biologisch abbaubar.

Nach der Bestrahlung von Oxolinsäure, sowohl mit als auch ohne Katalysator, und nach der Photokatalyse von Naldixinsäure wurde eine deutlich gesteigerte biologische Abbaubarkeit gemessen. Auch die Photokatalyse von Marbofloxacin und Prulifloxacin steigerte die Abbaubarkeit des Substanzgemisches (vgl. Tabelle 3). Mit Ausnahme von Lomefloxacin führte eine Photokatalyse immer zu einer deutlicheren Steigerung der Bioabbaubarkeit des Photo-TP-Gemisches im Vergleich zur Photolyse. Von den untersuchten Grundstrukturen zeigte nur die TP-Mischung von CG03 eine verbesserte Bioabbaubarkeit. Weiterhin ist deutlich zu sehen, dass die höhere Bakteriendichte und -diversität im MRT nicht zwangsläufig auch zu höheren Abbauwerten im Vergleich zum CBT führte (vgl. Tabelle 4).

Tabelle 3: Abbauwerte in Prozent im CBT der Muttersubstanzen (unbestrahlt) und der erzeugten Photo-TP-Mischungen (bestrahlt) (n=4)

Muttersubstanz	**Biologischer Abbau[%]**			
	Photolyse		Photokatalyse	
	unbestrahlt	bestrahlt	unbestrahlt	bestrahlt
Oxolinsäure	1,1 ± 6,2	29,0 ± 1,2	2,0 ± 1,0	29,8 ± 1,4
Nalidixinsäure	1,7 ± 0,3	13,2 ± 0,7	4,1 ± 3,9	35,3 ± 0,4
Marbofloxacin	-0,4 ± 1,3	9,8 ± 0,05	4,0 ± 0,9	16,8 ± 4,2
Moxifloxacin	1,9 ± 0,5	6,5 ± 0,7	-5,7 ± 2,0	-0,7 ± 0,5
Lomefloxacin	-2,6 ± 0,1	5,0 ± 0,6	-1,4 ± 10,2	8,5 ± 5,4
Prulifloxacin	7,8 ± 0,1	5,0 ± 0,6	-1,4 ± 10,2	8,5 ± 5,4
CG01	-3,0 ± 0,5	4,4 ± 0,5	-	-
CG02	0,9 ± 0,8	2,9 ± 0,8	-	-
CG03	-0,1 ± 2,6	10,45 ± 0,8	-	-

Tabelle 4: Abbauwerte in Prozent im MRT der Muttersubstanzen (unbestrahlt) und der erzeugten Photo-TP-Mischungen aus der UV-Photolyse (bestrahlt) (n=2)

Muttersubstanz	**Biologischer Abbau [%]**	
	unbestrahlt	bestrahlt
Nalidixinsäure	1,0 ± 6,6	9,5 ± 2,6
Lomefloxacin	-1,9 ± 2,6	10,4 ± 6,8
Moxifloxacin	-7,6 ± 2,6	-3,8 ± 2,8

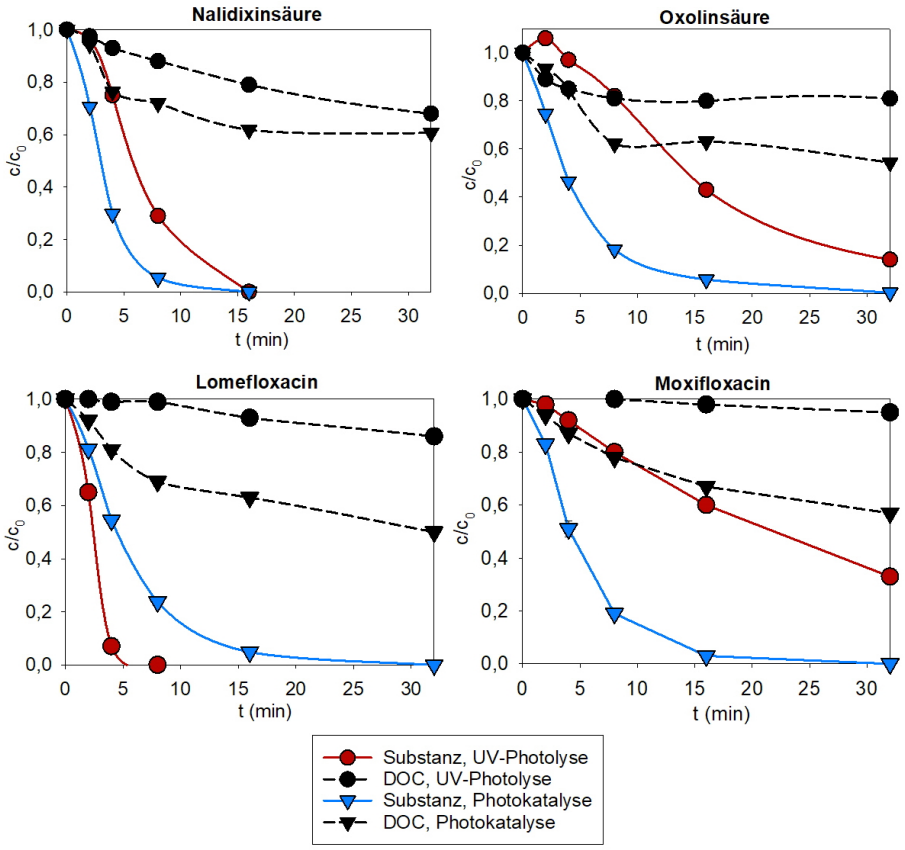
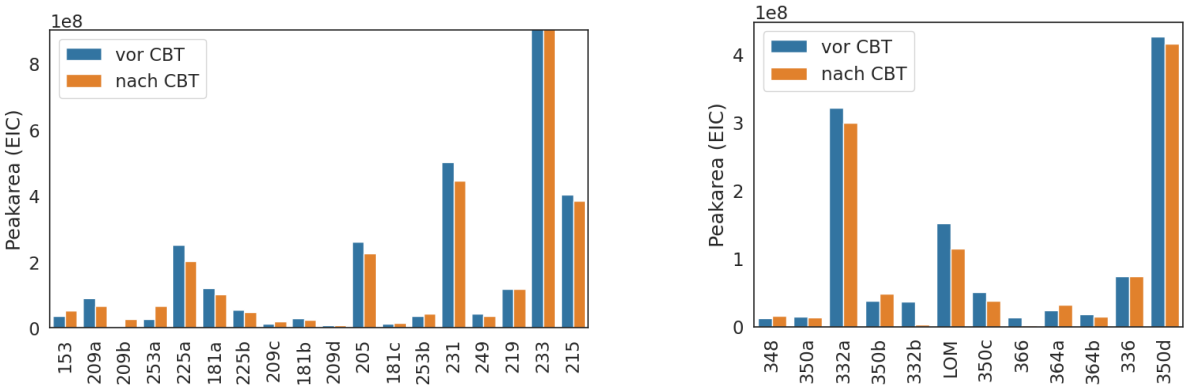


Abbildung 2: Relative Substanzkonzentration und gelöster Kohlenstoff über die Bestrahlungszeit bei Photolyse und Photokatalyse von Nalidixinsäure, Oxolinsäure, Lomefloxacin und Moxifloxacin (n=1)

In Abbildung 3 wurde beispielhaft dargestellt, wie eine Analyse der Peakflächen der gemessenen TP vor und nach dem Bioabbau aussehen kann. Mittels Massenspektrometrie (MS) wurden Masse-Ladungsverhältnisse gemessen und die gemessenen Peaks integriert. Die Peakflächen vor und nach dem Bioabbau-Experiment wurden anschließend verglichen, um ggf. biologisch abbaubare TP zu identifizieren. Eine Strukturaufklärung erfolgte dann für die als interessant identifizierten Massen mittels hochauflösender MS und MS^2 Messungen. Wie in Abbildung 3 dargestellt, unterscheiden sich die gemessenen Peakflächen vor und nach dem CBT oft nur minimal. Daher waren Rückschlüsse auf die für den gemessenen Abbau verantwortlichen TP nur bedingt möglich.

Einige Strukturvorschläge sind in Abbildung 4 dargestellt. Anhand der vorläufig vorgeschlagenen Strukturen ist hauptsächlich eine Defluorierung und Hydroxylierung an



(a) Nalidixinsäure nach UV-Photolyse

(b) Lomefloxacin nach UV-Photolyse

Abbildung 3: Vergleich der im MS detektierten Peakflächen der Photo-TPs von Nalidixinsäure und Lomefloxacin vor und nach dem CBT zur Identifikation von abbaubaren TP (n=1)

verschiedenen Stellen des Moleküles zu erkennen. Die exakte Stelle der Hydroxylierung lässt sich anhand der MS-Ergebnisse jedoch nicht bestimmen. Da zudem keine Referenzsubstanzen verfügbar waren, handelt es sich bei den dargestellten Strukturen nur um mögliche Isomere, die nicht eindeutig bestätigt werden konnten.

Bei der MS Analyse der Photo-TP-Mischungen zeigte sich, dass je nach verwendeter Muttersubstanz die resultierenden TP unterschiedlich gut analytisch erfassbar und identifizierbar sind. Die Aufklärung der TP von z.B. Nalidixinsäure und Lomefloxacin gestaltet sich aufgrund der guten Ionisierbarkeit der TP und vielen aus der Literatur bekannten Strukturen einfacher als dies z.B. für Oxolinsäure oder Marbofloxacin der Fall war. Eine Bestätigung der Strukturvorschläge war größtenteils nicht möglich, da die TP nicht als Standard erhältlich waren. Dabei zeigte sich eine erste Limiterung des verfolgten non-targeted Re-Design Ansatzes. Es wurden zwar viele TP gebildet und teilweise auch eine Steigerung der biologischen Abbaubarkeit der Photo-TP-Mischungen nachgewiesen, die Identifikation der jeweils dafür verantwortlichen Strukturen in diesem Gemisch war jedoch nicht eindeutig möglich. Eine Aussage darüber, ob die potentiell abbaubaren Strukturen nur teilweise abgebaut oder vollständig mineralisiert wurden, oder wie die Abbauwege aussehen, ist aufgrund der vielen gemessenen Signale nicht möglich. Es wäre daher nötig gewesen, die experimentellen Screening-Ergebnisse mit Einzelsubstanzen zu verifizieren.

Die unterstützende Verwendung von *in silico* Modellen zur Vorhersage der Bioabbau-

barkeit und die Identifikation von structural alerts, wie von Rastogi et al. (2014a,b, 2015) durchgeführt, konnte für die Transformationsprodukte der untersuchten Fluorchinolone nicht angewandt werden, da die Strukturen alle außerhalb der Anwendungsdomäne der verfügbaren Modelle lagen.

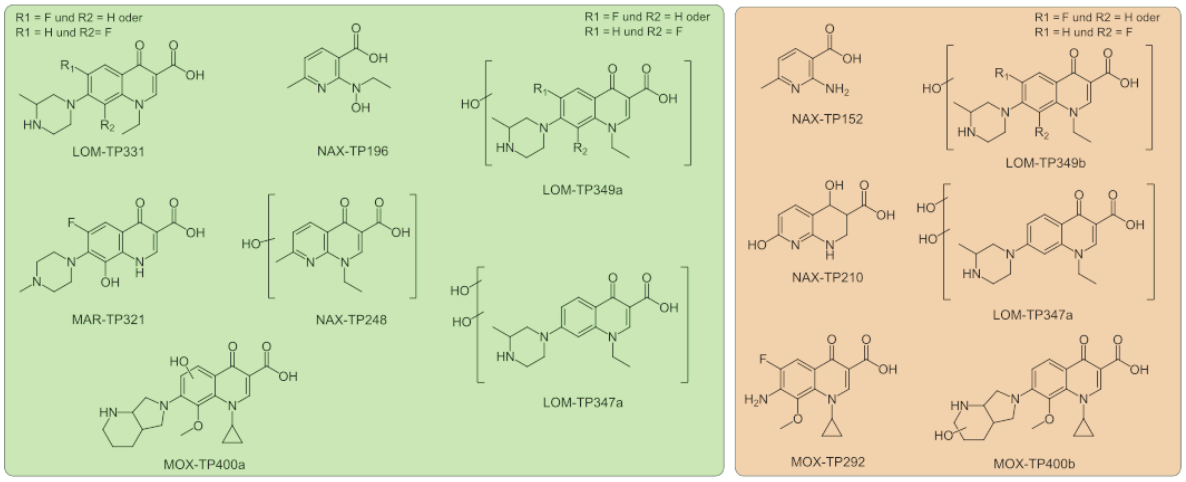


Abbildung 4: Strukturvorschläge für Transformationsprodukte, die im CBT abgebaut wurden (grün) oder während der 28 Tage als Metabolite entstanden sind (rot)

In den Leuchtbakterientests wurde die Hemmwirkung auf die Umweltbakterien im Laufe der Bestrahlungszeit von 0 min bis 64 min untersucht. Für die chronischen Endpunkte der Zellvermehrungshemmung nach 14 h und der Leuchthemmung nach 24 h wurde die gemessene Hemmung der erwarteten Hemmung der verbleibenden Muttersubstanz gegenübergestellt (Abbildungen A2, A3). Die Hemmwirkung der untersuchten Bestrahlungsproben nimmt gleichermaßen mit der Konzentration der Muttersubstanz ab, sodass sich die gemessene Hemmwirkung vollständig durch die Konzentration der Muttersubstanz erklären lässt. Für alle untersuchten (Fluor)Chinolone wurden vergleichbare Ergebnisse ermittelt. Anhand dieser Ergebnisse lassen sicher daher keine Hinweise auf toxische oder aktive Transformationsprodukte finden. Es lässt sich jedoch aufgrund dieser Untersuchung nicht vollständig ausschließen, dass trotzdem aktive TP in dem Substanzgemisch enthalten waren, diese aufgrund ihrer sehr geringen Konzentration jedoch unter den gewählten Testbedingungen keinen messbaren Effekt zeigten.

Der Reaktionsweg des abiotischen Abbaus mittels Photolyse (non-targeted Synthese) sowie der Bioabbau der (Fluor)Chinolone scheinen für alle untersuchten Substanzen sehr

ähnlich zu sein. Vermutlich findet oft eine Hydroxylierung am Piperazinring und an der Doppelringstruktur statt, meist an Position 6 oder 8, dabei auch einhergehend mit einer Substitution des Fluor-Atoms (van Doorslaer et al., 2014; Wetzstein et al., 2012).

Die Ergebnisse und Literaturdaten legen nahe, dass die Substitution durch eine oder mehrere Hydroxylgruppen an der Chinolon-Grundstruktur potentiell zu einer verbesserten biologischen Abbaubarkeit führt. Dies deckt sich zum Beispiel mit den Literaturergebnissen bezüglich des Abbaumechanismus durch verschiedene Pilze und anderen Mikroorganismen, die in der Lage sind (Fluor)Chinolone abzubauen (Cruz-Morató et al., 2013; Wetzstein et al., 2012). Dabei sind mehrere Positionen für Hydroxylgruppen vorstellbar, die zu einer verbesserten Abbaubarkeit führen können. Zum einen ist bekannt, dass durch eine Hydroxylgruppe an C2 die Stabilität des Moleküls verloren geht und dieses somit leichter von Mikroorganismen abgebaut werden kann (Felczak et al., 2014). Durch den Abbau der C7 Seitenkette und einer Hydroxylgruppe an Position C6 könnte bei einigen Strukturen zudem eine Hydroxylgruppe in ortho-Position zu der entstandenen Aminogruppe vorliegen. Somit würde eine reaktive 2-Aminophenol-Struktur entstehen, welche potentiell leicht biologisch abbaubar ist (Arora, 2015).

4.4 Schlussfolgerung

Die Fragestellung war, inwiefern das für β-Blocker erfolgreich angewandte Vorgehen des non-targeted Re-Designs auf die Stoffgruppe der Fluorchinolone übertragen werden kann. Die non-targeted Synthese konnte für FQ gut umgesetzt werden und durch verschiedene Bestrahlungsmethoden konnte eine Vielzahl an neuen Verbindungen erzeugt werden. Die resultierenden Substanzgemische konnten, wie von Rastogi et al. (2014a,b, 2015) gezeigt, in einem Screeningtest auf ihre biologische Abbaubarkeit untersucht werden. Die eindeutige Identifikation der TP war jedoch aufgrund fehlender Referenzsubstanzen nicht eindeutig möglich. Dadurch war es auch nicht möglich, die ersten experimentellen Ergebnisse aus den Screening-Tests mit Reinsubstanzen zu verifizieren.

Die Herausforderungen bei diesem Vorgehen bezogen sich vor allem auf die Arbeit mit den Substanzgemischen. Da sich alle gemessenen Effekte, wie Bioabbaubarkeit oder Toxizität, auf ein Substanzgemisch bezogen, war es kaum möglich diese Effekte einzelnen Strukturen zuzurechnen. Die Messung der TP-Massen und der Vergleich ihrer Peakflächen vor und nach dem Bioabbautest allein genügten nicht, um eine eindeutige Aussage

darüber zu treffen, ob die hier erzeugten Strukturen tatsächlich vollständig mineralisiert werden konnten. Die unterstützende Analyse mithilfe von *in silico* Bioabbau-Modellen konnte nicht durchgeführt werden, da die vorgeschlagenen Strukturen nicht in der Anwendungsdomäne der verfügbaren Modelle enthalten waren. Daher ist es für weitere Untersuchungen unumgänglich einzelne, in dem Substanzgemisch vermutete Strukturen zu synthetisieren und als Reinsubstanz weiter zu untersuchen. Eine Synthese des Transformationsproduktes 6-Hydroxy-Moxifloxacin wurde in Auftrag gegeben, da, wie oben bereits diskutiert, die Hydroxylierung an Position 6 eine besser abbaubare Struktur vermuten lässt. Die Synthese konnte technisch jedoch nicht in einem annehmbaren zeitlichen und finanziellen Rahmen umgesetzt werden und konnte daher von verschiedenen, angefragten Dienstleistern nicht geliefert werden.

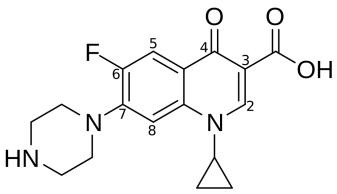
Abschließend lässt sich zusammenfassen, dass das non-targeted Re-Design wie von Rastogi et. al. beschrieben auf Fluorchinolone nur bedingt übertragbar ist. Dies ist vor allem auf die schlechte Synthetisierbarkeit der möglichen Transformationsprodukte zurückzuführen, was die Verifikation der Ergebnisse einschränkt. Auch wenn der hier verfolgte non-targeted Ansatz viele Vorteile für das Re-Design von Strukturen bietet, wie z.B. das schnelle und kostengünstige Screening vieler verschiedener Strukturen, scheint die Anwendbarkeit nicht auf alle Substanzklassen gleichermaßen gegeben zu sein. Die Ergebnisse zeigen, dass der Erfolg des non-targeted Re-Designs sehr abhängig von der Grundstruktur der untersuchten Stoffgruppe ist und schwer abbaubare und hoch substituierte Ausgangssubstanzen hier zu Schwierigkeiten führen können.

5 Targeted Re-Design von Fluorchinolonen: Untersuchung von Glucosamin-Derivaten

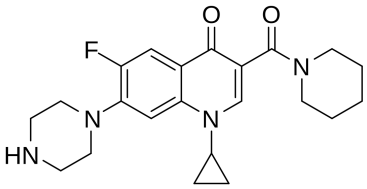
5.1 Problemstellung

Seit der Entdeckung der Nalidixinsäure als erstes Chinolon durchlief die Gruppe der Fluorchinolone verschiedene Entwicklungs- und Optimierungszyklen. Die Optimierung dieser Strukturen bezieht sich bisher vor allem auf die Verbesserung der Aufnahme, der Dosierung und dem Spektrum ihrer Aktivität, insbesondere gegen bereits resistente Pathogene. Dafür wurden verschiedene Seitenketten der Grundstruktur verändert und optimiert (Abb. 5, oben). Modifikationen an der Carboxylgruppe in Position 3 wurden bisher kaum durchgeführt. Die Carboxylgruppe gilt als verantwortlich für die Aktivität der Strukturen, da sie vermutlich als Bindungsstelle für die DNA Gyrase fungiert (Aldred et al., 2014). Es existieren jedoch einige wenige Publikationen, in denen an der Carboxylgruppe neue funktionelle Gruppen eingeführt wurden (Abb. 5, unten). Dabei konnte auch eine gesteigerte Aktivität dieser Derivate im Vergleich zur Ausgangssubstanz nachgewiesen werden, unter anderem auch gegen eigentlich bereits resistente Mikroorganismen (Mohammed et al., 2019, 2020).

Die von Mohammed et al. (2019, 2020) synthetisierten Derivate enthalten acetylierte und nicht-acetylierte Glucosamin-Fragmente in Position 3. Die Grundstruktur bildeten verschiedene bekannte (Fluor)Chinolone (Abb. 6). Diese Strukturen wurden hinsichtlich ihrer Aktivität und Cytotoxizität untersucht, ihre Umwelteigenschaften wurden bisher nicht näher beleuchtet. Da funktionelle Gruppen wie Ester, Amide, Hydroxylgruppen oder Phenoxy-Ringe dafür bekannt sind, die biologische Abbaubarkeit von Molekülen zu verbessern (Boethling et al., 2007), ist es aus Sicht des BbD interessant, näher zu untersu-



Struktur des Ciprofloxacins, eines häufig verwendeten Fluorchinolons der 3. Generation mit der typischen Carboxylgruppe an Position 3 und einer Ketogruppe an Position 4.



Beispiel eines an Position 3 substituierten Amin-Fluorchinolons, synthetisiert und publiziert von Kaur et al. (2020).

Abbildung 5: Struktur der FQ am Beispiel von Ciprofloxacin und einem substituierten Derivat von Kaur et al., 2020 (übersetzt aus **Publikation 2**)

chen, inwiefern die Glucosamin-Seitenketten die biologische Abbaubarkeit der Fluorchinolone beeinflussen. Daher wurde die biologische Abbaubarkeit der Derivate untersucht und die resultierenden Metabolite analysiert, um zu verstehen, inwiefern das Einfügen von Zuckerresten in einem Molekül zum gezielten Design von grüneren Fluorchinolonen beitragen kann.

5.2 Methoden

Acht Gluosamin-Derivate von Nalidixinsäure, Ciprofloxacin, Norfloxacin und Moxifloxacin, jeweils O-acetyliert (Substanzen 1, 3, 5 und 7) und O-deacetyliert (Substanzen 2, 4, 6 und 8) wurden von der University of Jordan von Prof. Ghadeer Suaifan (Department of Pharmaceutical Sciences) und Aya A. M. Mohammed zur Verfügung gestellt (Abb. 6).

Alle acht Substanzen wurden im CBT entsprechend der OECD Richtlinie hinsichtlich ihrer leichten biologischen Abbaubarkeit untersucht (OECD, 1992). Alle Testflaschen enthielten 1 % Dimethylsulfoxid (DMSO), welches in diesen Tests nicht abgebaut wird, um die Löslichkeit der Substanzen zu verbessern. Zum Testende nach 28 Tagen wurden Proben für eine massenspektrometrische Analyse entnommen.

Die verfügbare Menge der Substanzen erlaubte nur eine begrenzte Anzahl an biologischen Abbautests. Deshalb wurden für die folgenden Tests nur jeweils zwei Vertreter aus

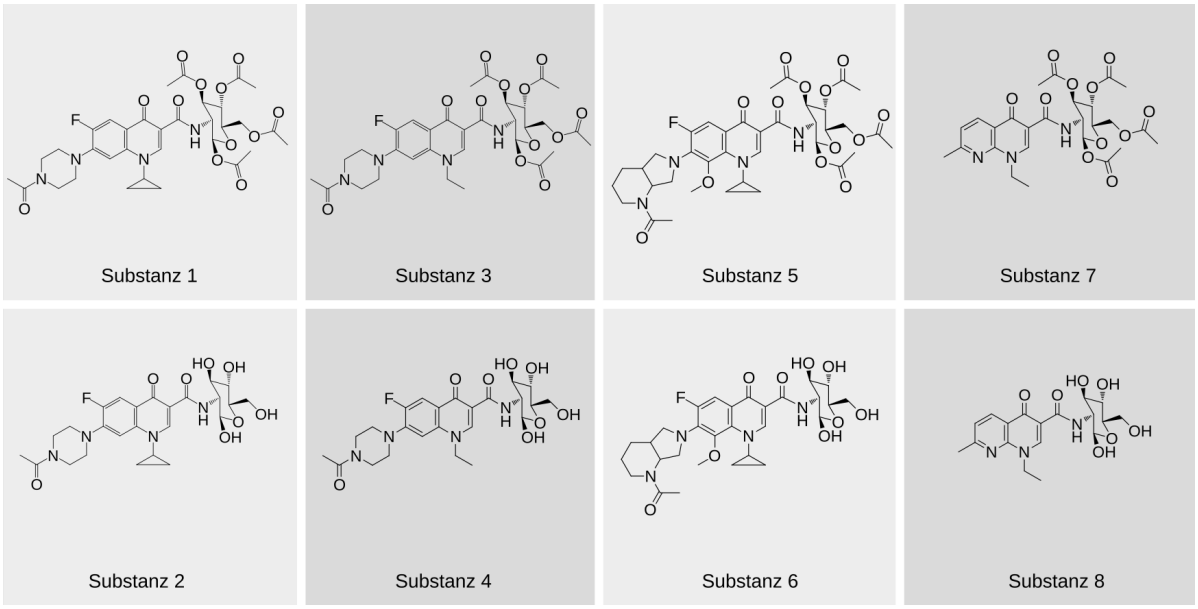


Abbildung 6: Strukturformeln der untersuchten Glucosamin Derivate verschiedener Chinolone und Fluorochinolone (aus **Publikation 2**)

den insgesamt acht verschiedenen Derivaten ausgewählt. Um die Entwicklung der Metabolite im Verlauf des Abbautests zu untersuchen, wurde ein an den CBT angelehnter Test für die Substanzen 1 und 2 über 48 Tage durchgeführt und alle 5-6 Tage eine Probe für eine Analyse entnommen. Für die Substanzen 5 und 6 wurde der MRT nach OECD 301F Richtlinie durchgeführt (OECD, 1992). Nach 28 Tagen wurden ebenfalls Proben für weitere analytische Untersuchungen entnommen.

Die entstandenen Transformationsprodukte wurden anschließend mittels LC-MS im positiven Ionisierungsmodus gemessen. Die methodischen Details können **Publikation 2** entnommen werden. Zur Identifikation der TP wurden die gemessenen Masse-Ladungsverhältnisse, Retentionszeiten und Fragmente (MS^2) herangezogen. Mögliche TP wurden mit Hilfe von enviPath und dem EAWAG-BBD Pathway Predicition System vorhergesagt und mit den gemessenen Masse-Ladungsverhältnissen verglichen, um Informationen über mögliche Abbauwege ableiten zu können.

5.3 Ergebnisse und Diskussion

Keine der untersuchten Substanzen konnte nach OECD Richtlinie als leicht biologisch abbaubar klassifiziert werden (Abbau über 60 % nach 28 Tagen). Von den acetylierten

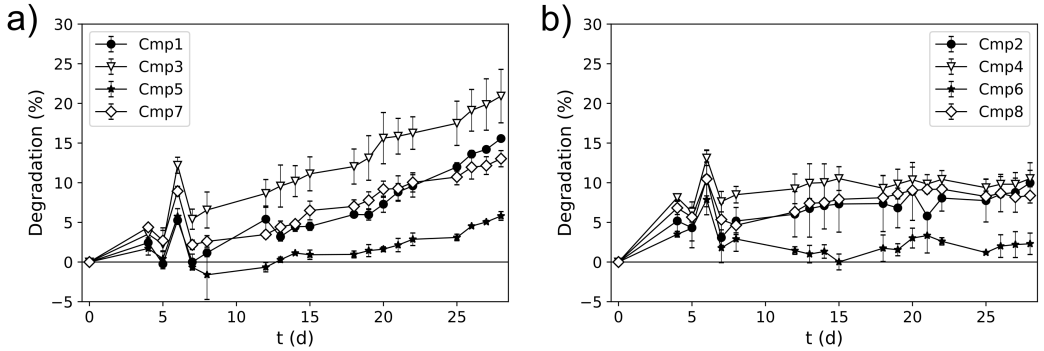


Abbildung 7: Verlauf des Bioabbaus im CBT für a) die acetylierten Derivate und b) die deacetylierten Derivate (n=2) (aus **Publikation 2**)

Substanzen war Substanz 3 mit 20 % am besten abbaubar, unter den deacetylierten Substanzen erreichten die Substanzen 2 und 4 den höchsten Bioabbauwert von 10 %. Vergleicht man den Verlauf des Abbaus über 28 Tage, ist deutlich sichtbar, dass die acetylierten Strukturen besser abgebaut wurden als die deacetylierten (Abb. 7). Dieser Trend zeigte sich sowohl im CBT als auch im MRT. In beiden Tests stagnierte der Abbau bei den deacetylierten Strukturen nach einer Adaptionsphase, bei den acetylierten Strukturen hingegen war ein stetiger Sauerstoffverbrauch bis zum Tag 28 zu beobachten.

Die LC-MS Analysen bestätigten die Unterschiede zwischen den beiden Substanzgruppen. Der Vergleich der Chromatogramme von Tag 0 und nach 28 Tagen der deacetylierten Derivate bestätigt, dass kein Primärabbau der Substanzen stattfand. Die Analyse der Testansätze mit den acetylierten Verbindungen hingegen zeigte, dass der Peak der Muttersubstanz über die Zeit kleiner wird und neue Peaks von Zwischenprodukten entstehen (**Publikation 2**, Abbildung 3). Die Endprodukte des Abbaus waren vermutlich persistente N-acetyl-Fluorchinolon-3-Carboxamid Derivate. Mit Hilfe von enviPath und EAWAG-PPS, sowie den gemessenen Zwischenprodukten, konnte zudem ein Abbauweg vorgeschlagen werden, der mit einem partiellen Abbau der Acetylreste am Glucosamin startet, bis letztendlich die komplette Glucosamin-Seitenkette entfernt wurde. Der Abbauweg der deacetylierten Substanzen folgt gemäß der Vorhersage einem anderen Schema und benötigt die Anwesenheit anderer Enzyme, was eine Erklärung für den Unterschied in den experimentellen Ergebnisse darstellen kann.

Wie bereits diskutiert, ist die Grundstruktur der Fluorchinolone in der Regel nicht leicht biologisch abbaubar. Dies bestätigt sich in diesen Versuchen durch die persistenten

Abbauprodukte, welche noch immer die FQ-Grundstruktur aufweisen. Der gemessene Sauerstoffverbrauch kann daher in diesem Fall dem (partiellen) Abbau der Glucosamin-Reste zugeordnet werden. Die Ergebnisse zeigen, dass ein Glucosamin-Rest die partielle Bioabbaubarkeit des Moleküls positiv beeinflussen kann, wenn dieser acetyliert vorliegt.

In der Literatur gibt es Beispiele die zeigen, dass Zuckersubstituenten die allgemeine Bioabbaubarkeit von Molekülen verbessern können (Kümmerer und Al-Ahmad, 1997; Kümmerer et al., 2000). Auch der Grad an Acetylierung zeigte einen Einfluss auf die Bioabbaubarkeit von zum Beispiel Chitosan (Yang et al., 2007). Viele verschiedene Faktoren beeinflussen die Bioabbaubarkeit von Molekülen, wie z.B. ihre Löslichkeit, Toxizität oder Aktivität gegen abbauende Mikroorganismen, ihr Adsorptionsverhalten, ihr Transport in die Zelle und verfügbare Enzyme (Boethling et al., 2007). Bioabbautests die nicht als *single-strain* Experimente, sondern mit Inokulum aus Kläranlagen durchgeführt werden, sind eher als „Black Box“ zu betrachten, da die enthaltenen Enzyme und Mikroorganismen nicht bekannt sind und je nach Jahreszeit oder aufgrund anderer äußere Einflüsse unterschiedlich sein können. Das macht es besonders schwer, Abbauwege und verantwortliche Mikroorganismen vollumfänglich zu verstehen und zu identifizieren.

5.4 Schlussfolgerung

Das Ziel von Benign by Design ist es, Moleküle so zu designen, dass diese während der Applikation stabil und aktiv sind, aber nach ihrer Verwendung in der Umwelt abgebaut werden können. Mohammed et al. (2019, 2020) konnten bereits nachweisen, dass die getesteten Moleküle stabil und aktiv gegen verschiedene pathogene Mikroorganismen sind. Die hier vorgestellten Ergebnisse legen außerdem nahe, dass zumindest die acetylierten Verbindungen teilweise in der Umwelt abgebaut werden können. Da es sich dabei allerdings nicht um eine vollständige Mineralisierung handelt, sollten die resultierenden Transformationsprodukte zukünftig unbedingt hinsichtlich ihrer Eigenschaften näher untersucht werden.

Auch wenn in diesem Fall persistente Abbauprodukte zurück bleiben, können diese Ergebnisse für das gezielte Design von abbaubaren Strukturen verwendet werden. Acetylierte Glucosamin-Substituenten könnten im Rahmen eines fragment-basierten Design Ansatzes genutzt werden, um zum Einen die Aktivität von Wirkstoffen zu erhöhen, während gleichzeitig eine definierte „Bruchstelle“ in dem Molekül entsteht. Dies könnte

so gestaltet werden, dass dieser partielle Abbau zu einer Deaktivierung des verbleibenden Moleküls führt, was somit wiederum den Selektionsdruck in der Umwelt reduziert. Weiterhin könnte die Kombination der Substituenten mit abbaubaren Leitstrukturen (**Publikation 3**) ein vielversprechender Ansatz für das Design von grüneren Wirkstoffen darstellen.

Die Ergebnisse legen nahe, dass eine gezielte Einführung von Substituenten, die potentiell die Abbaubarkeit des Moleküls verbessern, gleichzeitig auch zu einer Verbesserung der Aktivität führen können. Das zeigt, dass Aktivität und Bioabbaubarkeit nicht zwangsläufig als widersprüchliche Eigenschaften beim Wirkstoffdesign behandelt werden müssen.

6 Struktur-Bioabbau-Beziehungen von Chinolonen

6.1 Problemstellung

In **Publikation 1** wurde bereits diskutiert, wie wichtig *in silico* Modelle sind, um das Design von neuen Molekülen zu unterstützen. Insbesondere um die biologische Abbaubarkeit als Kriterium in den Wirkstoffentwicklungsprozess zu implementieren, werden entsprechende Modelle benötigt (Puhlmann et al., 2021). Aufgrund der Komplexität des Endpunkts performen Modelle zur Vorhersage der Bioabbaubarkeit oft vergleichsweise schlecht (Benfenati, 2012; Rücker und Kümmerer, 2012). Außerdem können globale Modelle aufgrund ihrer Generalisierung nicht immer gute Ergebnisse für alle Stoffklassen liefern. FQ sind zudem meist außerhalb der Anwendungsdomäne der vorhandenen Modelle, da bisher keine entsprechende Trainingsdaten verfügbar sind.

Während die Bioabbaubarkeit von einfachen Chinolinen in der Literatur bereits untersucht wurde (Lin und Jianlong, 2010), liegen keine umfassenden experimentellen Daten für komplexere N-Heterozyklen vor. Um den Wissensstand über die Bioabbaubarkeit verschiedener N-Heterozyklen zu verbessern, bioabbaubare Leitstrukturen zu identifizieren und Struktur-Bioabbau-Beziehungen abzuleiten, wurden 84 verschiedene Verbindungen aus der Gruppe der N-Heterozyklen nach OECD Testguidlines hinsichtlich ihrer leichten biologischen Abbaubarkeit untersucht. Der Fokus lag dabei auf Verbindungen die eine chelatbildende β-Ketonsäure enthalten, um die gewonnen Ergebnisse vor allem zum grünen Design von neuen Topoisomerase II und IV Inhibitoren zu nutzen. Weiterhin wurde der Einfluss von Halogenierung, Alkylierung, Hydroxylierung und Veretherung auf die biologische Abbaubarkeit der Leitstrukturen untersucht. Basierend auf diesen Ergebnissen sollten Regeln und Empfehlungen entwickelt werden, welche genutzt werden können um besser biologisch abbaubare N-Heterozyklen zu designen. Außerdem können die ex-

perimentellen Ergebnisse genutzt werden, um vorhandene QSAR Modelle zur Vorhersage der Bioabbaubarkeit zu ergänzen.

6.2 Methoden

Die untersuchten Strukturen gehören zu den Substanzklassen der Chinoline, Chinolone, Pyrimidine, Isochinoline, Fluorchinolone, Napthyridone und Quinazoldione. Ihre leichte biologische Abbaubarkeit wurde im CBT (OECD 301D) und MRT (OECD 301F) untersucht. Die potentiellen Metabolite der Muttersubstanzen wurden nach Testende mittels LC-MS untersucht.

Um die Struktur-Bioabbau-Beziehungen näher zu beleuchten, wurde ein field-based QSAR Model in Maestro (Schrödinger Inc., New York) erstellt. Dafür wurden die Ergebnisse aus CBT und MRT zusammengeführt und der jeweils höhere Bioabbau-Wert verwendet. Alle negativen Abbauwerte wurden auf null gesetzt. Der Datensatz enthielt insgesamt 79 Strukturen. Mit Hilfe der verschiedenen Module in Maestro wurden die 3D Strukturen entsprechend vorbereitet und anschließend mittels field-based QSAR Modul das Model erstellt und optimiert. Für die weitere Interpretation der Ergebnisse wurden die dadurch erzeugten Contour Maps verwendet.

6.3 Ergebnisse und Diskussion

Von den 20 untersuchten Chinolinen wurden 11 als leicht biologisch abbaubar klassifiziert. Damit sind Chinoline die getestete Substanzklasse mit dem höchsten Anteil an biologisch abbaubaren Strukturen. Auch fluorierte Chinoline waren abbaubar, wenn Aktivschlamm als Inokulum verwendet wurde. Allerdings war in diesen Fällen eine deutlich längere Lag-Phase zu beobachten. Dies deutet darauf hin, dass eine Adaption an die Substanzen nötig war, bis ein gewisser Anteil spezifischerer Mikroorganismen vorhanden ist. Anschließend folgte eine recht schnelle Mineralisierung.

Einen besonders positiven Einfluss auf die Bioabbau-Rate, sowohl der nicht-fluorierten als auch der fluorierten Chinoline, hatten Hydroxyl-Gruppen, inbesondere an Position C2. Dihydroxylierte Verbindungen zeigten eine deutlich geringere Lag-Phase als Monohydroxylierte. Die Aufklärung der Metabolite während des Abbaus bestätigte dies. Verschiedene monohydroxylierte Verbindungen zeigten als Zwischenprodukt eine dihydroxy-

lierte Verbindung mit einer zusätzlichen Hydroxylgruppe an Position C2 (**Publikation 3**, Abbildung 2 & 3). Dies legt nahe, dass eine Hydroxylierung an dieser Position einer der geschwindigkeitsbestimmenden Schritte beim Abbau der Chinoline ist. Diese Ergebnisse decken sich mit den bekannten Abbauwegen der Chinoline aus der Literatur.

Im Gegensatz dazu waren keine der getesteten Isochinoline leicht biologisch abbaubar. Die Position des Stickstoffs in den N-Heterozyklen scheint daher eine wichtige Rolle für die biologische Abbaubarkeit der Moleküle zu haben.

Auch aus der Gruppe der Chinolone, Napthyridone und Pyrimidine, welche alle eine β-Ketonsäure enthalten, war lediglich eine der 27 getesteten Strukturen abbaubar. Bei der einzigen abbaubaren Struktur handelte es sich um 4-Hydroxy-3-chinolincarbonsäure. Dies bedeutet, dass bei den Strukturen mit einer β-Ketonsäure jeder weitere Substituent, egal welcher Art, zu einer deutlichen Verschlechterung der biologischen Abbaubarkeit führt. Der Einfluss einer Hydroxylgruppe an Position C2 (2,4-Dihydroxy-3-chinolincarbonsäure) konnte in diesem Fall nicht näher untersucht werden, da die resultierende Struktur zu instabil ist.

Die untersuchten und in der Human- oder Veterinärmedizin eingesetzten Chinolone und Fluorchinolone waren weder im CBT noch im MRT leicht biologisch abbaubar, wie aus der Literatur bereits bekannt ist (Alexy et al., 2004).

Von den sieben untersuchten Chinazolinen waren zwei Strukturen im MRT leicht biologisch abbaubar. Bei diesen beiden Strukturen handelt es sich um vielversprechende Kandidaten für biologisch abbaubare Leitstrukturen, die als Grundlage für das Design verschiedener neuer Verbindungen genutzt werden könnten. Allerdings zeigte sich auch hier, dass gewisse Substituenten die Elektronendichte so verändern, dass die Strukturen nicht mehr abgebaut werden konnten.

Das field-based QSAR Modell der untersuchten Strukturen lieferte weitere Erkenntnisse über die Struktur-Bioabbau-Beziehungen der untersuchten Verbindungen. Dabei wurde deutlich, dass sterisch anspruchsvolle Gruppen an nahezu allen Positionen des Heterozyklen einen negativen Einfluss auf die Bioabbaubarkeit der Substanzen haben. Nur an Position 2 und in nicht-planarer Orientierung an Position 7 können Substituenten die Bioabbaubarkeit positiv beeinflussen. Diese Ergebnisse sind vor allem darauf zurückführen, dass hauptsächlich Strukturen mit einer Hydroxylgruppe an Position 2 untersucht wurden, die wie bereits diskutiert, einen deutlich positiven Einfluss auf die Abbaubarkeit hat. Ob auch andere Substituenten an Position 2 ähnliche Auswirkungen haben, kann anhand der vorliegenden Daten nicht extrapoliert werden. Weitere Struktur-

Bioabbau-Beziehungen können anhand der Contour Maps (**Publikation 3**, Abbildung 5) abgelesen werden.

6.4 Schlussfolgerung

Die Ergebnisse zeigen, dass ein Großteil der untersuchten N-Heterozyklen nicht leicht biologisch abbaubar ist. Von 84 untersuchten Strukturen konnten 14 als leicht biologisch abbaubar klassifiziert werden, darunter vor allem Chinoline. Die Chinolingrundstruktur, als auch verschiedene Derivate dieser, zum Beispiel mit Hydroxylgruppen an spezifischen Positionen, wurden leicht abgebaut. Auch Fluor als Substituent hinderte in diesen Fällen nicht den Bioabbau. Auf diesen Erkenntnissen kann in Zukunft weiter aufgebaut werden, um die Leitstruktur weiter so zu modifizieren, dass sie leicht biologisch abbaubar bleibt, aber auch ein entsprechendes Wirkungsspektrum zeigt. Die Chinolonleitstruktur ohne Substituenten war ebenfalls abbaubar. Allerdings führten alle weiteren Substituenten zu einer starken Verschlechterung der Bioabbaubarkeit. Vor allem die zwei abbaubaren Strukturen aus der Gruppe der Chinazoline stellen vielversprechende Kandidaten für das Design von Topoisomerasen Inhibitoren und auch anderen Verbindungen dar. Die drei identifizierten, leicht biologisch abbaubaren Leitstrukturen als auch die abgeleiteten Struktur-Bioabbau-Beziehungen bilden eine erste Grundlage, um in Zukunft potentiell grünere Wirkstoffe zu designen und diese anschließend zu synthetisieren und weiter zu untersuchen.

Viele der QSAR Modelle zur Vorhersage der Bioabbaubarkeit stützen sich vor allem auf die zweidimensionale Repräsentation der Moleküle und entsprechende Structural Alerts. Die hier vorgestellten experimentellen Ergebnisse zeigen jedoch noch einmal deutlich, wie groß der Einfluss der Stereochemie auf die Abbaubarkeit ist und welchen Einfluss bestimmte funktionelle Gruppen an ganz spezifischen Stellen haben können. Dies deckt sich mit den Erkenntnissen, dass die Generalisierungen die in globalen Modellen vorgenommen werden, nicht immer zufriedenstellend alle Zusammenhänge abbilden können. Daher können die hier präsentierten Ergebnisse zum einen genutzt werden, um vorhandene QSAR Modelle und ihre Anwendungsdomäne zu erweitern, aber gleichermaßen sollen die Ergebnisse verdeutlichen, dass auch die Verwendung lokaler Modelle einen wichtigen Beitrag beim Design hin zu besserer biologischen Abbaubarkeit liefern können.

7 Diskussion

Im Rahmen dieser Dissertation sollten die verschiedenen BbD Ansätze identifiziert und deren Anwendbarkeit für die Antibiotikaklasse der Fluorchinolone untersucht werden. Das Ziel war es, besser zu verstehen, wie Fluorchinolone zukünftig umweltfreundlicher und besser biologisch abbaubar designt werden können, sowie dabei Wissenslücken bezüglich der Struktur-Bioabbau-Beziehungen von FQ zu schließen. Ausgangspunkt dafür waren die von Leder et al. (2015) und Rastogi et al. (2014a,b, 2015) veröffentlichten Frameworks, deren Anwendbarkeit über die bisherigen Beispiele hinaus untersucht werden sollten. Zudem sollen die gewonnenen Erkenntnisse und erarbeiteten Workflows dazu beitragen, BbD weiter in der Forschung und Entwicklung zu etablieren.

In **Publikation 3** wurden biologisch abbaubare Leitstrukturen identifiziert, sowie der Einfluss verschiedener Substituenten auf die Abbaubarkeit ermittelt. Dabei wurde deutlich, dass eine Hydroxylgruppe an der Chinolinstruktur bereits zu einer wesentlich besseren Abbaubarkeit des gesamten Moleküls führen kann. Eine Hydroxylgruppe an einem Fluorchinolon hingegen konnte dessen Abbaubarkeit nicht verbessern. Darin liegt auch der deutliche Unterschied zu den von Rastogi et al. (2014a,b, 2015) untersuchten β-Blockern, in deren Fall bereits eine einzelne Hydroxylgruppe zu einer deutlichen Veränderung der Umwelteigenschaften geführt hat. Da die Erzeugung neuer Strukturen mittels Photolyse oft zur Hydroxylierung der Ausgangssubstanz führt, wird deutlich, warum das Vorgehen des non-targeted Re-Designs, welches für β-Blocker mehrfach erfolgreich umgesetzt werden konnte, auf die Substanzklasse der Fluorochinolone nicht problemlos übertragbar war. Aufgrund dessen müssen für diese Substanzklasse weitere Ansätze untersucht werden, um vollständig mineralisierbare Fluorchinolone zu entwickeln.

Das Beispiel Cipro-Hemi (Leder et al., 2021) und die hier untersuchten Glucosamin-Derivate (**Publikation 2**) haben gezeigt, dass durch gezielt eingeführte Substituenten ein partieller Abbau erreicht werden kann, ohne dass die Aktivität des Ausgangsmoleküls dabei verloren geht. Anhand dieser Beispiele wird deutlich, dass der partielle Abbau

von Fluorchinolon-Derivaten und eine damit einhergehende Inaktivierung umsetzbar ist. Die verbleibende Grundstruktur ist in beiden Fällen jedoch weiterhin persistent in der Umwelt. Als nächstes ist daher wichtig, eine abbaubare Leitstruktur zu nutzen und mit den Substituenten, die zu einem partiellen Abbau führen, zu kombinieren, um ein in der Umwelt vollständig abbaubares Derivat zu entwickeln. Dafür wurde mit den in **Publikation 3** präsentierten abbaubaren Leitstrukturen und Struktur-Bioabbau-Beziehungen ein wichtiger Grundstein gelegt.

Das in **Publikation 1** vorgeschlagenen Vorgehen des Re-Designs mit Hilfe von *in silico* Modellen konnte für FQ im Rahmen dieser Dissertation nur bedingt angewandt werden, da die Substanzen in der Regel außerhalb der Anwendungsdomäne der Modelle lagen. Mit den in **Publikation 2 & 3** gewonnen Struktur-Abbau-Beziehungen könnten in Zukunft vorhandene und etablierte Bioabbau-Modelle erweitert werden. Diese könnten dann in dem Workflow (**Publikation 1**) zum Design von in der Umwelt besser abbaubaren Strukturen eingesetzt werden. Ein mögliches Vorgehen könnte sein, mit Hilfe der hier gewonnen Erkenntnisse (z.B. über die abbaubaren Leitstrukturen und Fragmente) digital verschiedenste neue Strukturen zu entwerfen und mit Hilfe von Modellen zur Abbaubarkeit, Aktivität und Toxizität eine Vorauswahl aus diesen Vorschlägen zu treffen. Diese vielversprechenden Strukturen sollten dann synthetisiert und im Labor experimentell hinsichtlich ihrer Eigenschaften untersucht werden. Die daraus gewonnen Erkenntnisse könnten dann wieder in entsprechende Modelle und Designentscheidungen einfließen, um damit eine immer bessere Wissensgrundlage, sowie Modelle mit größerer Anwendungsdomäne für das Design von in der Umwelt abbaubaren Fluorchinolonen zu gewinnen.

Die Ergebnisse legen nahe, dass nicht jeder identifizierte BbD-Ansatz (**Publikation 1**) oder jede generalisierte Struktur-Abbau-Beziehung (Boethling et al., 2007) ohne Probleme auf eine beliebige Stoffgruppe anwendbar sind. Umso wichtiger ist es hervorzuheben, dass die vier in **Publikation 1** vorgestellten Herangehensweisen (sowohl *in silico* als auch im *wet-lab*) auf die jeweiligen Bedürfnisse und Gegebenheiten angepasst werden müssen. Für das Benign by Design gibt es keine „one fits it all“ Herangehensweise. Weiterhin wurde deutlich, dass Moleküle immer mehr als die Summe ihrer Fragmente sind. Selbst wenn vermeintlich abbaubare Fragmente zu einer neuen Struktur kombiniert werden, muss dies nicht zwangsläufig zu einem ebenfalls vollständig biologisch abbaubaren Molekül führen (**Publikation 3**). Dies macht das Design von abbaubaren Strukturen besonders herausfordernd und zeigt zum einen, wie wichtig es ist, die Struktur-Bioabbau-Beziehungen

umfänglich zu verstehen, und zum Anderen, dass eine experimentelle Validierung von *in silico* Ergebnissen unbedingt notwendig ist.

In der Literatur lassen sich auch andere Ansätze zur Umsetzung des Benign by Design Prinzips finden. Eine Kombination der unterschiedlichen Herangehensweisen könnten in Zukunft ebenfalls interessant sein. Zumstein und Fenner (2021) diskutierten einen Ansatz für Peptid-basierte Antibiotika, welche ebenfalls so designt werden sollen, dass sie während der Behandlung im Blut stabil bleiben, aber in der Umwelt durch Peptidasen inaktiviert werden können. Die Idee dabei ist, zu identifizieren, welche extrazellulären Peptidasen im Abwasser und der Umwelt, aber nicht im menschlichen Blut, vorliegen. Ein entsprechend designtes Antibiotikum müsste dann also von den Peptidasen im Blut nicht abbaubar sein, Peptidasen im Abwasser sollten aber in der Lage sein, die Substanzen mittels Hydrolyse zu inaktivieren. In einem weiteren Beispiel aus der Literatur wurde eine „programmierte Inaktivierung“ durch die Bestrahlung mit UV-Licht in der Trinkwasseraufbereitung eingeführt (Espinosa et al., 2022). Dabei handelt es sich ebenfalls um eine vielversprechende Herangehensweise, da in diesem Fall nicht die Präsenz bestimmter abbauender Mikroorganismen oder Enzyme ausschlaggebend ist, sondern die Inaktivierung auf eine abiotische Reaktion, ähnlich wie bei der Inaktivierung des Cipro-Hemi (Leder et al., 2021), zurückzuführen ist. Damit vermeidet man beispielsweise die Gefahr, dass Resistenzen entstehen und durch horizontalen Gentransfer auf möglicherweise pathogene Bakterien übertragen werden können. Auf der anderen Seite wird ein Großteil des Wassers global oft keiner Trinkwasserbehandlung in Form von UV-Strahlung unterzogen (WHO, 2023). Inwieweit dieser Ansatz daher eine umfassende, nachhaltige Lösung darstellt, sollte in zukünftiger Forschung betrachtet werden.

Das in dieser Arbeit vorgestellte Re-Design kann auch *in silico* durchgeführt werden, wie zum Beispiel von van Dijk et al. (2022) gezeigt. Auch wenn durch die experimentelle non-targeted Synthese eine Vielzahl an Strukturen erzeugt werden kann, bleibt der Strukturraum, je nach unterliegenden Reaktionsmechanismen, doch recht limitiert. Zudem kann die Strukturidentifikation herausfordernd sein, sodass die konkreten Strukturen oft nicht eindeutig bekannt sind. Durch computergestützte Verfahren kann eine wesentlich größere Anzahl an Strukturen erzeugt werden, deren Strukturformeln in diesen Fällen eindeutig vorliegen (van Dijk et al., 2022). Die digital erzeugten Strukturen können mit Hilfe verschiedener (QSAR-)Methoden gefiltert werden, um die vielversprechendsten zu identifizieren, welche dann synthetisiert und experimentell untersucht werden können, wie in dem in **Publikation 1** ausgearbeiteten Workflow dargestellt.

Das Design von biologisch abbaubaren Arzneimitteln und Chemikalien ist natürlich nicht die alleinige Lösung für eine nachhaltigere Zukunft. Daher sollten immer auch weitere Maßnahmen mitgedacht und ergriffen werden, wie die Reduzierung von off-target Effekten, reduzierte Anwendung von Arzneimitteln oder die Verabreichung von geringeren Konzentrationen durch personalisierte Medizin oder verbesserte Wirkstoffabgabesysteme (Moermond et al., 2022). Für eine grünere und nachhaltigere Zukunft ist es daher umso wichtiger, dass verschiedene Disziplinen übergreifend zusammenarbeiten und das Problem von Arzneimitteln in der Umwelt von multidisziplinären Perspektiven betrachtet wird.

8 Fazit

Benign by Design ist ein wichtiges Konzept, welches wir für eine nachhaltigere Zukunft und die Umsetzung der SDGs und anderer Strategien z.B. im Rahmen des Green Deal der EU, als wichtigen Baustein benötigen. Daher ist es wichtig zu verstehen, wo und wie es Anwendung finden kann, inwiefern es übertragbar auf verschiedene Substanzklassen ist und welche Limitierungen zu erwarten sind. Erfolgreiche Beispiele der Umsetzung des BbD Konzeptes helfen, die Machbarkeit zu beweisen und eine Umsetzung über die akademische Forschung hinaus voran zu treiben.

Die Betrachtung der verschiedenen Benign by Design Ansätze, die Entwicklung eines *in silico* Workflows, sowie die Machbarkeitsstudien am Beispiel von Fluorochinolonen haben demonstriert, dass die Umsetzung von BbD im Wirkstoffdesign möglich ist und wie mögliche Vorgehensweisen aussehen können. Es wurde jedoch auch deutlich, dass es keine allgemeingültige Herangehensweise gibt und die Methoden immer den entsprechenden Substanzgruppen und Bedürfnissen angepasst werden müssen. Daher ist es wichtig, die vorgestellten BbD Ansätze weiter zu untersuchen und ihre Übertragbarkeit auf andere Substanzklassen zu erforschen.

Die durchgeführten Studien haben gezeigt, dass selbst bei den bisher immer als besonders persistent angesehenen Fluorochinolonen das Potential für ein Design hin zu besser abbaubaren Verbindungen möglich ist. Entweder, indem die hier identifizierten Leitstrukturen genutzt und weiterentwickelt werden, oder verschiedene Ansätze des partiellen Abbaus kombiniert werden. Diese Vorgehensweisen lassen sich auch über Fluorchinolone hinaus für das Design von grünen Wirkstoffen verwenden. Zuckersubstituenten, wie im Beispiel der Glucosamin-Derivate, stellen unabhängig von der Grundstruktur mit der sie kombiniert werden, eine Option dar, um einen partiellen Abbau der Struktur zu erreichen. Weiterhin basieren viele Substanzgruppen auf N-heterozyklischen Grundstrukturen. Die veröffentlichten Struktur-Bioabbau-Beziehungen können daher auch zum Design anderer Verbindungen genutzt werden.

Weitere Forschung an Universitäten und in Entwicklungsabteilungen von Pharmaun-

ternehmen können von den in **Publikation 2** und **3** vorgestellten Struktur-Bioabbau-Beziehungen profitieren, um zukünftig besser biologisch abbaubare Verbindungen zu entwickeln. Insbesondere die Integration von *in silico* Tools sollte die Implementation von BbD in der Pharma-Industrie erleichtern, da Modelle zur Vorhersage der Bioabbaubarkeit gut in bereits etablierte Arbeitsabläufe integriert werden können. Durch den Einsatz von computergestützten Methoden können zudem Ressourcen eingespart, aber auch frühzeitig eine Vorbereitung auf zukünftige regulatorische Vorgaben erreicht werden. Die erzeugten Daten können zum Beispiel genutzt werden, um generalistische Bioabbau-QSAR-Modelle zu erweitern und verbessern.

Aus regulatorischer Sicht wird es immer wichtiger, umweltfreundlichere Wirkstoffe zu designen, da Strategien wie die „Chemicals Strategy for Sustainability towards a Toxic-Free Environemnt“ oder der „European Union Strategic Approach to Pharmaceuticals in the environment“ fordern, zukünftig grünere und nachhaltigere Chemikalien zu entwickeln. Die im Rahmen dieser Arbeit vorgestellten Ansätze zeigen mögliche Lösungswege, um diese regulatorischen Vorgaben zukünftig insbesondere für Substanzen mit einer „environmentally open“ Anwendung umzusetzen.

Zukünftige Forschung sollte die Anwendung der identifizierten BbD Ansätze auf verschiedenste Substanzklassen untersuchen, insbesondere solcher, die in der Umwelt bereits jetzt oder zukünftig zu Problemen führen können. Der Erfolg von BbD Ansätzen beruht zum Großteil auf der Verfügbarkeit von Struktur-Bioabbau-Beziehungen sowie entsprechenden *in silico* Tools zur Vorhersage der Abbaubarkeit von Strukturen. Daten von hoher Qualität werden dafür dringend benötigt und sollten daher Gegenstand zukünftiger Forschung und Publikationen sein. Die Förderung der Zusammenarbeit von Industrie, Forschung und Behörden spielt in diesem Zusammenhang ebenfalls eine wichtige Rolle.

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Anhang

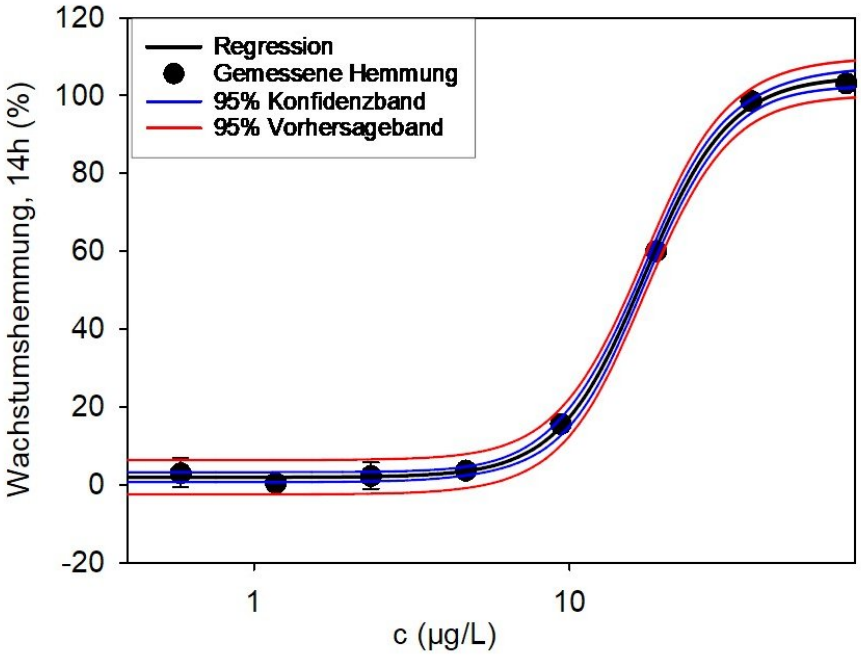

Abbildung A1: Dosis-Wirkungskurve der Wachstumshemmung von Lomefloxacin

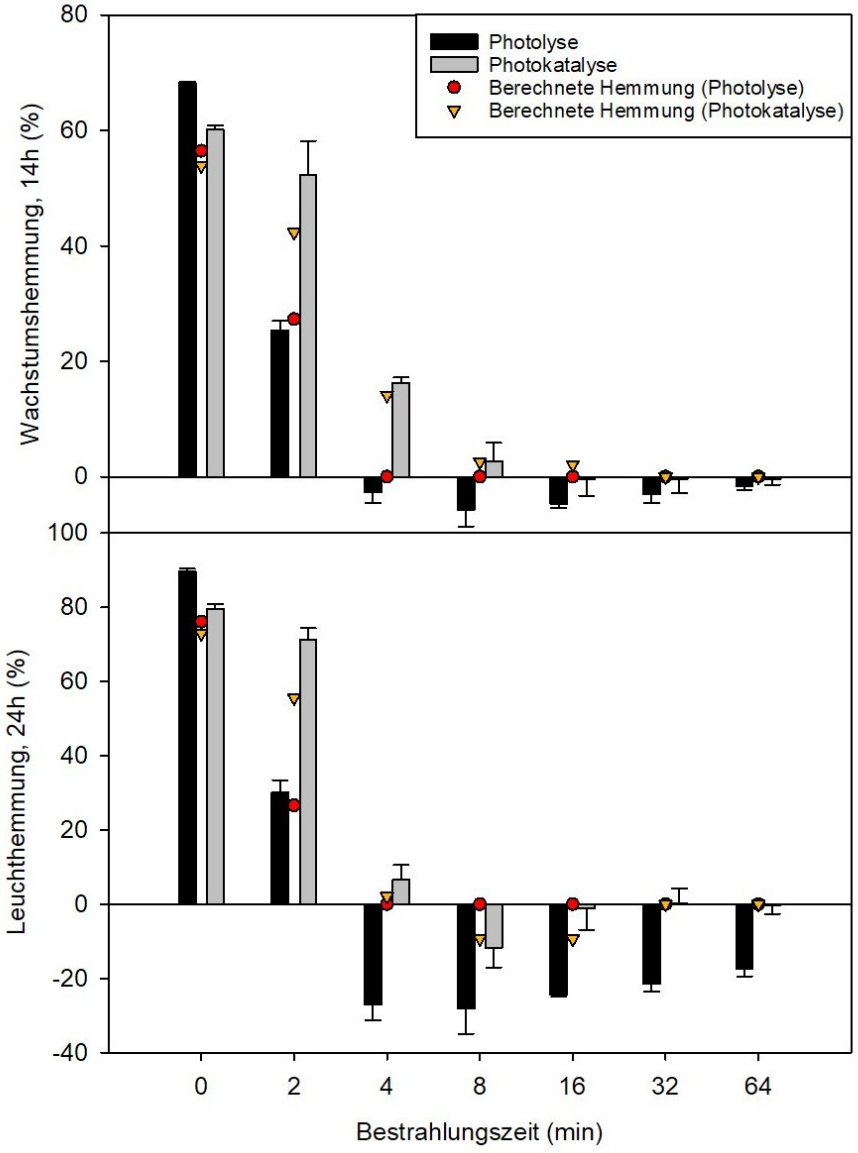

Abbildung A2: Wachstums- und Leuchthemmung von Lomefloxacin, gemessen und berechnet auf Basis der Konzentration der Muttersubstanz

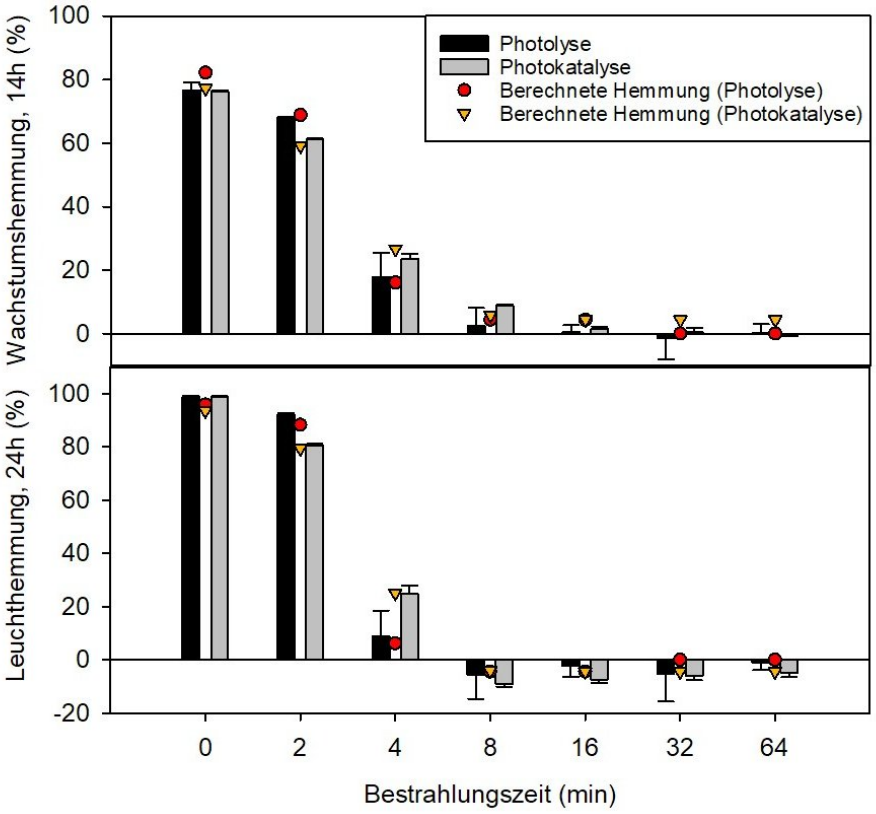

Abbildung A3: Wachstums- und Leuchthemmung von Marbofloxacin, gemessen und berechnet auf Basis der Konzentration der Muttersubstanz

Publikationen zur kummulativen Dissertation

Publikation 1	Lorenz, S., Amsel, A.K., Puhlmann, N., Reich, M., Olsson, O., Kümmerer, K. (2021): Toward Application and Implementation of in Silico Tools and Workflows within Benign by Design Approaches. *ACS Sustainable Chemsitry and Engineering*. 9 (37), 12461-12475. DOI: https://doi.org/10.1021/acssuschemeng.1c03070.
Publikation 2	Lorenz, S., Suaifan, G., Kümmer, K. (2022): Designing benign molecules: The influence of O-acetylated glucosamine-substituents on the environmental biodegradability of fluoroquinolones. *Chemosphere*. 309 (2), 136724. DOI: https://doi.org/10.1016/j.chemosphere.2022.136724
Publikation 3	Suk, M., Lorenz, S., Kümmerer, K. (2023): Identification of environmentally biodegradable scaffolds for the benign design of quinolones and related substances. *Sustainable Chemistry and Pharmacy*. 31, 100947. DOI: https://doi.org/10.1016/j.scp.2022.100947.

Publikation 1

Stefanie Lorenz, Ann-Kathrin Amsel, Neele Puhlmann, Marco Reich, Oliver Olsson, Klaus Kümmerer (2021).

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

ACS Sustainable Chemsitry and Engineering. 9(37), 12461-12475.

DOI: https://doi.org/10.1021/acssuschemeng.1c03070.

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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*

Cite This: https://doi.org/10.1021/acssuschemeng.1c03070

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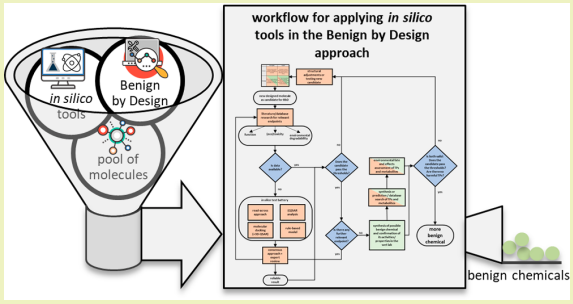
ABSTRACT: To avoid adverse side effects of chemicals, pharmaceuticals, and their transformation products (TPs) in the environment, substances should be designed to fully mineralize in the environment at their end-of-life while ensuring a degree of stability as needed for their application. These considerations 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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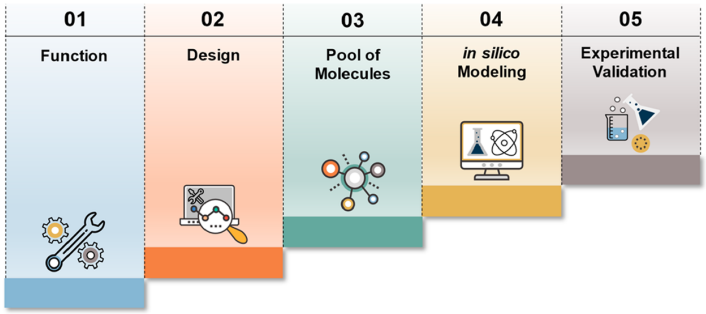

Figure 1. General procedure for the benign design of chemicals using *in silico* tools.

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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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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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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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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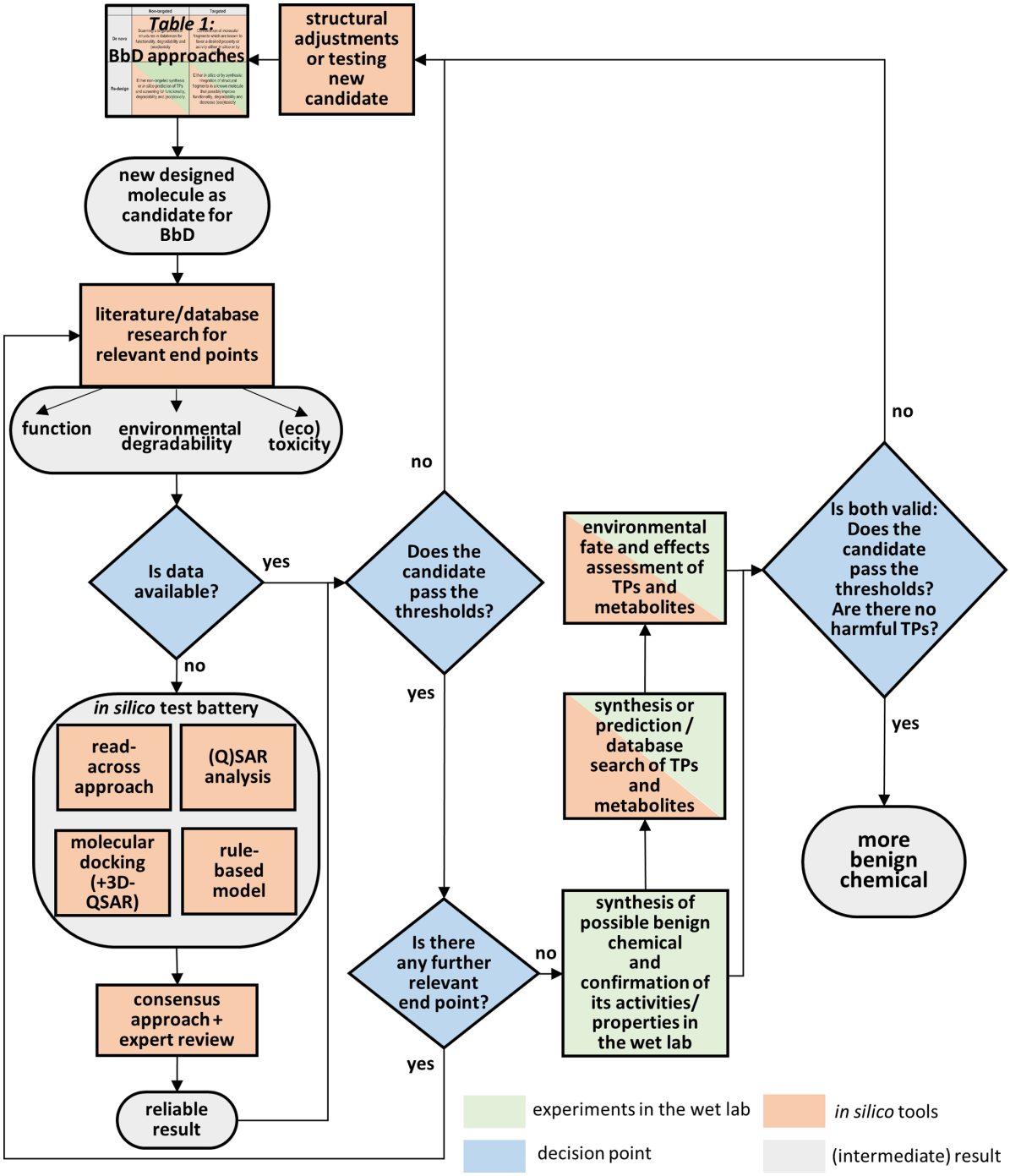

Figure 2. Workflow for the *in silico* assessment of newly or redesigned molecules according to the presented BbD approaches. It starts with the design of new molecules, followed by literature research and an evaluation with the help of a before defined *in silico* test battery. After the identification 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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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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■ 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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Supporting Information

Towards application and implementation of *in silico* tools and workflows within Benign by Design approaches

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Algorithms and methods

According to Chapter 6 of the "Guidance on information requirements and chemical safety assessment" (ECHA, 2008) *in silico* tools can be classified into three main approaches:

a) Grouping approaches, which includes read-across (RA) techniques;

b) (Quantitative) structure-activity relationships ((Q)SAR) and

c) Expert systems like rule-based models (RB).

All predictions of properties of chemicals by those approaches are based on the "similarity principle", which means that they predict similar properties/activities for similar compounds. The advantages and limitations of those different approaches in the context of BbD are summarized in the Supporting Information Material, table S1 and exemplary software packages are listed.

The read-across approach (RA) uses information about known chemicals from the same group (also called chemical category) as the test chemical to predict its unknown properties. It is based on the assumption that chemicals within the same group follow a similar pattern regarding their properties or (toxic) effects. Read-across predictions can lead to qualitative or quantitative results, depending on the input data (see table S1). There are several methods to calculate and define such chemical categories. Therefore, the predictions for the substance of interest can differ, depending on the assumptions, which were used to find similar chemicals.[1,2]

(Quantitative) Structure-activity relationships ((Q)SARs) make use of statistical tools used for data mining. The used algorithms for linear models are i.e. linear regression analysis or multiple linear regression analysis for continues endpoints as well as linear discriminant analysis for categorical endpoints. For non-linear models, techniques like artificial neutral networks, support vector machines, decision trees, clustering or K-nearest neighbor approaches are used.[3] (Q)SAR models and their prediction power do not just differ by the algorithms and descriptors,

which were used for the model development, but also by the structural diversity and number of chemicals that are used to build and to train the model. If a relatively small number of similar chemicals are used it is called a "local model", while it is referred to as "global model" if a large set of diverse substances was used.[3] The applicability domain (AD) describes the most appropriate prediction space of a certain model and depends on the used training data.[4] The prediction of a property of a chemical, which lies within the AD is an interpolation, whereas the prediction of properties of molecules outside the AD are extrapolations and therefore less reliable. The degree of generalization of a model is dependent on how broad and diverse the AD is.[5] Since the reliability of predictions is heavily depending on the AD of a model it is important to carefully define it. Sahigara et al.[5] presented a detailed comparison of different approaches to calculate an AD.

Matched molecular pair analysis (MMPA) is a special case of (Q)SAR and is not based on the similarity principle. Matched molecular pairs (MMP) are pairs of chemicals that differ structurally only through a well-defined transformation at a single site (one-point MMP) or sometimes more than one side (multiple point MMP). Therefore, small and well-defined transformations in a molecular structure can be directly linked to the change in properties. Such insights can be directly used for the optimization of molecules.

Rule based models (RB) are usually developed in combination with structural alerts. They are often used in the form of "if A is present/absent than T", where A is a certain substructure and T a certain type of toxicity or any other endpoint. The rules can be either derived from field expert knowledge (human-based rules) or derived computationally by statistical methods (induction-based rules)[3] Rule based models have been developed for the prediction of ready biodegradability[6] and have been used to predict different toxicological endpoints.[7]

The prediction of environmental properties via read-across techniques plays an important role in the REACH registration process, where RA techniques are recommended.[8–10] It has also been used in different studies to predict ready biodegradability[11] or for toxicity risk assessment.[12]

There are even more *in silico* techniques and endpoints from the field of drug development, which could be utilized for other purposes in the context of BbD, like the prediction of ADME properties[13] or molecular docking, depending on the substance of interest.

Molecular docking is a method, which tries to find out the preferred orientation of molecules to the active site of proteins, receptors or enzymes. This information can be further used to predict and improve binding affinities.[14]

Table S1: Comparison of different *in silico* prediction approaches and their advantages and limitations for the application within the BbD concept (*Different approaches may be part of bigger software packages and therefore may appear multiple times)

Prediction model	Advantages	Limitations	Examples*
Statistical (Q)SAR	• Easy to interpret if the descriptors used are meaningful • Can model categorical, ordinal and continuous endpoints • Could enlarge the space of knowledge by identifying rules by itself (without explanations of the underlying mode of action)	• Activity cliffs cannot be modeled accurately, they are either discarded as outliers or cause overfitting • Requires large datasets for model development • Requires feature selection to identify the most significant molecular descriptors • Provides little or no insights into the underlying mode of	CASE Ultra[15], OECD QSAR Toolbox[16], Toxtree[17], VEGA[18], ECOSAR via EpiSuite[19], Leadscope[20], ChemProp[21], QSARINS[22]

		action (“black box”)	
Rule-based models	• Usually based on expert knowledge • Easy to interpret and therefore useful to use in the design of molecules • Most often based on known molecular mechanism	• Mostly limited to human knowledge and therefore it could be incomplete or biased, which would lead to false negative results • Cannot enlarge the space of knowledge • Uses only binary features (present/absent) • Can often be used only for qualitative endpoints or potency classes	OECD QSAR Toolbox[16], Toxtree[17], Derek Nexkus[23]
Read across	• Can be qualitative or quantitative based on the used data • It is transparent and easy to interpret • Requires less data points than typical statistical evaluations (QSAR)	• Depends on the type of similarity scoring (parameters are very subjective and can depend on the endpoints, therefore expert opinions may be required) • May provide insights into the pathways or mechanisms if it is known for the used analogues • May be wrong if analogs have conflicting toxicity profiles	OECD QSAR Toolbox[16], Toxtree[17], ToxRead[24], ChemProp[21], VEGA[18]

Matched Molecular Pair Analysis	• Activity cliffs can be identified • By analyzing a large dataset observed property changes as a consequence of a given transformation can be found • Can lead to the formulation of simple transformation rules to derive a certain property	• Large datasets with similar molecules and their properties are needed • The results depend on the structural similarity criterion • There are almost no trained models available yet	MedChemTK[25], OEMedChem Toolkit[26], mmpdb[27]
Docking	• Can provide more detailed information about molecule-enzyme interactions and possible mode of actions (toxicity) or metabolism (biodegradation) • Docking scores can be further used for (Q)SAR investigations	• Docking can be time consuming as for one molecule only at a time • Detailed information about enzymes of interest are necessary (e.g. crystal structure) • Highly specific and therefore just useful to a limited extent for screening large amounts of molecules • Some expertise in handling the used software and performing molecular docking is necessary	Maestro by Schroedinger[28], AutoDock[29], CABS-dock[30], FlexAID[31]

Descriptors

The classical descriptor based (Q)SAR approaches correlate properties or activities with certain structural features. Descriptors are numerical representations of chemical structures or their properties (i.e. physio-chemical properties like $\log K_{ow}$). Today several hundred descriptors are in use.[32] An overview of common descriptors can be found elsewhere.[33] The advantages and limitations for the usage of different types of descriptors in the field of Benign by Design are summarized in table S2.

Depending on the underlying descriptors in different (Q)SAR models or other *in silico* techniques we distinguish between chemical descriptor-based models, fragment-based models and 3D-(Q)SAR-models.

Chemical descriptors are computed values usually based on the whole structure, mainly quantifying various electronic, geometric or steric properties, e.g. by using quantum chemical approaches. They can be based on one or two-dimensional representations of a chemical's structure. However, since there are a lot of different 1D- and 2D-descriptors available, it is quite challenging to choose uncorrelated and meaningful ones.[3]

In the context of biodegradation and toxicity predictions popular descriptors are fragment-based descriptors and (binary) fingerprints to represent a molecule's structure as a binary string for example by the absence or presence of certain fragments or substructures.[6] The correlation of that information with a certain dependent variable leads to so called structural alerts (SA). This approach has become very popular since it is straightforward when it comes to the interpretability by the user, is very in line with chemists' thinking e.g. in functional groups, and the direct usage of such information to utilize it in the design process.[32]

All the so far presented descriptors lack of the consideration of the 3D structure of molecules and therefore their stereochemistry and chirality which is of importance for the interaction of molecules e.g. with enzymes (e.g. biodegradability) and receptors (e.g. efficacy, toxicity). 3D-

(Q)SAR includes all *in silico* methods, which correlate properties with computed descriptors derived from the spatial representation of the molecular structure and its force field[34]. One of the most used method for 3D-(Q)SAR[35] is the comparative molecular field analysis (CoMFA). It focuses mainly on steric and electrostatic properties of the molecule, which result in favorable and unfavorable receptor-ligand interactions. The computed interaction energies are than considered as independent variables to be correlated to biological responses or other endpoints.

Chemical descriptors are common in a lot of models which are used to investigate environmental endpoints,[36,37] fragment-based methods can be found in a lot of models, which predict toxicity, mutagenicity or even biodegradability.[18,38] 3D-descriptors are not that common yet with regard to the endpoints of interest here. However, there are some studies where they have been used to model the enzymatic degradation in the environment.[14]

Table S2: Comparison of different descriptor-types and their advantages and limitations from a user perspective in the field of green and sustainable chemistry.

Prediction model	Advantages	Limitations	Examples
Chemical descriptor-based-(Q)SAR (1D- and 2D-descriptors)	• Usage of different types of descriptors allows for modeling complex endpoints • Performs best for a small focused sets with known mechanisms	• Results and interactions of the descriptors can be hard to interpret • The decision of molecular descriptors is very important, too few and they won't represent the mechanism of the endpoint but too many render the multidimensional space very complex and the	OECD QSAR Toolbox[16], VEGA[18], EPI Suite and ECOSAR[19], ChemProp[21]

		results hard to interpret • For some models the AD is not or only poorly assessed and the output may not give enough insights to properly interpret the results	
Fragment-based-(Q)SAR/ Structural Alerts (SAs)	• Can propose relationships between an endpoint and structural features that may be not have been identified by humans • Easy to interpret and they can give insights how a structure should be altered to achieve a certain property	• SAs use only binary features (substructure is present or absent) • No insights in pathways or mechanisms behind the investigated endpoint • The list of rules could be incomplete and therefore result in a lot of false negative results • The structural rules identified are dependent on the set they are derived from and may, therefore, not be transferable from one setting to another. • No continuous representation of the molecule due to the use of fragments and loses sight of the	CASE Ultra[15], OECD QSAR Toolbox[16], Toxtree[17], VEGA[18], Leadscope[20]

		molecule as a whole	
3D-(Q)SAR (e.g. CoMFA)	• Allows to understand receptor-ligand interactions	• Requires the alignment of molecules, which is time consuming and requires experience	Maestro by Schroedinger[28], 3D-QSAR.com (py-CoMFA)[39]

Endpoints and models

In the following table S3 endpoints and respective models are listed as well as their specifications complementing the discussion in the article on the five preliminary considerations for an *in silico* assessment. This table S3 is not meant to be comprehensive and complete, but rather be a first starting point, which models for which endpoints are available in the mentioned software. If the user would like to assess the mutagenic potential of impurities in pharmaceuticals according to the ICH M7 guideline[40] two different model methodologies should be chosen. One model should be based on expert rules and the other on statistics. If these models do not indicate any structural alert for mutagenicity, then there is no concern for mutagenicity. It is recommended to do an expert review for supportive evidence.[40] In addition, the models should be in compliance with the OECD validation criteria.[41]

Table S3: Endpoints and corresponding available *in silico* models of interest for BbD. The software mentioned here include also further models that have not been listed here. Artifical Neural Network (ANN), k-Nearest Neighbor (kNN), Multiple Linear regression (MLR), Super vector Machine (SVM), Weight of Evidence (WoE).

endpoint	model specification	software	Lit.
Physicochemical properties			
n-Octanol/Water partition coefficient			
$\log K_{ow}$	continuous, regression (descriptor: structural fragments)	EPI Suite, VEGA	[18,42]
K_{ow}	continuous, read-across	ChemProp	[21]
Soil adsorption coefficient			
K_{oc} (L/kg)	continuous, regression (descriptor: Molecular Connectivity Index)	EPI Suite	[19]
K_{oc} (L/kg)	continuous, regression (descriptor: $\log K_{ow}$)	EPI Suite	[19]
Environmental degradation			
Biodegradability			
Ready biodegradability	classification, rule-based (fragment-based)	VEGA	[6,18]
Ready biodegradability, general aerobic and anaerobic biodegradability	classification, statistical/regression (descriptor: structural fragments)	EPI Suite, in Biowin1-7 (also available in the OECD QSAR tool box)	[16,19,43,44]
Ready biodegradability (%)	classification and continuous, statistical (fragment-based)	CASE Ultra	[15,45]
Ready biodegradability (%)	continuous, expert system	Catalogic	[46]
Ready biodegradability	classification, knn (molecular and physicochemical descriptors	Opera	[47]

Biodegradation half-life	continuous, knn (molecular and physicochemical descriptors	Opera	[47]
Microbial catabolic reactions (i.e. pathways)	rule-based	EAWAG-BBD Pathway Prediction System	[48,49]
Biodegradation (aerobic, anaerobic)	classification, fragment-based	ChemProp	[21]
Hydrolysis			
Hydrolysis rate (l/mol-sec) and half-life	continuous, statistical/regression (descriptor: structural fragments)	EPI Suite (also available in the OECD QSAR tool box)	[16,19]
Photodegradation			
OH radical rate constant in gas phase (cm^3/molecules·sec) and half-life	continuous, structural fragments (SAR)	EPISuite	[19,50]
O_3 rate constant in gas phase (cm^3/molecules·sec) and half-life	continuous, structural fragments (SAR)	EPISuite	[19,50]
OH radical rate constant in gas phase (cm^3/molecules·sec)	continuous, knn (molecular and physochemical descriptors	Opera	[47]
Persistence			
Persistence in sediment	classification, knn (structural alerts)	VEGA	[18,51]
Persistence in water	classification, knn (structural alerts)	VEGA	[18], implementation as in [51]
Persistence in soil	classification, knn (structural alerts)[18]	VEGA	[18], implementation as in [51]

Bioconcentration factor			
BCF in fish (log(L/kg))	continuous, read-across	VEGA	[18]
BCF in fish (log(L/kg))	continuous, statistical/regression (descriptor: logP)	VEGA	[18,52]
BCF in fish (log(L/kg))	hybrid model (MLR, neural network, SVM)	VEGA, CAESAR	[18,53,54]
BCF in fish (log(L/kg))	continuous, statistical/regression (descriptor: logP)	EPI Suite	[19,52]
BCF in fish (log(L/kg)) (three trophic levels)	continuous, statistical/regression (descriptor: logP)	EPI Suite	[19,55]
BCF for Cyprinus Carpio (logBCF)	classification and continuous, statistical (fragment-based)	CASE Ultra	[15]
BCF for Bluegill(logBCF)	classification and continuous, statistical (fragment-based)	CASE Ultra	[15]
BCF in fish (logof the ratio of a contaminant concentration in biota to its concentration in the surrounding medium (water).)	continuous, knn (molecular and physicochemical descriptors	Opera	[47]
BCF in fish	quantitative, read-across	ChemProp	[21]
mg/L, bioaccumulation: aquatic / sediment	statistical, partial logistic regression	Leadscope	[56]

Bioaccumulation factor			
BAF in fish (log(L/kg)) (three trophic levels)	continuous, statistical/regression (descriptor: logP)	EPI Suite	[19,55]
Toxicity			
Mutagenicity			
Salmonella typhimurium (Ames test)	classification, hybrid model (SVM and rule-based (structural alerts))	VEGA (extends the original Casear model)	[18,57]
Salmonella typhimurium (Ames test)	classification, rule-based (structural alerts)	VEGA	[18,58]
Salmonella typhimurium (Ames test)	classification, rule-based	VEGA	[18]
Salmonella typhimurium (Ames test)	classification, read-across	VEGA	[18]
Salmonella typhimurium 5-strains	classification, statistical (fragment-based)	CASE Ultra	[59]
E. coli composite	classification, statistical (fragment-based)	CASE Ultra	[59]
E. coli WP strains	classification, statistical (fragment-based)	CASE Ultra	[59]
Mammal in vivo composite	classification, statistical (fragment-based)	CASE Ultra	[59]
Mammal in vivo dominant lethal	classification, statistical (fragment-based)	CASE Ultra	[59]

Mammal in vitro, Chinese hamster ovary	classification, statistical (fragment-based)	CASE Ultra	[59]
Mammal in vitro, mouse lymphoma	classification, statistical (fragment-based)	CASE Ultra	[59]
Induction of in vivo micronucleus in rodents	classification, decision tree (structural alerts)	ToxTree	[17,60]
In vitro mutagenicity (Ames test)	classification, decision tree (structural alerts)	ToxTree	[17]
Salmonella typhimurium (Ames test)	classification, read across and consensus model of four QSAR models from VEGA	ToxRead	[24]
Microbial in vitro, OECD 471 Bacterial Reverse Mutation Test (Salmonella and E. coli.)	classification, rule-based	Leadscope	[61]
Mammalian in vitro, mouse lymphoma activated	classification, partial logistic regression	Leadscope	[62]
Microbial in vitro, combination of results from E. coli WP2 uvrA, E. coli WP2 uvrA (pKM101), and S. typhimurium TA102	classification, statistical, partial logistic regression	Leadscope	[63]
Microbial in vitro, Salmonella typhimurium (strains TA97, TA98, TA100, TA1535, TA1536, TA1537, and TA1538)	classification, statistical, partial logistic regression	Leadscope	[64]

Microbial in vitro, Salmonella typhimurium (strains TA97, TA97a, TA1537, TA98, TA100, TA1535, TA102) and E. coli	classification, statistical, partial logistic regression	Leadscope	[65]
Mammalian in vitro, Chinese hamster	classification, statistical, partial logistic regression	Leadscope	[66]
Mammalian in vivo, rat, mice, and other rodents	classification, statistical, partial logistic regression	Leadscope	[67]
Mammalian in vivo, rat, mice, and other rodents	classification, statistical, partial logistic regression	Leadscope	[68]
In vitro chromosome aberration, Chinese hamster lung cells	classification, statistical, partial logistic regression	Leadscope	[69]
In vitro chromosome aberration, Chinese hamster ovary cells	classification, statistical, partial logistic regression	Leadscope	[70]
In vitro sister chromatid exchange, Chinese hamster	classification, statistical, partial logistic regression	Leadscope	[71]
In vitro sister chromatid exchange, mammalian cell cultures	classification, statistical, partial logistic regression	Leadscope	[72]
Chromosome aberrations in vivo, rats, mice, and other undefined	classification, statistical, partial logistic regression	Leadscope	[73]
Chromosome aberrations in vivo, rats	classification, statistical, partial logistic regression	Leadscope	[74]

In vivo micronucleus, mice	classification, statistical, partial logistic regression	Leadscope	[75]
Carcinogenicity			
Carcinogenicity	classification, ANN	VEGA (extends the original Casear model)	[18,76]
Carcinogenicity	classification, rule-based	VEGA	[18]
Carcinogenicity in male or female rats	classification, rule-based	VEGA	[18]
Carcinogenicity (expert assessment based on carcinogenic effects in different species)	classification, rule-based (structural alerts)	VEGA	[18]
Carcinogenicity in male rats	classification, statistical (fragment-based)	CASE Ultra	[59]
Carcinogenicity in female rats	classification, statistical (fragment-based)	CASE Ultra	[59]
Carcinogenicity in rats	classification, statistical (fragment-based)	CASE Ultra	[59]
Carcinogenicity in male mice	classification, statistical (fragment-based)	CASE Ultra	[59]
Carcinogenicity in female mice	classification, statistical (fragment-based)	CASE Ultra	[59]

Carcinogenicity in mice	classification, statistical (fragment-based)	CASE Ultra	[59]
Carcinogenicity	classification, decision tree (structural alerts)	ToxTree	[17]
Carcinogenicity	classification, WoE, consensus model of ChemProp models	ChemProp	[21]
Carcinogenicity in vitro C3H10T1-2 (rodent)	classification, partial logistic regression	Leadscope	[77]
Carcinogenicity in vitro, Balb/c-3T3	classification, partial logistic regression	Leadscope	[78]
Carcinogenicity in vitro, Syrian hamster embryo, Balb/c-3T3, and C3H10T1/2	classification, partial logistic regression	Leadscope	[79]
Carcinogenicity in vivo, female mouse	classification, partial logistic regression	Leadscope	[80]
Carcinogenicity in vivo, female rat	classification, partial logistic regression	Leadscope	[81]
Carcinogenicity in vivo, male mouse	classification, partial logistic regression	Leadscope	[82]
Carcinogenicity in vivo, male rat	classification, partial logistic regression	Leadscope	[83]

Reproductive toxicity			
Developmental toxicity	classification, random forest (regression and decision trees)	Vega (extends the original Casear model)	[84]
Developmental and reproductive toxicity	classification, decision tree	VEGA	[18,85]
Fertility female rats (3 sub models)	classification, statistical (fragment-based)	CASE Ultra	[59]
Fertility female mice	classification, statistical (fragment-based)	CASE Ultra	[59]
Fertility male rats (2 sub models)	classification, statistical (fragment-based)	CASE Ultra	[59]
Fertility male mice	classification, statistical (fragment-based)	CASE Ultra	[59]
Fertility male sperm rats (2 sub models)	classification, statistical (fragment-based)	CASE Ultra	[59]
Fertility male sperm mice	classification, statistical (fragment-based)	CASE Ultra	[59]
Dysmorphogenesis in rats (2 sub models)	classification, statistical (fragment-based)	CASE Ultra	[59]
Dysmorphogenesis in mice (2 sub models)	classification, statistical (fragment-based)	CASE Ultra	[59]

Dysmorphogenesis in rabbits (4 sub models)	classification, statistical (fragment-based)	CASE Ultra	[59]
Behavioral toxicity in rats (2 sub models)	classification, statistical (fragment-based)	CASE Ultra	[59]
Behavioral toxicity in mice	classification, statistical (fragment-based)	CASE Ultra	[59]
In vivo pre-, peri-, post natal development and / or fertility (mouse)	classification, partial logistic regression	Leadscope	[86]
In vivo pre-, peri-, post natal development and / or fertility (female rat)	classification, statistical, partial logistic regression	Leadscope	[87]
In vivo pre-, peri-, post natal development and / or fertility (female mouse)	classification, statistical, partial logistic regression	Leadscope	[88]
In vivo pre-, peri-, post natal development and / or fertility (male mouse)	classification, statistical, partial logistic regression	Leadscope	[89]
In vivo pre-, peri-, post natal development and / or fertility (male rat)	classification, statistical, partial logistic regression	Leadscope	[90]
In vivo pre-, peri-, post natal development and / or fertility, fetal growth, mouse	classification, statistical, partial logistic regression	Leadscope	[91]
In vivo pre-, peri-, post natal development and / or fertility, fetal weight decrease, rabbit	classification, statistical, partial logistic regression	Leadscope	[92]
In vivo pre-, peri-, post natal development and / or fertility, fetal death, rodent	classification, statistical, partial logistic regression	Leadscope	[93]

Endocrine disrupting			
relative binding affinity	classification, decision tree	VEGA, CASE Ultra	[18,94]
estrogen receptor mediated effect	classification, rule-based	VEGA	[18]
Ecotoxicity			
toxicity to environmental bacteria (mg/kg)	classification and continous, statistical (fragment-based)	CASE Ultra	[95]
mg/L, toxicity to microorganisms (Tetrahymena pyriformis)	continuous, statistical, partial logistic regression	Leadscope	[96]
Aquatic toxicity			
LC_{50} (mg/L) for fish (96 hr)	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LC_{50} (mg/L) for Daphnids (48 hr)	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
EC_{50} (mg/L) for green algae (96 hr)	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LC_{50} (mg/L) for Mysida (96 hr)	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LC_{50} (mg/L) for fish in salt water (96 hr)	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
toxicity for gold fish	classification, statistical (fragment-based)	CASE Ultra	[15]

toxicity for rainbow trout	classification, statistical (fragment-based)	CASE Ultra	[15]
LC_{50} for fish	classification, rule-based (structural alerts)	VEGA	[18]
LC_{50} (mg/L and -log(mg/L)) for fish	continuous, read-across	VEGA	[18]
LC_{50} (mg/L and log(1/(mmol/L)) for fish	continuous, ANN	VEGA	[18]
LC_{50} (mg/L and -log(mol/L)) for fathead minnow (96 hr)	continuous, regression (21 molecular descriptors)	VEGA	[18]
LC_{50} (mg/L and -log(mol/L)) for Daphnia Magna (48 hr)	continuous, regression (17 molecular descriptors)	VEGA	[18]
LC_{50} (mg/L and -log(mol/L)) for Daphnia Magna (48 hr)	continuous, hybrid model based on MLR (16 molecular descriptors)	VEGA	[18,97]
Algae acute toxicity (biocides)	classification, rule-based, read-across, fragment-based	ToxRead	[24]
Daphnia magna acute toxicity (biocides)	classification, rule-based, read-across, fragment-based	ToxRead	[24]
Fish acute toxicity (biocides)	classification, rule-based, read-across, fragment-based	ToxRead	[24]
Microbial toxicity (biocides)	classification, rule-based, read-across, fragment-based	ToxRead	[24]

GHS categories, toxicity to aquatic algae and cyanobacteria	categories, expert alerts	Leascope	[98]
mg/L, toxicity to aquatic algae and cyanobacteria	categories, statistical, partial logistic regression	Leadscope	[99]
GHS categories, short-term toxicity to fish (fathead minnow)	categories, expert alerts	Leadscope	[100]
mg/L, GHS category, short-term toxicity to fish (fathead minnow)	categories, statistical, partial logistic regression	Leadscope	[101]
mg/L, GHS category, short-term toxicity to fish (bluegill)	categories, statistical, partial logistic regression	Leadscope	[102]
Toxicity to terrestrial organisms			
acute toxicity in honeybees	classification, k-NN	VEGA	[18,103]
LC_{50} (mg/L) for earthworm (14 d)	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LD_{50} (mg/kg) for rats	classification and continuous, statistical (fragment-based)	CASE Ultra	[15]
LD_{50} (mg/kg) for rats (oral), (GHS category 1)	classification, statistical (fragment-based)	CASE Ultra	[15]
LD_{50} (mg/kg) for rats (oral), (GHS category 1-2)	classification, statistical (fragment-based)	CASE Ultra	[15]

LD_{50} (mg/kg) for rats (oral), (GHS category 1-3)	classification, statistical (fragment-based)	CASE Ultra	[15]
LD_{50} (mg/kg) for rats (oral), (GHS category 1-4)	classification, statistical (fragment-based)	CASE Ultra	[15]
LD_{50} (mg/kg) for rats (oral), (GHS category 1-5)	classification, statistical (fragment-based)	CASE Ultra	[15]
GHS categories for acute toxicity – oral (rats)	categories, expert alerts	Leadscope	[104]
GHS categories for acute toxicity – oral (rats)	classification, statistical, partial logistic regression	Leadscope	[105]
Chronic aquatic toxicity			
LC_{50} (mg/L) for fish	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LC_{50} (mg/L) for Daphnid	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LC_{50} (mg/L) for green algae	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LC_{50} (mg/L) for Mysida in salt water	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]
LC_{50} (mg/L) for fish in salt water	continuous, statistical/regression (descriptor: logP)	ECOSAR (via EPI Suite)	[19]

PBT profiler			
persistence, bioconcentration potential and fish chronic toxicity	classification, uses ECOSAR and EpiSuite models	software by EPA (website of the profiler currently under construction)	[106]

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Publikation 2

Stefanie Lorenz, Ghadeer Suaifan, Klaus Kümmerer (2022).

Designing benign molecules: The influence of O-acetylated glucosamine-substituents on the environmental biodegradability of fluoroquinolones.

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Designing benign molecules: The influence of O-acetylated glucosamine-substituents on the environmental biodegradability of fluoroquinolones

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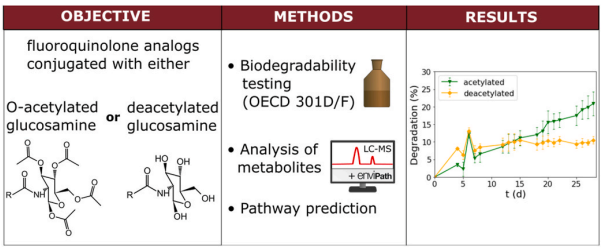
HIGHLIGHTS

- Novel FQ analogs conjugated with glucosamine have been investigated regarding their ready biodegradability (OECD 301D/F).
- Degradation products have been identified via LC-MS.
- It could be shown that the acetylation of the glucosamine moiety has a major influence on the biodegradability.
- Results could be further used for a fragment-based design of benign molecules.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotics are detected worldwide in the aquatic environment, with continuously rising concentrations. Antibiotics in the environment have the potential to damage ecosystems and contribute to the development of resistance. Whilst a few antibiotics, such as some β-lactams, are eliminated by effluent treatment, others, such as fluoroquinolones, are not or just partially removed and enter the environment. Therefore, approaches are needed to tackle those problems at the compound level. Benign by design (BbD), an important part of green pharmacy, has the goal to integrate environmental fate and end-of-use considerations at the very beginning, i.e., into the design of active pharmaceutical ingredients. Hence, pharmaceuticals should be designed to be sufficiently active and stable during storage and usage but should degrade after excretion into the environment, so that they cannot cause any adverse effects.

Fluoroquinolones (FQs) are important broad-spectrum antibiotics. They are known to be persistent in the environment and to be neither inactivated nor degraded or even mineralized during sewage treatment. The addition of new substituents via amidation, like glucosamine moieties, at the carboxylic group of FQs, led to better antimicrobial activity compared to its parent compounds against various microorganisms. To investigate if the addition of sugar moieties could improve the overall environmental biodegradability of FQs, eight novel quinolone and fluoroquinolone analogs conjugated with 1,3,4,6-Tetra-O-acetyl-β-D-glucosamine and 2-deoxy-D-glucopyranose have been investigated regarding their ready biodegradability (OECD 301D/F) and their

Abbreviations: API, Active pharmaceutical ingredient; BbD, Benign by Design; DOC, Dissolved organic carbon; FQ, Fluoroquinolone; ThOD, Theoretical oxygen demand; TP, Transformation product.

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degradation pathways have been analyzed. According to the OECD 301D test, none of the substances could be classified as readily biodegradable. However, the O-acetyl analogs did undergo a partial degradation of the O-acetyl glucosamine moiety, via stepwise deacetylation and the degradation of the whole glucosamine moiety. The degradation resulted in Fluoroquinolone-3-carboxamide derivatives.

Those insights could be further used as input for fragment-based design of benign APIs that will degrade once they reached the environment.

1. Introduction

1.1. Antibiotics in the environment

The worldwide consumption of antibiotics did significantly increase over the last decades (Browne et al., 2021). Consequentially, antibiotics can be found in environmental water bodies and soils and their environmental concentrations are continuously rising (Chow et al., 2021; Kümmerer, 2009a, 2009b; Schwarzenbach et al., 2006). Active pharmaceutical ingredients (APIs) have been optimized regarding high stability to ensure easy and safe storage and application. However, high chemical stability comes along with high persistence in the environment (Leder et al., 2015). Environmental degradation or mineralization of APIs is often not intended or even seen as a contradicting property.

There are some antibiotics, like some β-lactams, which can be removed in sewage treatment plants (Längin et al., 2009). However, full elimination of APIs, also if advanced oxidation processes (AOPs) are used, is often not achieved. Furthermore, the methods of AOP lead to multiple transformation products often with unknown properties, which are released into the environment (Fatta-Kassinos et al., 2011). If antibiotics are released into the environment, they have the potential to damage ecosystems and their functions (Jordan and Gathergood, 2013). Antibiotics in the environment and sewage treatment plants also significantly contribute to the development of resistant microorganisms (Davies and Davies, 2010; Gullberg et al., 2011; Tello et al., 2012).

To delimit the development of resistances against antibiotics and therefore achieve the Sustainable Development Goals (SDGs) extensive solutions are needed (Jasovský et al., 2016). It is also emphasized by the "Chemicals Strategy for Sustainability – Towards a Toxic-Free Environment" and the "European Union Strategic Approach to Pharmaceuticals in the Environment" by the European Commission that it is urgent to tackle these issues from multiple perspectives (European Comission, 2019; 2020).

According to the concept of Benign by Design (BbD), which is based on the 10th principle of green chemistry (Anastas and Eghbali, 2010), chemicals that necessarily end up in the environment must be designed to mineralize during wastewater treatment or in the environment, or at least are transformed into less harmful degradation products (Kümmerer, 2019). Benign by Design is an important part of sustainable pharmacy. The goal of sustainable pharmacy is to integrate environmental behavior and end-of-use considerations already into the stage of drug development (Kümmerer and Hempel, 2010). The key to this approach is that pharmaceuticals have no need to be stable under all circumstances but only if it is necessary (e.g. during application or storage). Therefore, it could be benefited from the different physio-chemical conditions and present microorganisms at different life stages (e.g. during application and after release into the environment). There are some rules of thumb, which can be used to design better biodegradable molecules (Boethling et al., 2007). Workflows, which show how such considerations could be implemented into the design process (Leder et al., 2015; Lorenz et al., 2021; Puhlmann et al., 2021) as well as some successful examples of the application of the BbD concept (Beil et al., 2021; Kümmerer, 2019; Leder et al., 2021; Rastogi et al., 2015; Suk et al., 2020; Zumstein and Fenner, 2021) have been published so far.

1.2. Fluoroquinolones

Fluoroquinolones (FQs) are widely used broad-spectrum antibiotics in human and veterinary medicine, animal production, and in some countries also in aquacultures. They are considered one of the most successful antimicrobials regarding their spectrum, administration, and tissue distribution (Millanao et al., 2021). Their clinical and medical importance has been approved several times, being an active pharmacophore with various activities and applications (Suaifan and Mohammed, 2019). They belong to the 'Watch' group according to the WHO AWaRe classification and are considered as Highest Priority Critically Important Antimicrobials in human medicine. Furthermore, they are also classified as Veterinary (Highly) Important Antimicrobial Agents (European Centre for Disease Prevention and Control (ECDC) et al., 2021).

FQs are considered the third largest drug class since they accounted for 17% of the antibiotic market in 2009 with sales of 7.1 billion dollars globally (Hamad, 2010). In 2013 the usage of FQs for food-producing animals was around 200 tons in the USA and Europe, and alone 22, 210 tons in China (Millanao et al., 2021). The EFSA reports a population-weighted mean consumption of FQs in humans and food-producing animals in 2017 of 7.6 and 2.4 mg per kg biomass, respectively (European Centre for Disease Prevention and Control (ECDC) et al., 2021). FQs in humans and animals are mainly eliminated through the kidneys without any changes (Millanao et al., 2021). They are metabolized by a fraction of 30–70% (Kümmerer and Henninger, 2003). However, FQ metabolites often are still active compounds (Millanao et al., 2021).

FQs can be found in all environmental aquatic compartments (Felis et al., 2020). Environmental concentrations of fluoroquinolones have been detected at up to 100 μg L^{-1} in sewage treatment plant effluents and from 5 to 120 μg L^{-1} in surface water (Kümmerer, 2009a). Ciprofloxacin, one of the most commonly used FQs, exceeded the predicted no effect concentration at more than 30% of the tested environmental matrices from 47 different countries (Booth et al., 2020). The identified sources are municipal wastewater, hospital wastewater, industrial wastewater, and dispersion of manure onto agricultural soils (Millanao et al., 2021; Sukul and Spiteller, 2007). Industrial wastewater did reach concentrations of Ciprofloxacin of up to 3548 μg L^{-1}. But there are also high average concentrations in municipal wastewater (577 μg L^{-1}) or even surface water (384 μg L^{-1}) (Booth et al., 2020). FQs are known to be non-biodegradable and very persistent in the environment (Alexy et al., 2004; Li and Zhang, 2010; Suaifan et al., 2022). In sewage treatment plants they are at most eliminated via sorption but never mineralized (Wang et al., 2017).

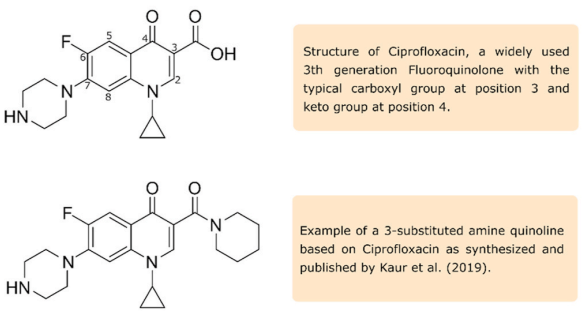
As FQs are produced to be highly biologically effective, their presence in the environment is expected to have ecotoxicological effects (Sukul and Spiteller, 2007). In fact, fluoroquinolones are among those antibiotics which show the greatest toxicity to aquatic organisms (Felis et al., 2020). They produce short-term effects by directly affecting microbial communities as well as long-term indirect effects, such as the selection of resistant mutant bacteria. The correlation between FQ consumption and use in agriculture, and the development of resistant bacteria strains has been proven several times (Booth et al., 2020; Kenyon, 2021).

Since the discovery of the first quinolone Nalidixic acid, this group of substances did undergo several developments and optimizations.

Research in the field of fluoroquinolone optimization usually aims to improve drug oral and parenteral dosing or the spectrum of activity, particularly against resistant pathogens. Several positions at the pharmacophore have been altered to improve the properties of the whole molecule (Suaifan and Mohammed, 2019). Modifications on the 3-carboxylic acid group have been rarely attempted since it is, among others, responsible for the mode of action and provides the binding side for DNA gyrase (Aldred et al., 2014).

However, there are several publications dealing with the development and synthesis of new analogs introducing different new functional groups and substituents at the position of the 3-carboxylic acid group (Scheme 1). Arayne et al. (2010), Haroon et al. (2012), and Sultana et al. (2011) developed new analogs of Ciprofloxacin, Ofloxacin, and Enoxacin by introducing new functionalities at position 3 via ester aminolysis reactions. Therefore, the 3-carboxylic acid group was first esterified and later subjected to nucleophilic attacks by various (aromatic) amines like urea, aminophenol, acetamide or aniline. They did show that some of those compounds had either comparable or even better antimicrobial activities than the parent substance. Jubie et al. (2012) introduced several heterocyclic compounds like oxadiazole or triazole into the 3-carboxylic group. This lead to increased activity especially in gram-positive bacteria. Kant et al. (2016) synthesized and tested new bis-1,2,4-triazole-linked ciprofloxacin conjugates, which showed enhanced activity against both gram-positive and gram-negative species compared to the parent compound. Akhtar et al. (2019) added phenol and alkyl halide via esterification at the third position of Moxifloxacin, resulting in Moxifloxacin-ester derivatives with a significant antibacterial and antifungal profile with similar or enhanced activity compared to the parent drug. Kaur et al. (2020) developed molecular hybrids of Norfloxacin and Ciprofloxacin by the substitution of various amines at the third position, which also resulted in even a 10-fold increase in their antibacterial activities *in vitro*.

Mohammed et al. (2019, 2020) used glucosamine moieties for modifications at position 3 to synthesize novel glycosylated fluoroquinolone derivatives. They modified Nalidixic acid, Ciprofloxacin, Norfloxacin, and Moxifloxacin featuring an amide functional group at position 3. The coupling with a glucosamine moiety resulted in hybrid derivatives with improved antibacterial and antifungal activities. They have shown that the amidation with glucosamine could improve the antimicrobial activity compared to the reference drugs. Moreover, the new Ciprofloxacin and Norfloxacin conjugates even showed antibacterial activity against a quinolone-resistant *E. Coli* clinical isolate (Mohammed et al., 2020). Most of the tested conjugates were less cytotoxic than their corresponding parent drug. Glucosamine amino sugar moieties were therefore proposed to promote drug uptake and thus altering their antibacterial activity, as well as lowering their cytotoxicity level because of their selective uptake via the sugar-dependent phosphotransferase system in bacteria (Mohammed et al., 2019).



Scheme 1. Example structures of fluoroquinolones.

However, those newly designed analogs were mainly tested regarding their antimicrobial activity against various microorganisms, but their environmental properties have not been studied yet. Some of these new analogs are of interest regarding their effects (selection of resistant bacteria) and fate in the aquatic environment. Esters and amides, as well as hydroxyl or aldehyde functionalities and phenoxy rings, are known to be able to improve the biodegradability of molecules (Boethling et al., 2007). The influence of sugar moieties on the overall degradability of molecules has been poorly studied yet. However, it has been proposed that they can lead to an overall improvement in biodegradability (Kümmerer et al., 2000; Kümmerer and Al-Ahmad, 1997).

To overcome the environmental pollution with pharmaceuticals and their negative impacts such as the selective pressure on bacteria in the environment, the design of environmentally degrading and mineralizing APIs is of interest. Therefore, the novel glucosamine conjugates of Nalidixic acid, Ciprofloxacin, Norfloxacin, and Moxifloxacin as published by Mohammed et al. (2019, 2020) have been investigated regarding their ready environmental biodegradability (OECD 301) and resulting transformation products have been analyzed. The aim was to get insights into how sugar moieties and their derivatives (degree of acetylation) influence the breakdown of fluoroquinolones in the aquatic environment. Such insights are of importance for the targeted design of future benign APIs.

2. Materials and methods

2.1. Substances

Eight novel glycosylated derivatives of Nalidixic acid, Ciprofloxacin, Norfloxacin, and Moxifloxacin, i.e., one O-acetylated and one deacetylated each (Fig. 1) have been synthesized as described by Mohammed et al. (2019, 2020). The compounds **1, 3, 5,** and **7** are the O-acetylated ones and **2, 4, 6,** and **8** are the deacetylated ones, respectively. As a reference for the possible dead-end transformation product of all compounds, Quinoline-3-carboxamide (CAS-Nr. 6480-67-7) has been purchased from abcr GmbH (Karlsruhe, Germany).

2.2. Biodegradation testing

Two different biodegradation tests according to the OECD 301 guideline have been performed. The Closed Bottle Test (CBT, 301D) is one of the most stringent tests, due to its relatively low bacterial density and diversity and low substance concentrations. It represents the conditions in surface waters. The OECD 301F test (Manometric Respiratory Test, MRT) on the other hand has higher bacterial density and diversity as well as higher substance concentrations, with active sludge being used as inoculum. To see if the microbial community has an influence on biodegradation and resulting transformation products, both tests have been performed for some of the substances.

2.2.1. Closed Bottle Test (OECD 301D)

All substances (compounds **1–8**) have been tested for ready biodegradability by an optode-based (Fibox 3, PreSens, Regensburg, Germany) Closed Bottle Test (Friedrich et al., 2013). The starting concentration of the test compounds corresponded to 5 mg L^{-1} theoretical oxygen demand (ThOD). The inoculum was derived from the secondary effluent from the local municipal sewage treatment plant (Abwasser Grün & Lüneburg Services GmbH, Lüneburg, Germany, 325, 000 equivalent inhabitants). The collected inoculum was filtered through a paper filter before use. The mineral media was prepared as described in the OECD 301D guideline (OECD, 1992). The tests consisted of a blank, quality control using sodium acetate as a readily biodegradable substance, the test series, and toxicity controls. All flasks contained 1% Dimethyl sulfoxide (DMSO), which is not degrading in the test, to enhance the solubility of the tested compounds. The tests were performed in the dark to inhibit photodegradation. The degree of

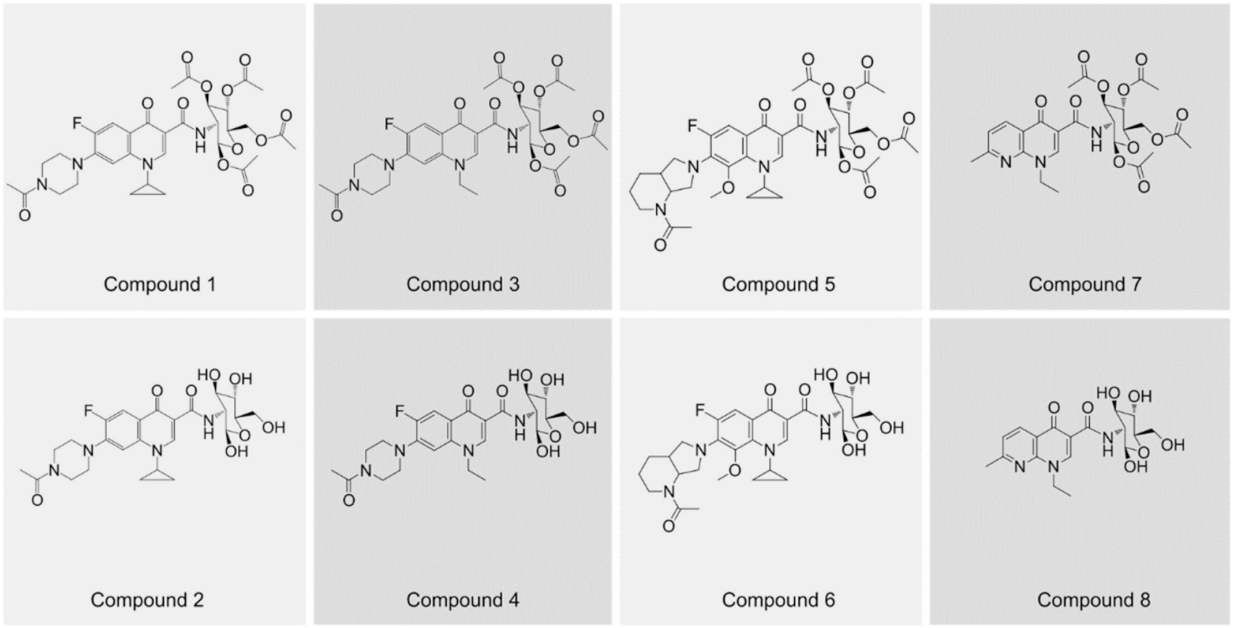


Fig. 1. Chemical structures of the investigated glycosylated quinolones and fluoroquinolones.

degradation was monitored via oxygen consumption. Samples were taken on day 0 and day 28 and stored at −20 °C before further analysis for identification of possibly formed products recalcitrant of incomplete mineralization (transformation products, TPs).

In parallel, the same inoculum and mineral media have been used for a long-term investigation of biodegradation over 48 d. Compounds **1** and **2** have been used as representatives for O-acetylated and O-deacetylated compounds, respectively. Ten bottles for each substance have been prepared, also with an initial test concentration which corresponded to 5 mg L^{-1} ThOD, as well as blank and quality controls (in total 40 bottles). Samples for further analysis have been taken every 5–6 d to monitor the development of intermediates over time.

2.2.2. Manometric Respiratory Test (OECD 301F)

Since the amount of the substances to investigate was limited, only two representatives (compounds **5** and **6**) have been used for another ready biodegradability test according to OECD 301F. The test was conducted using the OxiTop system (OC110-System, WTW GmbH, Weilheim, Germany). The starting concentration corresponded to 30 mg L^{-1} ThOD and the substances were directly weight into the test flasks. As inoculum 30 mg suspended solids L^{-1} of activated sludge from the above-mentioned sewage treatment plant was used. Before use, it was washed three times with tap water. The mineral media was prepared according to the OECD 301F guideline (OECD, 1992). The test consisted of blank and quality controls, the test series with corresponding toxicity controls, and an additional sterile control containing 320 mg L^{-1} NaN_3. The oxygen depletion, as well as the DOC elimination (ASI-V autosampler, TOC-VCPN analyzer, Shimadzu, Germany), were monitored. Since the substances were directly weighted into the test bottles there are no samples from day 0, which could be analyzed and compared to day 28. Therefore, no MRT samples were further analyzed via LC-MS.

2.3. LC-MS analysis of transformation products and in silico predictions

Identification of TPs formed during biodegradation testing was done by an Ultimate 3000 UHPLC (Thermo Scientific, Dreieich, Germany) coupled with an LTQ-Orbitrap-XL (Thermo Scientific, Dreieich, Germany). For ionization, a HESI source in positive mode was used (Table S20). For chromatographic separation, a NUCLEODUR C18 Gravity (125 × 4 mm, 5 mm, Macherey Nagel, Germany) column equipped with an EC 4/3 NUCLEODUR C18 Gravity guard column (Macherey Nagel, Germany) was used. The column temperature was set to 25 °C. A gradient mode at a 0.5 mL min^{-1} flow rate was used. The gradient consisted of 0.1% formic acid in water and acetonitrile. The injection volume was 25 μL.

Qual Browser (Thermo Xcalibur 2.2 SP1.48, Thermo Fisher Scientific Inc.) was used for the evaluation of the measured signals. MASS FRONTIER 7.0.3.1 (HighChem Ltd.) was used to predict MS^2 patterns. Furthermore, enviPath (http://envipath.org) (Wicker et al., 2016) and the EAWAG-BBD Pathway Prediction System (http://eawag-bbd.ethz.ch/) (Gao et al., 2010) were used to predict potential environmental TPs, which in turn were used as supporting information for the analysis and target masses. The measured mass-to-charge ratio (m/z), MS^2 patterns, and the *in silico* predicted structures were considered to develop the proposed structures of transformation products. The web tool SwissADME (Daina et al., 2017) has been used to predict a consensus log P based on different models for the proposed structures of resulting TPs to get more information on potential retention times. Furthermore, the rules for degradation from enviPath and EAWAG-PPS have been used to further understand the degradation pathways.

3. Results

3.1. Biodegradation according to OECD 301D, F

All CBT tests have been valid according to the OECD guideline (OECD, 1992). The degradation results after 28 d are summarized in Table 1. None of the tested substances can be classified as readily biodegradable as none of them reached a degradation of more than 60% after 28 d (OECD, 1992). Of the acetylated structures, compound **3** was the most biodegradable one, reaching about 20% oxygen consumption, followed by compounds **1**, **7,** and **5** being the least biodegradable acetylated derivative. The deacetylated structures with the highest degradation rate were compounds **2** and **4** with around 10% degradation. The least degradable one was compound **6** with less than 3% oxygen consumption. None of the substances had significant toxic effects

Table 1
Biodegradation results after 28 d under CBT conditions (n = 2).

	Degradation [%]	Tox Control Degradation [%]
Compound **1**	15.6±0.3	50.4±1.1
Compound **2**	10.0±2.5	48.4±0.1
Compound **3**	20.9±3.4	53.0±2.3
Compound **4**	10.5±1.0	51.0±0.8
Compound **5**	5.8±0.6	44.9±1.8
Compound **6**	2.3±1.4	39.3±0.1
Compound **7**	13.0±1.0	46.9±1.1
Compound **8**	8.4±0.2	44.9±1.3

on the inoculum since all toxicity controls reached a degradation rate of at least 40%. However, there are slight differences in toxicity controls, showing that compounds **5**, **6, 7,** and **8** seem to have at least some toxic effects on the inoculum, with compound **6** being the most toxic of the tested substances.

Fig. 2 shows the course of the degradation over the test duration of 28 d. After a typical adaptation phase of 4–6 d, the development of oxygen consumption for the acetylated (Fig. 2a) and deacetylated compounds (Fig. 2b) differs. For the acetylated compounds, it steadily increased over time, while it stagnated after the adaptation phase for the deacetylated ones.

The oxygen consumption during the degradation of Quinoline-3-carboxamide reached a level of 16.0% ± 5.3%. However, the adaption phase was around 22 d. The substance had no toxic effect on the inoculum.

The MRT has been valid according to the OECD guideline (OECD, 1992). The results for compound **5** with a degradation of around 28% and compound **6** with 15% degradation back up the trend that has been seen in the CBT (see Table S22). The acetylated compound **5** shows a significantly higher degradation rate than its deacetylated analog. Abiotic degradation seems to play a role since the removal of 10–15% has been measured also in the sterile controls (Tab. S22).

3.2. LC-MS analysis of transformation products and pathway prediction

The chromatograms of each substance at the start of the CBT and after 28 d can be found in the SI (Table S1-S19). For all the deacetylated compounds it could be shown that the chromatograms between the two sampling times do not differ, backing up the result that no degradation of the compounds happened, and no degradation products were formed. The analysis of all acetylated compounds revealed that the peak of the parent compound significantly decreased over time. Furthermore, the occurrence of several new peaks indicated the generation of degradation products. The retention times, mass-to-charge ratio, MS^2 fragments, and sum formula for compound **1** TPs are summarized in Table 2. The results for all other compounds can be found in the SI (Table S1-S19).

Fig. 3 shows the chromatograms of samples from compound **1** in the modified CBT after 5, 15, and 48 d. The parent compound peak at RT = 23.4 min decreased constantly over time. After 5 d TPs could already be detected with retention times between 20 and 22 min. Those same TPs are still present after 15 d, but also other intermediates with retention times around 18 min can be measured. Finally, after 48 d the parent compound peak and the peaks of the first TPs did decrease again, resulting in basically two dominant peaks at around 16 and 18 min, indicating that this may be the resulting products of the biodegradation process. This shows that the degradation of the acetylated compounds seemed to result in persistent *N*-acetyl-fluoroquinolone-3-carboxamide derivatives (m/z = 373.1661 and RT = 17.5 min for compound **1**). The calculated log P values of the proposed TP structures from Table 2 do also show, that the transformation products did become more polar than their parent compound, resulting in shorter retention times (Table S21).

The analysis of CBT samples of Quinolone-3-carboxamide after 28 d did show, that it was partially transformed to Quinoline, carrying a carboxylic acid group instead of the carboxamide group (see Table S19).

EnviPath and the EAWAG-PPS predicted the stepwise, partial degradation of acetyl groups as the main degradation pathway for the acetylated compounds (Fig. S1, according to rule bt00024). Furthermore, the removal of the acetylated glucosamine side chain was predicted, based on the underlying rules bt0067 and bt0243. For the deacetylated derivatives, a different degradation pathway has been predicted (Fig. S2). In this case, the generation of an aldehyde group (bt0434) and the generation of a carboxylate group (bt0003) at the glucosamine moiety were predicted as the first steps before the degradation of the glucosamine moiety could take place.

4. Discussion

The effects of acetylated and deacetylated glucosamine side chains at position 3 of fluoroquinolones on their overall environmental biodegradability have been investigated using OECD 301 tests and LC-MS analysis as well as biodegradation pathway prediction models to examine the resulting transformation products and degradation pathways.

4.1. Biodegradability and the influence of acetylation

Fluoroquinolones are well known to be not readily biodegradable (Alexy et al., 2004; Li and Zhang, 2010). This indicates that the herein measured oxygen consumption can be fully ascribed to the partial degradation of the (acetylated) glucose group. The results clearly showed that the acetylation of the glucosamine moiety influences the biodegradability results since the degradation rates of the acetylated compounds were approx. 1.5 to 2-times higher than for their deacetylated analogs (Table 1).

The stagnation of oxygen consumption after the adaption phase for the deacetylated compounds (Fig. 2b) suggests that no degradation of those compounds did take place. The initial oxygen consumption during the first days, esp. for the deacetylated compounds, may be due to the degradation of some readily degradable impurities from synthesis (data not shown). Their acetylated analogs on the other hand did show a

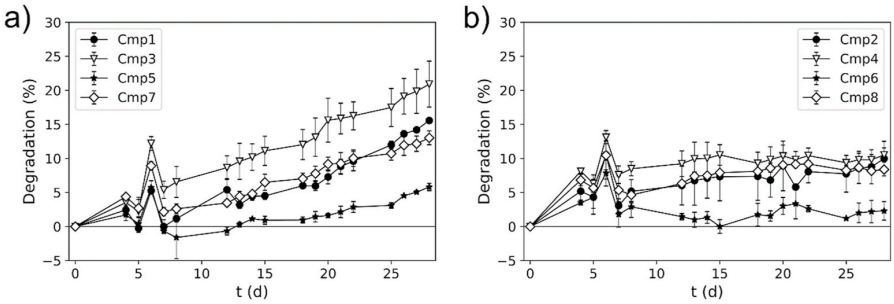
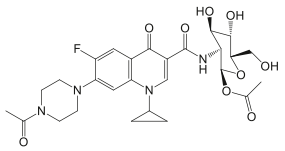
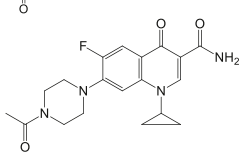
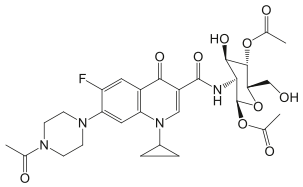
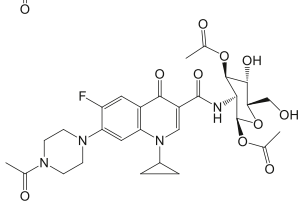
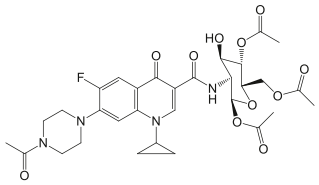
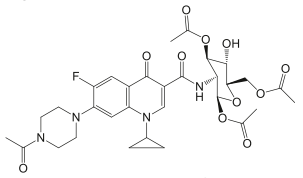
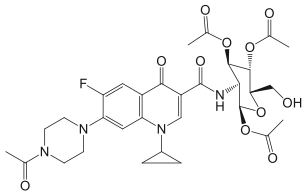
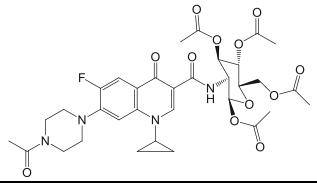


Fig. 2. Biodegradation curve under CBT conditions for a) the acetylated compounds and b) the deacetylated compounds (n = 2).

Table 2
Results of LC-MS analysis after 28 d in the CBT for Compound 1.

RT (min)	$[M+H]^+$	MS^2	Sum formula	Removed functionality	Structural formula of resulting TPs[a]
16.24	577.2292	559.2209 541.2108	$C_{27}H_{34}O_9N_4F$	3 x H_2C_2O	
17.59	373.1661	356.1406	$C_{19}H_{22}O_3N_4F$	1x Tetra-O-acetyl Glucosamine	
17.87	619.2399	601.2314 541.2101	$C_{29}H_{36}O_{10}N_4F$	2 x H_2C_2O	
18.26	619.2395	–	$C_{29}H_{36}O_{10}N_4F$	2 x H_2C_2O	
20.07	661.2498	601.2318 541.2106	$C_{31}H_{38}O_{11}N_4F$	1 x H_2C_2O	
20.52	661.2527	–	$C_{31}H_{38}O_{11}N_4F$	1 x H_2C_2O	
21.00	661.2523	643.2427 583.2215 523.2000	$C_{31}H_{38}O_{11}N_4F$	1 x H_2C_2O	
23.39	703.2767	643.2552 583.2327 523.2100	$C_{33}H_{40}O_{12}N_4F$		

[a] The proposed structural formulas are just one possible conformation of the resulting transformation products.

steadily increasing oxygen consumption over time (Fig. 2a). The degradation of one acetyl group would translate into around 7% oxygen consumption, while the whole acetylated glucosamine would consume around 44%, based on the theoretical oxygen demand as calculated according to the OECD guideline (OECD, 1992). This and the trend in Fig. 2a, where the oxygen consumption did not reach a plateau, suggest that the partial degradation of compounds **1, 3, 5,** and **7** was not completed after 28 d.

The degradation results from CBT (second effluent) and MRT (activated sludge) differ, showing that the higher bacterial diversity and

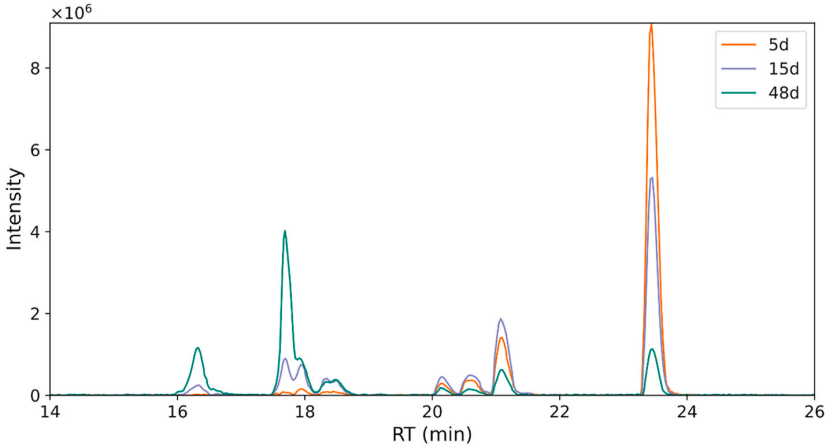


Fig. 3. Chromatograms of samples from compound 1 after 5, 15, and 48 d under CBT conditions (n = 1).

density of the used activated sludge resulted in overall higher degradation rates. The oxygen consumption in the sterile controls during the MRT suggests that also abiotic degradation processes may play a minor role. Taking the results of the sterile control as a blank to subtract from the test samples, shows that the microorganisms were able to degrade (parts of) the acetylated compound **5** (Tab. S22). The modification of fluoroquinolones by coupling with a glucosamine moiety has been described to improve their antibacterial and antifungal activity (Mohammed et al., 2019, 2020). This may be due to an increase in lipophilicity, which led to an enhancement in cell penetration. This could also correlate with their ability to be broken down by environmental bacteria.

There are examples, which did show that the addition of a sugar moiety led to an overall better biodegradable molecule. Kümmerer and Al-Ahmad (1997) did show that Cytarabine and Gemcitabine were better biodegradable than 5-Fluorouracil, which did not contain any sugar group. Also for other antineoplastic compounds, an influence of glucosidation could be shown (Kümmerer et al., 2000). However, there are also several examples, which show that a sugar moiety does not necessarily lead to better biodegradability. Aminoglycosides for example are considered non-biodegradable in aerobic and anaerobic degradation tests (Reis et al., 2020a).

Acetylated derivatives of chitosan, a polymer build of glucosamine monomers, show a correlation between the degree of acetylation and biodegradability. The degradation rate increases with an enhancement of the acetylation degree *in vitro* and *in vivo* (Yang et al., 2007). Responsible for the degradation, in that case, was lysozyme, which degraded the acetylated derivatives fully to their amino-glucose subunits or into chitosan oligosaccharides (depolymerization). However, the degradation by lysozyme works mainly due to the breakdown of O-glycosidic bonds. Since the herein investigated compounds have *N*-glycosidic bonds, it is questionable if the correlation of acetylation and biodegradation can be transferred to the glucosamine fluoroquinolone conjugates.

4.2. Degradation pathways

The difference in biodegradability of acetylated and deacetylated compounds has been verified via LC-MS analysis. The chromatograms showed that none of the deacetylated structures were degraded (see SI Tables S1-S19). The partial degradation of acetylated compounds however was verified by the detection of products of degradation via LC-MS (Table 2). While the underlying basic fluoroquinolone backbone seems to influence the absolute degradation values after 28 d (Fig. 2a), the resulting metabolites followed the same pattern of stepwise deacetylation for all investigated acetylated compounds. Since the peaks of the TPs with one or two removed acetyl groups were higher after 5 resp. 15 d of the degradation experiment than after 48 d (Fig. 3), it looks like the degradation pathway follows a stepwise removal and degradation of acetyl groups until the whole acetylated glucose group has been released, resulting in *N*-acetyl-fluoroquinolone-3-carboxamide derivatives as persistent degradation products.

Biodegradation via microorganisms usually follows the patterns of a stepwise partial degradation until it leads to an intermediate product, which would enter the central pathway of metabolism (Boethling et al., 2007). The biodegradability study of diatrizoic acid by Haiss and Kümmerer (2006) did show, that de-acetylation is a common step in biodegradation processes, resulting in some oxygen consumption by microorganisms but not in the full mineralization of the compound. The deacetylation of sulfonamide metabolites in the environment has also been observed (Göbel et al., 2005). The predicted degradation pathway by the EAWAG-PPS/enviPath also showed a difference between acetylated and deacetylated substances (Figs. S1 and S2). The main degradation pathway for the acetylated glucosamine derivatives was predicted as stepwise deacetylation, which could be catalyzed by carboxylic ester hydrolases (EC 3.1.1). The removal of the whole glucose side chain, resulting in the measured metabolites, may be mediated by demethylases (EC 1.13.12 or EC 1.14.13), hydrolases (EC 3.3.2) or dehydrogenases (EC 1.5.99), based on the rules in the enviPath database. According to the predictions, the generation of a carboxylate group is necessary before a further degradation of the deacetylated compounds can happen. This step would be catalyzed by isomerases (EC. 5.3.99). It could be possible that this group of enzymes was not present in the inoculum, which may be an explanation for why no degradation happened for the deacetylated compounds. However, further research is needed to back up this hypothesis.

There are multiple factors influencing the biodegradability of molecules, like their solubility, toxicity or activity against degrading microorganisms, adsorption to compartments, their transportation into the cell, the available enzymes, and so on (Boethling et al., 2007). Water-soluble substances usually biodegrade faster than insoluble ones. The latter tend to adsorb usually to solid phases. Biodegradation is more or less like a “black box” since the microorganisms present in an environmental sample such as wastewater effluent or sludge and related enzymes are not fully known and of course depend on the location, season, pre-exposure of the inoculum, etc. (Thouand et al., 2011). This makes it challenging to understand which enzymes could be responsible for the degradation and generation of different metabolites. Further research, for example with single strain degradation experiments, could help to better understand the underlying pathways for the herein studies

substances.

Since fluoroquinolones are very persistent, it is not surprising that the remaining Quinolone-3-carboxamide-analogs of compounds **1**, **3**, **5,** and **7** were not further degraded. However, the investigation of Quinoline-3-carboxamide in the CBT did show a partial transformation of the substance to Quinoline carrying a carboxylic acid group at position 3. Therefore, it might be even possible that the resulting degradation products are transformed back to corresponding fluoroquinolones after the elimination of the glucosamine moiety. However, most of the resulting degradation products still contained an additional acetyl group at the piperazine ring, compared to their fluoroquinolone counterparts.

4.3. Application in the context of BbD

BbD intends to design molecules, which are active and stable during application but can be fully mineralized once they are released into the environment. Mohammed et al. (2019, 2020) did already show that the newly developed fluoroquinolone conjugates have been stable and active against several pathogenic microorganisms *in vitro*. The herein presented results suggest that the acetylated compounds can be partially degraded by environmental microorganisms. It has also been shown that the kinetic of the degradation is rather slow, ensuring that the substances will not be metabolized too fast in the human body. Since acetylation at the piperazine ring is one of the most common fluoroquinolone transformations, resulting in a decrease in antimicrobial activity (Reis et al., 2020b), those resulting products still may be less harmful in the environment and may create less selection pressure. However, it is crucial to further investigate the behavior and toxicological profile of potential dead-end transformation products.

The usage of acetylated and deacetylated glucosamine substitutes to enhance the activity of APIs on one hand and to increase their environmental biodegradability, on the other hand, could be a promising tool for the benign design of chemicals and pharmaceuticals. This could be used to implement defined breaking points into a molecule so that molecules could be deactivated in the environment by environmental bacteria to reduce the selection pressure or other adverse effects.

Leder et al. (2021) published the result of another approach to fluoroquinolone re-design, resulting in a more environmentally friendly derivative. There, the cyclo-propyl substituent of Ciprofloxacin was replaced by a hemiaminal unit. This resulted in a molecule with a still high activity which can be partially degraded by abiotic hydrolysis after excretion. Even though there are multiple single strains known to partially degrade fluoroquinolones, their complete mineralization is usually not achieved because the quinolone nucleus is rarely cleaved (Reis et al., 2020b). The example by Leder et al. (2021) as well as the herein presented results do support this. In each case, just the partial degradation of side chains could be achieved. However, the combination of different side chains, which do not interfere with the activity of the molecule or even enhance it, but can be later broken down in the environment, could be an interesting approach for the further benign design of fluoroquinolones.

5. Conclusion

The presented work is embedded in the concept of green and sustainable pharmacy, which aims to implement end-of-life considerations already at the stage of drug development to reduce the concentration of pharmaceuticals in the environment. Pharmacophores and functional groups, which enhance the biodegradability of the whole molecule, are needed to be combined to new APIs. Fragment-based design can help to develop and design benign chemicals. However, the body of knowledge needed to design biodegradable APIs is still little.

Even though the underlying degradation mechanisms of the investigated substances could not be fully understood yet, the presented results can be used as one building block to develop and implement BbD further. The study results showed that a molecule is always more than the sum of its functional groups. As shown, even if the amide linkage between the glucosamine sugar and fluoroquinolone backbone is the same for all investigated substances, just for those with additional acetyl-groups a cleavage was possible. But it could also be shown that the targeted design for degradation does not necessarily mean a decrease in activity, since the investigated glycosylated derivatives were even more active than their corresponding fluoroquinolones, also against already resistant strains.

This study adds knowledge for the usage of glucosamine substituents for benign by design. However, further research is needed to better understand the underlying processes and to investigate how these results can be transferred to other classes of APIs. Single strain experiments could help to further elucidate the degradation pathways and to understand the differences between acetylated and deacetylated glucosamine conjugated compounds. As has been demonstrated in previous studies and herein too, *in silico* tools are important to complement experimental approaches.

Author contributions statement

Stefanie Lorenz: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization, Funding acquisition **Ghadeer Suaifan**: Resources, Visualization, Writing – review & editing, **Klaus Kümmerer**: Conceptualization, Resources, Writing – review & editing, Supervision

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136724.

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Supplementary Information

Designing benign molecules: The influence of O-acetylated glucosamine-substituents on the environmental biodegradability of fluoroquinolones

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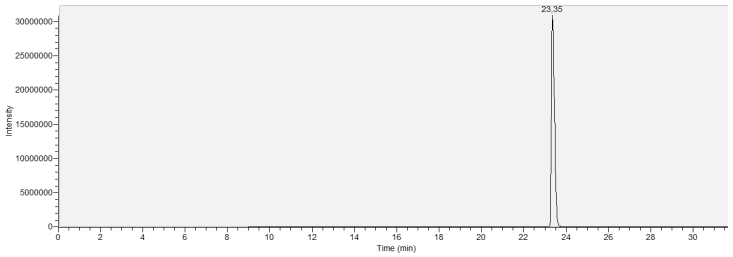
LC-MS results after biodegradation tests

Table S1: Chromatogram and MS results of compound 1

Chromatogram



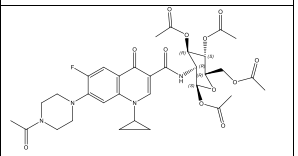
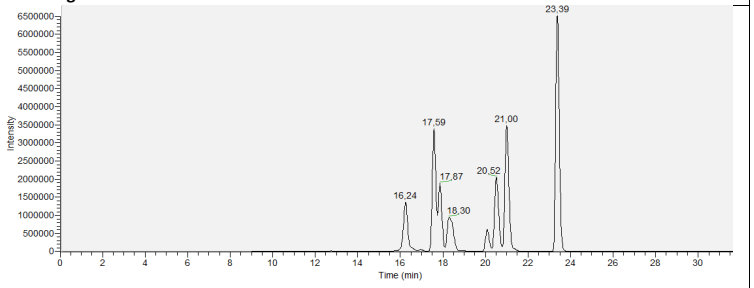
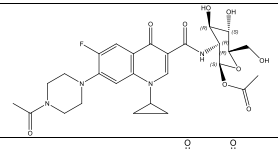
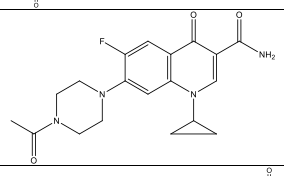
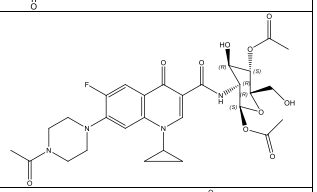
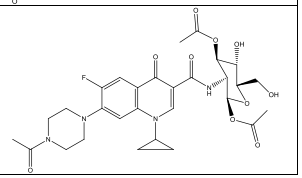
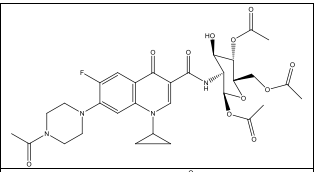
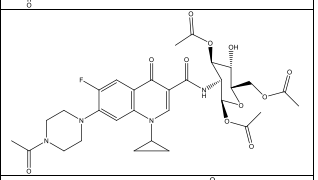
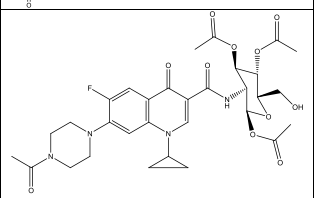
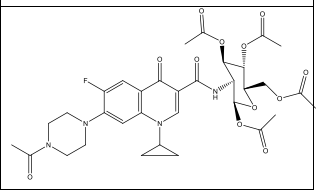
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
23.35	703.2617	643.2423 (- $C_2H_4O_2$) 583.2215 (- 2x $C_2H_4O_2$) 523.1992 (- 3x $C_2H_4O_2$)	$C_{33}H_{40}O_{12}N_4F$	-1.359	

Table S2: Chromatogram and MS results of compound 1 – 28 days after CBT

Chromatogram

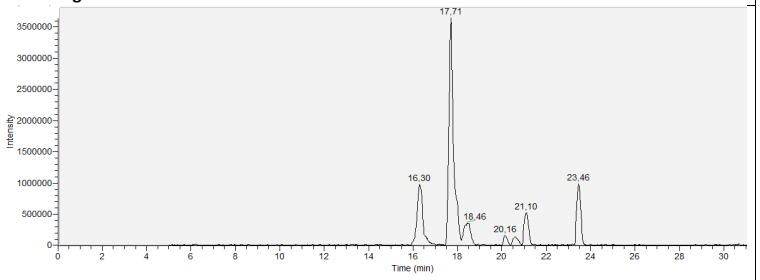


RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula[1]
16.24	577.2292	559.2209 (- H_2O) 541.2108 (- 2x H_2O)	$C_{27}H_{34}O_9N_4F$	-3.104	
17.59	373.1661	356.1406 (- H_3N)	$C_{19}H_{22}O_3N_4F$	-3.976	
17.87	619.2399	601.2314 (- H_2O) 541.2101 (- $C_2H_6O_3$)	$C_{29}H_{36}O_{10}N_4F$	-2.610	
18.30	619.2395	-	$C_{29}H_{36}O_{10}N_4F$	-3.305	
20.07	661.2498	601.2318 (- $C_2H_4O_2$) 541.2106 (- 2x $C_2H_4O_2$)	$C_{31}H_{38}O_{11}N_4F$	-3.555	
20.52	661.2527	-	$C_{31}H_{38}O_{11}N_4F$	0.876	
21.00	661.2523	643.2427 (- H_2O) 583.2215 (- H_2O + $C_2H_4O_2$) 523.2000 (- H_2O + 2x $C_2H_4O_2$)	$C_{31}H_{38}O_{11}N_4F$	0.225	
23.39	703.2767	643.2552 (- $C_2H_4O_2$) 583.2327 (- 2x $C_2H_4O_2$) 523.2100 (- 3x $C_2H_4O_2$)	$C_{33}H_{40}O_{12}N_4F$	19.899	

[1] The exact positions of the removed acetyl group(s) are not known. The shown structures are just for visualization.

Table S3: Chromatogram and MS results of compound 1 – 48 days after CBT

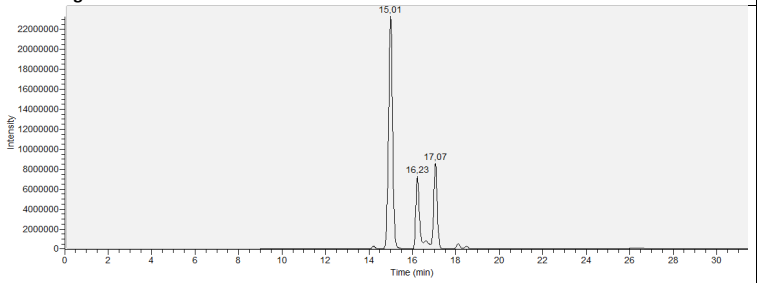
Chromatogram



RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)
16.30	577.2297	559.2208 (- H_2O) 541.2108 (- 2x H_2O)	$C_{27}H_{34}O_9N_4F$	-2.255
17.71	373.1664	356.1410 (- H_3N)	$C_{19}H_{22}O_3N_4F$	-3.092
18.46	619.2416	-	$C_{29}H_{36}O_{10}N_4F$	0.054
21.00	661.2518	643.2419 (- H_2O) 583.2208 (- H_2O + $C_2H_4O_2$) 523.1993 (- H_2O + 2x $C_2H_4O_2$)	$C_{31}H_{38}O_{11}N_4F$	-0.516
23.39	703.2629	643.2427 (- $C_2H_4O_2$) 583.2216 (- 2x $C_2H_4O_2$) 523.2998 (- 3x $C_2H_4O_2$)	$C_{33}H_{40}O_{12}N_4F$	0.291

Table S4: Chromatogram and MS results of compound 2

Chromatogram



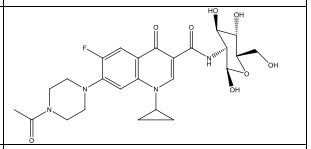
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
15.01	535.2205	517.2098 (- H_2O) 499.1996 (- 2x H_2O)	$C_{25}H_{32}O_8N_4F$	0.641	
16.23	439.1780	356.1400 (-83.04)			Unknown impurity
17.07	439.1778	356.1400 (-83.04)			Unknown impurity

Table S5: Chromatogram and MS results of compound 2 – 28 days after CBT

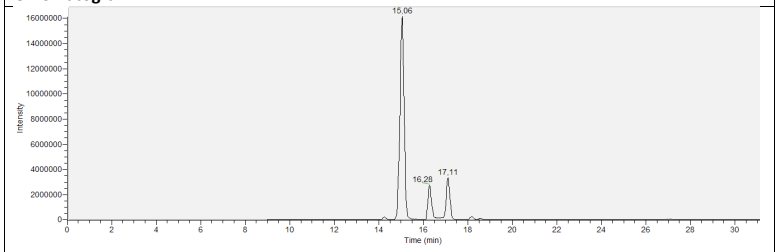
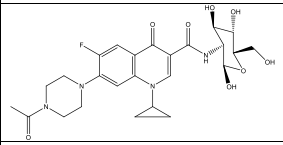
Chromatogram					
					
RT (min)	**[M+H]⁺**	**MS²**	**Sum formula**	**Delta (ppm)**	**Structural formula**
15.06	535.2207	517.2102 (- H_2O) 499.2000 (- 2x H_2O)	$C_{25}H_{32}O_8N_4F$	0.529	
16.28	439.1779	356.1405 (-83.04)			Unknown impurity
17.11	439.1775	-			Unknown impurity

Table S6: Chromatogram and MS results of compound 2 – after 48 days CBT

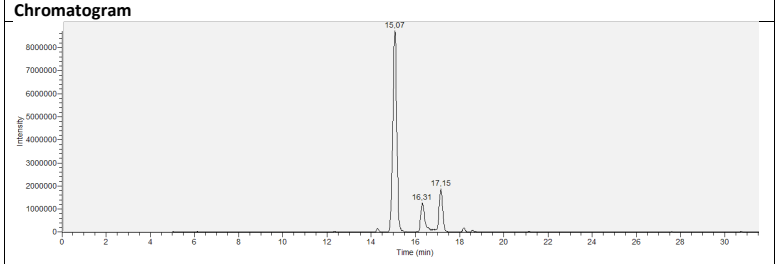
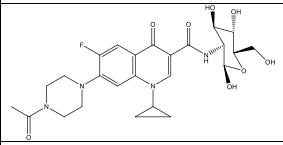
Chromatogram					
					
RT (min)	**[M+H]⁺**	**MS²**	**Sum formula**	**Delta (ppm)**	**Structural formula**
15.07	535.2202	517.2104 (- H_2O) 499.2003 (- 2x H_2O)	$C_{25}H_{32}O_8N_4F$	-0.387	
16.30	439.1773	356.1408 (-83.04)			Unknown impurity
17.14	439.1773	-			Unknown impurity

Table S7: Chromatogram and MS results of compound 3

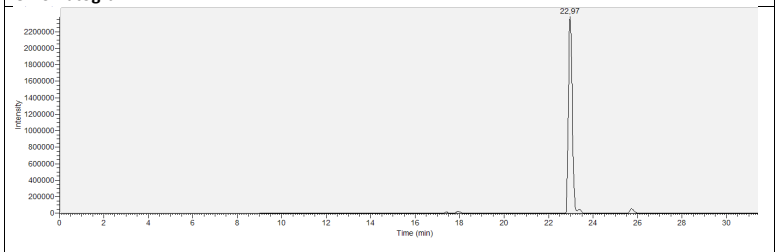
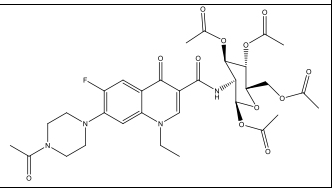
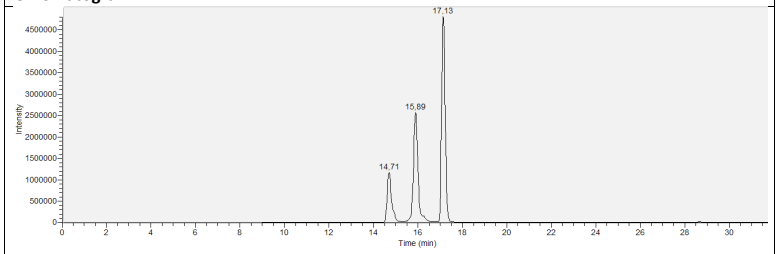
Chromatogram					
					
RT (min)	**[M+H]⁺**	**MS²**	**Sum formula**	**Delta (ppm)**	**Structural formula**
22.96	691.2771	631.2552 (- $C_2H_4O_2$) 571.2327 (- 2x $C_2H_4O_2$) 511.2106 (- 3x $C_2H_4O_2$)	$C_{32}H_{40}O_{12}N_4F$	20.866	

Table S7: Chromatogram and MS results of compound 3 – 28 days after CBT

Chromatogram



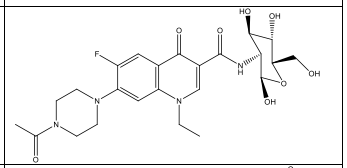
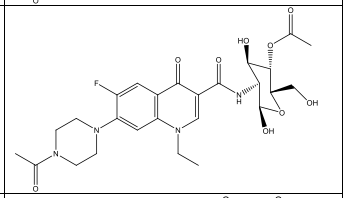
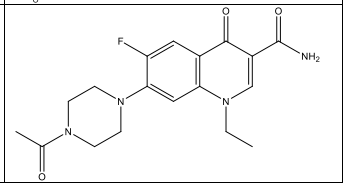
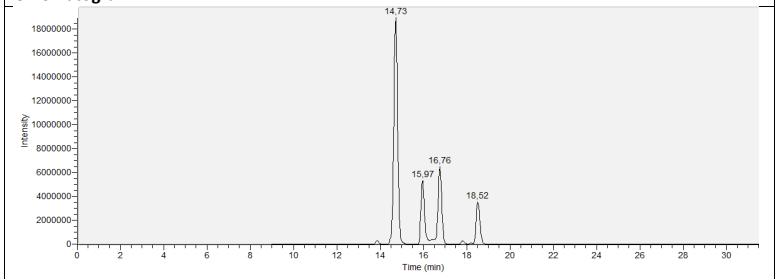
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula[1]
14.71	523.2293	505.2188 (- H_2O) 487.2081 (- 2x H_2O)	$C_{24}H_{32}O_8N_4F$	16.996	
15.89	565.2405	-	$C_{26}H_{34}O_9N_4F$	16.910	
17.14	361.1735	344.1469 (- NH_3)	$C_{18}H_{22}O_3N_4F$	16.242	

Table S8: Chromatogram and MS results of compound 4

Chromatogram



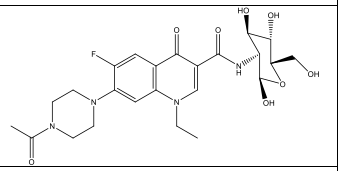
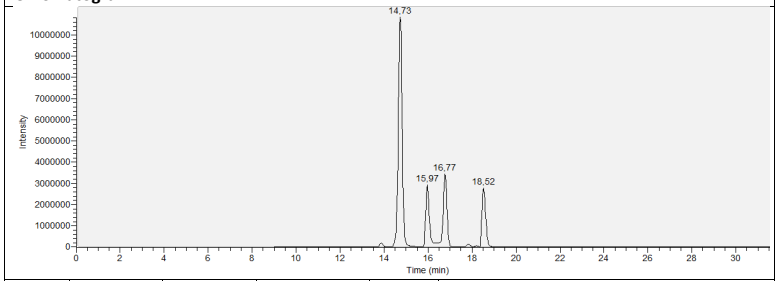
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
14.73	523.2208	505.2091 (- H_2O) 487.1989 (- 2x H_2O)	$C_{24}H_{32}O_8N_4F$	0.656	
15.97	427.1780	344.1399			Unknown impurity
16.76	427.1780	344.1399			Unknown impurity
18.52	390.1827	362.1507 344.1400			Unknown impurity

Table S9: Chromatogram and MS results of compound 4 – after 28 days CBT

Chromatogram



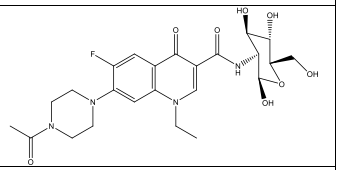
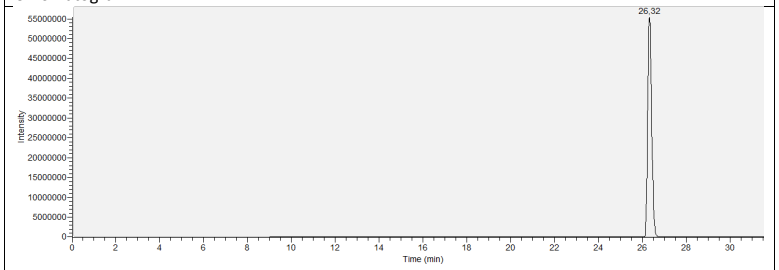
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
14.73	523.2208	505.2091 (- H_2O) 487.1989 (- 2x H_2O)	$C_{24}H_{32}O_8N_4F$	0.656	
15.97	427.1780	344.1399			Unknown impurity
16.77	427.1780	344.1399			Unknown impurity
18.52	390.1827	362.1507 344.1400			Unknown impurity

Table S10: Chromatogram and MS results of compound 5

Chromatogram



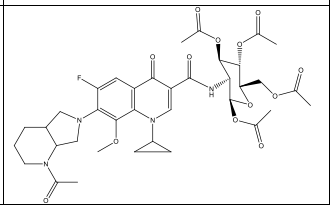
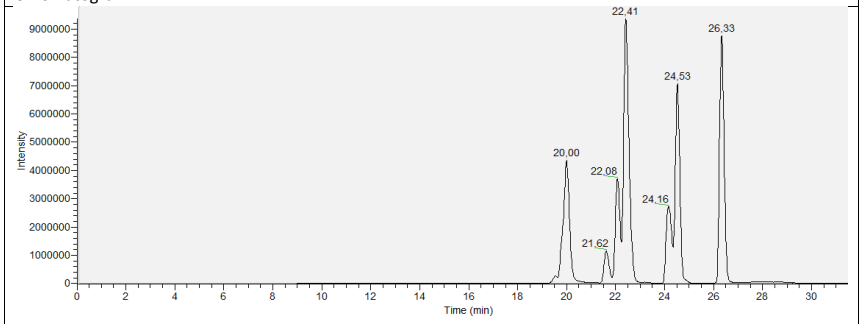
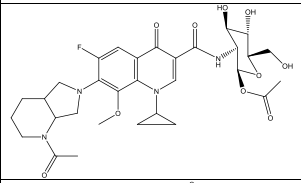
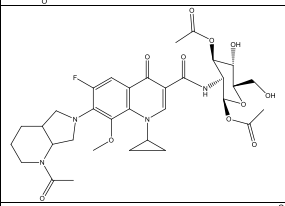
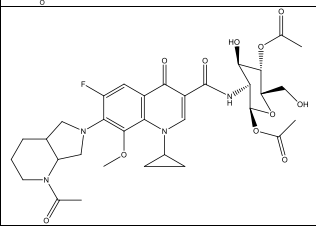
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
26.32	773.3088	713.2883 (- $C_2H_4O_2$) 653.2675 (- 2x $C_2H_4O_2$) 593.2442 (- 3x $C_2H_4O_2$)	$C_{37}H_{46}O_{13}N_4F$	5.560	

Table S11: Chromatogram and MS results of compound 5 – 28 days after CBT

Chromatogram



RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula[1]
20.00	647.2747	-	$C_{31}H_{40}O_{10}N_4F$	2.802	
21.62	689.2842	671.2752 (- H_2O) 629.2638 (- $C_2H_4O_2$) 611.2533 (- H_2O + $C_2H_4O_2$)	$C_{33}H_{42}O_{11}N_4F$	1.116	
22.08	689.2847	-	$C_{33}H_{42}O_{11}N_4F$	1.826	

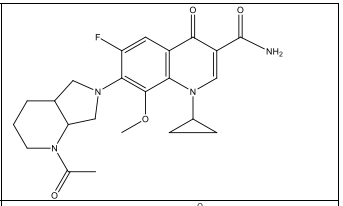
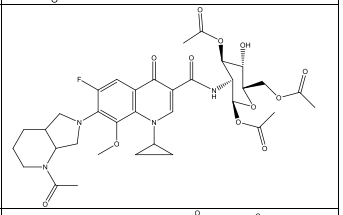
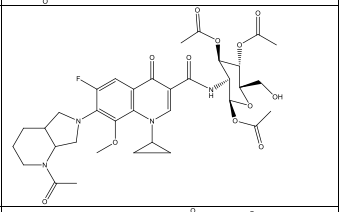
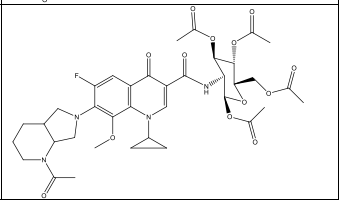
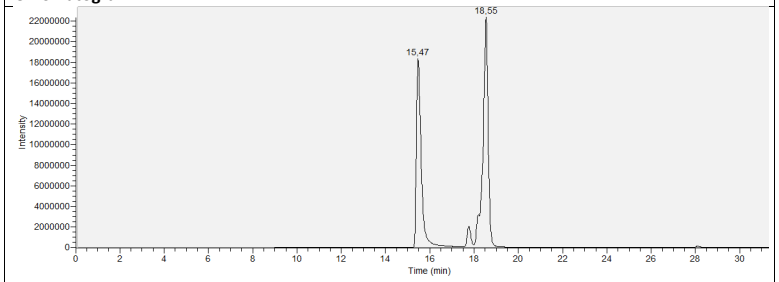
22.41	443.2088	426.1825 (- NH_3)	$C_{23}H_{28}O_4N_4F$	-1.418	
24.16	731.2968	-	$C_{35}H_{44}O_{12}N_4F$	3.876	
24.53	731.30	671.28 (- $C_2H_4O_2$) 611.25 (- 2x $C_2H_4O_2$)	$C_{35}H_{44}O_{12}N_4F$	2.744	
26.33	773.3080	713.2872 (- $C_2H_4O_2$) 653.2651 (- 2x $C_2H_4O_2$) 593.2432 (- 3x $C_2H_4O_2$)	$C_{37}H_{46}O_{13}N_4F$	4.448	

Table S12: Chromatogram and MS results of compound 6

Chromatogram



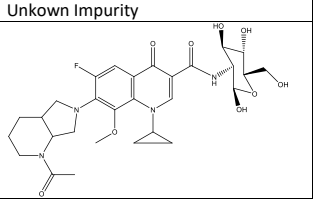
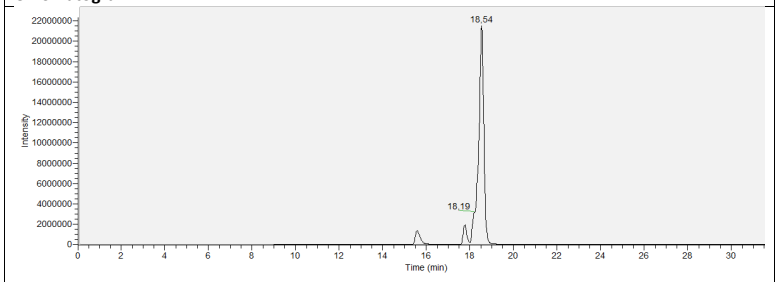
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
15.47	241.2036	342.2630			Unkown Impurity
18.55	605.2625	587.2523 (- H_2O) 569.2415 (- 2x H_2O) 509.2199(- 2x H_2O + $C_2H_4O_2$)	$C_{29}H_{38}O_9N_4F$	0.377	

Table S13: Chromatogram and MS results of compound 6 – 28 days after CBT

Chromatogram



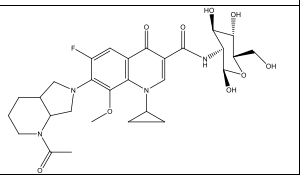
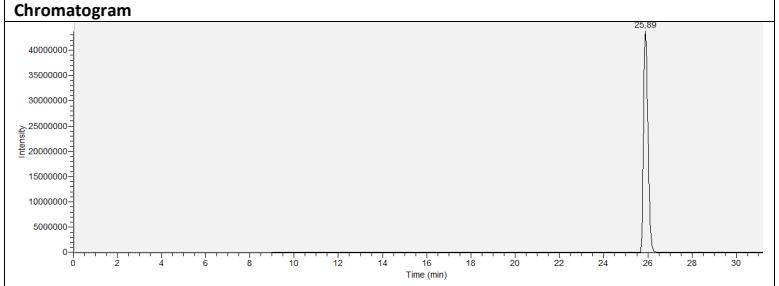
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
18.55	605.2625	587.2523 (- H_2O) 569.2415 (- 2x H_2O) 509.2199(- 2x H_2O + $C_2H_4O_2$)	$C_{29}H_{38}O_9N_4F$	0.377	

Table S14: Chromatogram and MS results of compound 7

Chromatogram



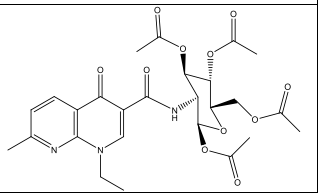
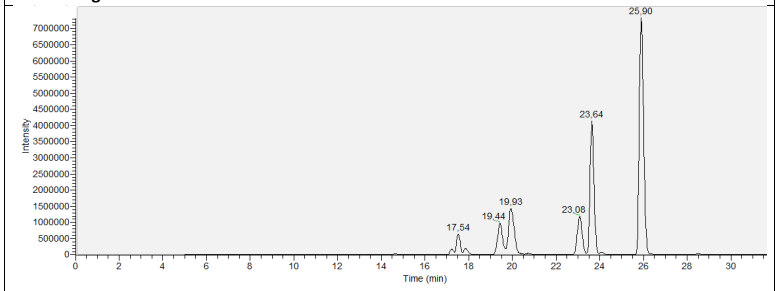
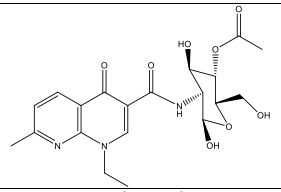
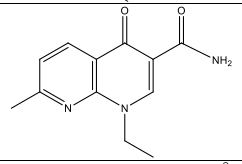
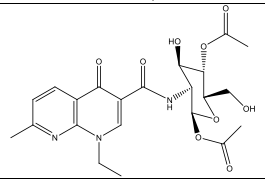
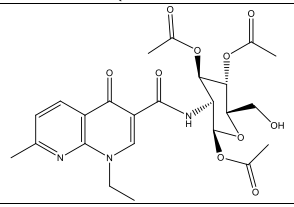
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
25.89	562.2037	502.1825 (- $C_2H_4O_2$) 442.1613 (- 2x $C_2H_4O_2$) 382.1402 (- 3x $C_2H_4O_2$)	$C_{26}H_{32}O_{11}N_3$	1.399	

Table S15: Chromatogram and MS results of compound 7 – 28 days after CBT

Chromatogram					
					
RT (min)	**$[M+H]^+$**	**MS^2**	**Sum formula**	**Delta (ppm)**	**Structural formula[1]**
17.5	436.1719	-	$C_{20}H_{26}O_8N_3$	0.024	
19.4	232.1082	215.0813 (- H_3N)	$C_{12}H_{14}O_2N_3$	-1.816	
19.9	478.1822	-	$C_{22}H_{28}O_9N_3$	-0.699	
23.08	520.1927	502.1825 (- H_2O) 460.1718 (- $C_2H_4O_2$) 382.1402 (- H_2O + $C_2H_4O_2$)	$C_{24}H_{30}O_{10}N_3$	-0.709	

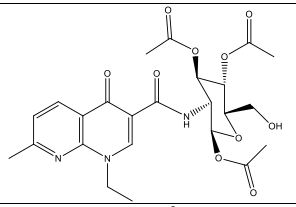
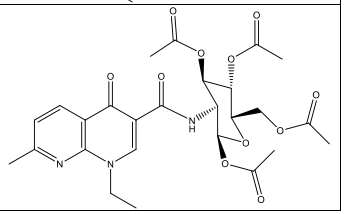
23.63	520.1927	502.1825 (- H_2O) 460.1718 (- $C_2H_4O_2$) 382.1402 (- H_2O + $C_2H_4O_2$)	$C_{24}H_{30}O_{10}N_3$	-0.709	
25.9	562.2031	502.1823 (- $C_2H_4O_2$) 442.1611 (- 2x $C_2H_4O_2$) 382.1399 (- 3x $C_2H_4O_2$)	$C_{26}H_{32}O_{11}N_3$	-0.985	

Table S16: Chromatogram and MS results of compound 8

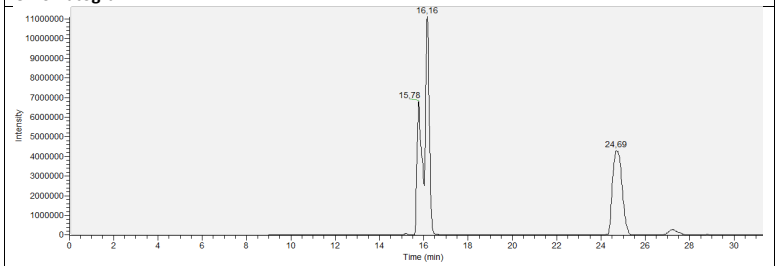
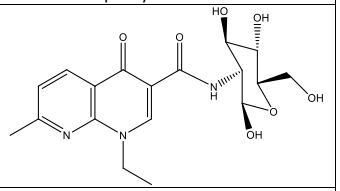
Chromatogram					
					
RT (min)	**$[M+H]^+$**	**MS^2**	**Sum formula**	**Delta (ppm)**	**Structural formula**
15.78	218.2114				Unknown Impurity
16.16	394.1611	376.1504 (- H_2O) 358.1395 (- 2x H_2O)	$C_{18}H_{24}O_7N_3$	-0.175	
24.69	354.2849				Unknown Impurity

Table S17: Chromatogram and MS results of compound 8 – after 28 days

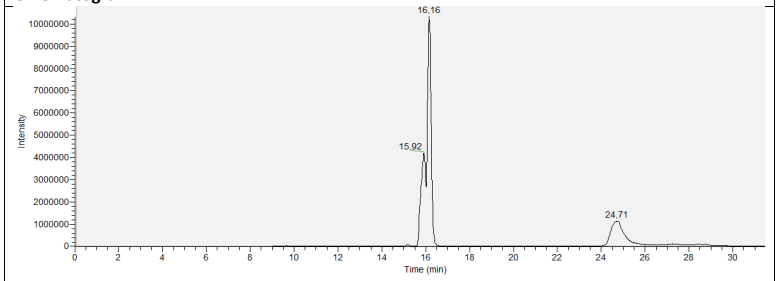
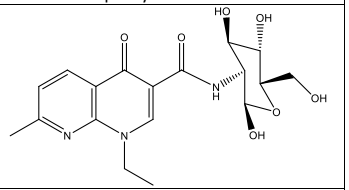
Chromatogram					
					
RT (min)	**$[M+H]^+$**	**MS^2**	**Sum formula**	**Delta (ppm)**	**Structural formula**
15.78	218.2114				Unknown Impurity
16.16	394.1611	376.1504 (- H_2O) 358.1395 (- 2x H_2O)	$C_{18}H_{24}O_7N_3$	-0.175	
24.69	354.2849				Unknown Impurity

Table S18: Chromatogram and MS results of Quinoline-3-carboxamide

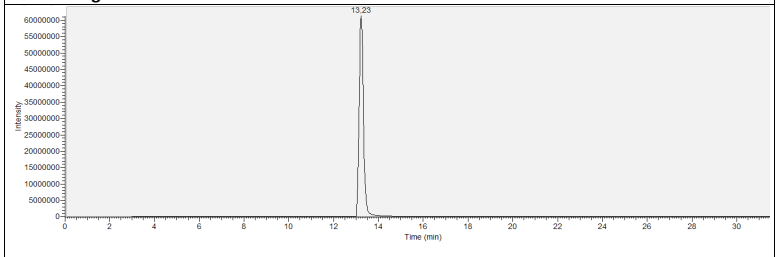
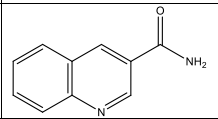
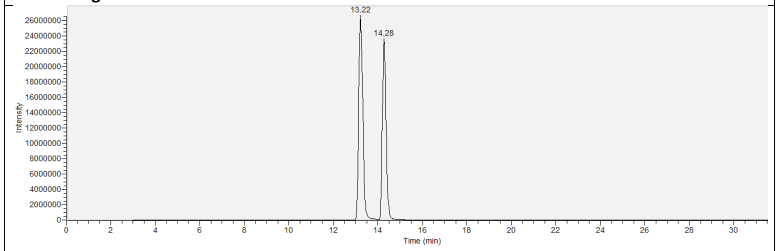
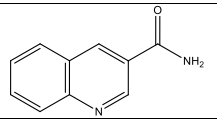
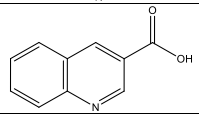
Chromatogram					
					
RT (min)	**$[M+H]^+$**	**MS^2**	**Sum formula**	**Delta (ppm)**	**Structural formula**
13.2	173.0708	-	$C_{10}H_9ON_2$	-4.032	

Table S19: Chromatogram and MS results of Quinoline-3-carboxamide – after 28 days

Chromatogram



RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
13.2	173.0708	-	$C_{10}H_9ON_2$	-4.032	
14.3	174.0550	-	$C_{10}H_8O_2N$	-2.950	

EAWAG BBD predictions

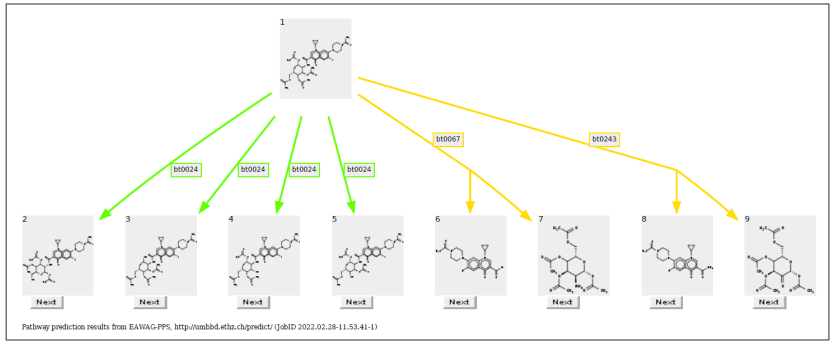


Figure S1: Predicted degradation pathway from EAWAG-PPS for compound 1 (as representative for acetylated compounds)

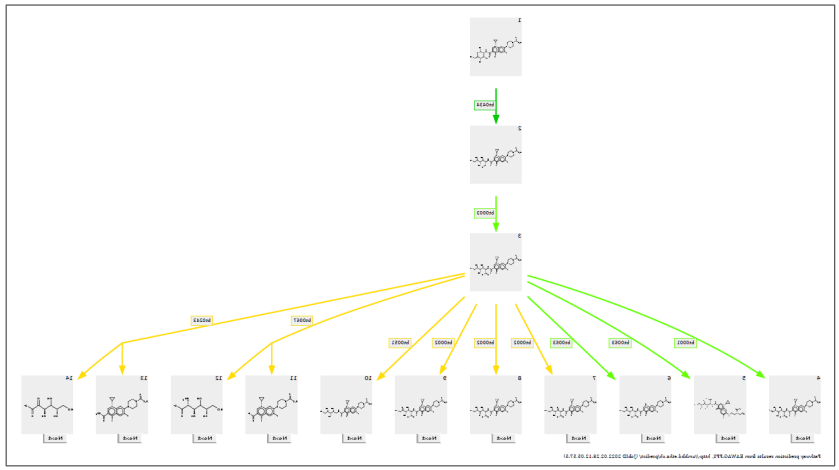


Figure S2: Predicted degradation pathway from EAWAG-PPS for compound 2 (as representative for non-acetylated compounds)

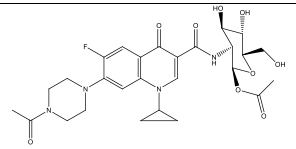
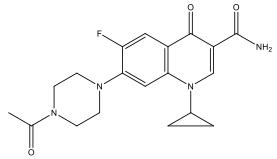
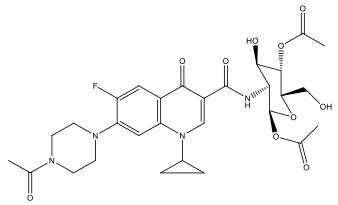
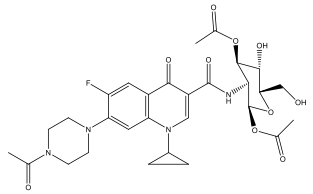
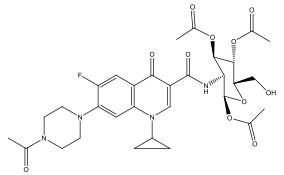
HESI conditions

Table S20: HESI source and ion trap mass analyzer parameters

HESI source	
Heater Temp.	350 °C
Sheath gas flow rate	49 arb
Aux gas flow rate	5 arb
Sweep gas flow rate	0 arb
Spray Voltage	3.5 kV
Capillary Temp.	275 °C
Capillary Voltage	11.5 V
Tube Lens	70 V
Ion optics	
Multipole 00 Offset	-1.75 V
Lens 0 Voltage	- 5.5 V
Multipole 0 Offset	- 5 V
Lens 1 Voltage	- 34 V
Gate Lens Voltage	- 74 V
Multipole 1 Offset	- 6 V
Multipole RF Amplitude	660 V p-p
Front Lens	- 7 V

log P predictions for TPs of compound 1

Table S21: Measured retention times and m/z-ratios of compound **1** transformation products and their predicted consensus log P by SwissADME (* the proposed structural formulas are just one possible conformation of the resulting transformation products)

RT (min)	**m/z**	**predicted log P**	**Structural formula of resulting TPs***
16.24	577.2292	-0.24	
17.59	373.1661	1.11	
17.87	619.2399	0.26	
18.26	619.2395	0.26	
20.07	661.2498	0.38	

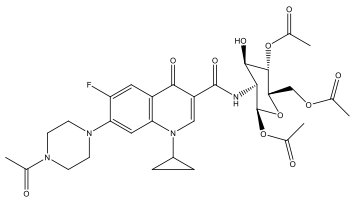
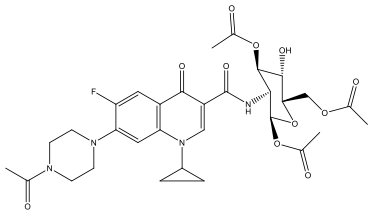
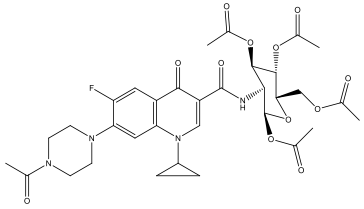
20.52	661.2527	0.82	
21.00	661.2523	0.94	
23.39	703.2767	1.38	

Table S22: Biodegradation results after 28 days under MRT conditions

	Degradation [%]	**Tox control [%]**	**Sterile control [%]**
	(n=2)	(n=1)	(n=1)
Compound 5	28.2 ± 5.2	37.6	15.0
Compound 6	15.1 ± 5.4	34.8	11.3

Publikation 3

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Identification of environmentally biodegradable scaffolds for the benign design of quinolones and related substances

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ABSTRACT

Many micropollutants, such as pharmaceuticals and other chemicals, are present globally in the aquatic environment. During effluent treatment and in the environment, transformations result in new chemicals of often unknown structure, fate, and toxicity. The design of chemicals, that can be fully mineralized or broken down into non-hazardous fragments, is considered a green chemistry approach avoiding such problems from the very beginning ("benign by design"). N-heterocycles are central lead scaffolds for many important chemicals and pharmaceuticals such as quinolines, isoquinolines, quinolones, fluoroquinolones, naphthyridones, and quinazolinones. Understanding their environmental biodegradability is mandatory for the design of greener derivatives. While the biodegradability of simple quinolines has already been reported in the literature, information on more complex azaarenes and other N-heterocycles is rather scarce.

The goal of this study was to investigate the ready biodegradability of several N-heterocycles to identify biodegradable lead scaffolds. LC-HRMS studies were performed to identify possible metabolites. Out of the 84 tested substances, only 14 were readily biodegradable in either the closed bottle test (OECD 301D) or the manometric respiratory test (OECD 301F). Hydroxylation at the C2 position increased the biodegradation level of the quinolines generally and tolerated even fluorine in the molecule. Moreover, 4-oxo-1,4-dihydroquinoline-3-carboxylic acid has been tested as readily biodegradable. It is an important bioactive lead scaffold with many different applications, i.e., in antibiotics. All other quinolones containing the β-keto-carboxylic acid moiety were persistent, including their bioisosteres. The identified biodegradable scaffolds can be used to design new environmentally biodegradable molecules following green fragment-based design.

1. Introduction

The presence of micropollutants in the environment and their persistence against degradation processes, either in the environment or sewage treatment processes, is a serious threat to the environment and human health. Pharmaceuticals, e.g. antibiotics can be found already in most environmental compartments (Schwarzenbach et al., 2006; Kümmerer, 2009a, 2009b). Antibiotics in the environment contribute to the development of resistant microorganisms (Gullberg et al., 2011). Consequences are that more and more drugs will become inefficient to treat infections and therefore leading to serious health problems (Davies and Davies, 2010).

Green and sustainable chemicals and processes are considered to be the foundation for a sustainable society. However, it is challenging to design greener and more sustainable molecules (Zimmerman et al., 2020). One important property of such molecules is

Abbreviations: QSAR, Quantitative Structure Activity Relationship; PLS, Partial Least Squares.

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their, in the best case, complete biodegradability, i.e. full mineralization as it directly relates to the effects and fate of the compound in the environment. Biodegradation in general describes the break down of organic substances by microorganisms. Mineralization is the process of complete degradation into a compound's mineral components, i.e. carbon dioxide and water. Design for degradation is addressed by the 10th principle of *Green Chemistry*. It states that chemicals should be designed to break down in the environment into innocuous products, which means either full mineralization or decomposition into non-hazardous fragments, after their application. This is also an essential element of 'Benign By Design chemistry' (Anastas, 1994). However, there are just a few examples of completely biodegradable molecules, which are specially designed for this by intention (Kümmerer, 2019).

Benign by design is based on the assumption that even small changes in a chemical's structure can lead to significant changes in its properties and behavior, including environmental biodegradability (Leder et al., 2015). However, sometimes the alteration of substituents leads only to partial degradation of molecules (Leder et al., 2021). This shows how important the search for readily and fully biodegradable lead scaffolds is to implement the concept of Benign by Design further.

Quinolines (Fig. 1) are common environmental pollutants emitted by the processing of fossil fuel and coal gasification. However, quinolines and isoquinolines are also versatile scaffolds in many natural (e.g., morphine, papaverine, quinine) and synthetic (e.g., chloroquine, elvitegravir) bioactive molecules. Besides bioactive molecules, quinolines are also important bulk chemicals and intermediates in the industry. Among these scaffolds, especially 4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Fig. 1, quinoline scaffold with hydrogen at R_1-R_8) has received increased popularity as a synthetic lead scaffold in many pharmaceuticals with a broad spectrum of biological activities. The 4-oxo-1,4-dihydroquinoline-3-carboxylic acid scaffold was used to design potent inhibitors of the human protein kinase CK2, which has become of increased therapeutic interest since it participates in cancer proliferation, viral infections, and inflammatory failures (Golub et al., 2006). An amide analog of this scaffold was used to develop CB_2-selective cannabinoid receptor ligands (Stern et al., 2007). The most prominent use of this scaffold is in the design of quinolinone and fluoroquinolone antibiotics. These are an important group of antibiotics, that bloc bacterial DNA synthesis by inhibiting the enzymes topoisomerase II and IV in many pathogenic organisms. However, due to biological persistence in sewage treatment plants and their potency, fluoroquinolones are of high environmental concern as they can induce selection pressure promoting the dissemination of resistant organisms (Gartiser et al., 2007; Bergheim et al., 2015; Chow et al., 2021). For the prominent fluoroquinolone ciprofloxacin this selection pressure was already verified at 100 ng L^{-1}, a typical concentration of ciprofloxacin in surface waters (Gullberg et al., 2011; Ferrando-Climent et al., 2014).

Quinazolinones (Fig. 1) received attention as alternative topoisomerase inhibitors which are unaffected by common fluoroquinolone-resistant mutations (Drlica et al., 2014; Tran et al., 2007; Aldred et al., 2013). 3-aminoquinazolidiones already showed high activity against quinolone-resistant *Bacillius anthracis*, yet prevention of cross-reactivity against human topoisomerase IIα remains challenging (Aldred et al., 2013).

While the biodegradability of simple quinolines has already been reported in the literature, information on more complex azaarenes is rather scarce. To improve the data basis of biodegradable N-heterocycles, to identify biodegradable lead scaffolds for a green design, and to derive structure biodegradability relationships (SBR), the ready biodegradability of 84 N-heterocycles was examined in OECD standard tests. Several substance groups (quinolines, quinolones, 4H-pyrido[1,2-*a*]pyrimidines, isoquinolines, fluoroquinolones, naphthyridones, and quinazolidiones, Fig. 1) were investigated employing a modified Closed Bottle Test (CBT, OECD 301D) and the manometric respiratory test (MRT, OECD 301F). The focus of this study were N-heterocycles, which contain the chelating β-keto carboxylic acid pharmacophore to design mineralizable topoisomerase II and IV inhibitors. In addition, the influence of halogenation, alkylation, hydroxylation, and etherification on the environmental biodegradability of these scaffolds was studied. Based on the biodegradation results the study derives rules to design environmentally better biodegradable N-heterocycles, which can be used to design new biodegradable pharmaceuticals and chemicals in general.

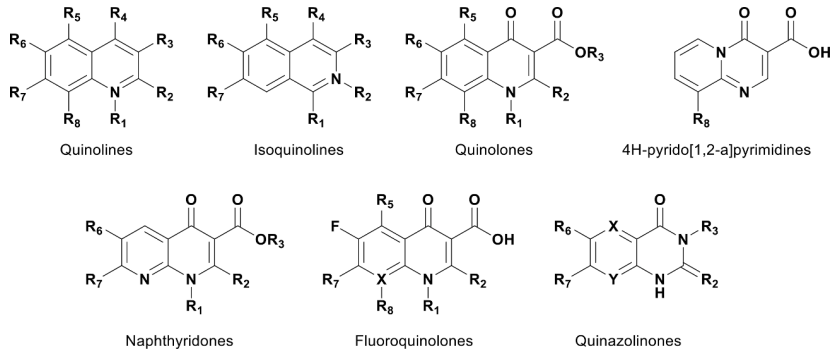


Fig. 1. Substructures of the investigated N-heterocycles.

2. Materials and methods

2.1. Chemicals

The tested substances were purchased from Sigma Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), ASCA GmbH (Berlin, Germany), TCI (Eschborn, Germany), Chempur (Karlsruhe, Germany), Santa Cruz Biotechnology (Heidelberg, Germany), Biozol (Eching, Germany), ABCR GmbH (Karlsruhe, Germany), Appollo Scientific Ltd (Bredbury, UK) and Key Organics (Camelford, UK) all with >95% purity. The formulas of all N-heterocycles are listed in Table S7. Acetonitrile was purchased from VWR (Darmstadt, Germany). Sodium azide and sodium acetate were purchased from Sigma Aldrich (Steinheim, Germany). Formic acid was purchased from Merck KGaA (Darmstadt, Germany).

2.2. Ready biodegradability – closed botte test (CBT) and manometric respiratory test (MRT)

Ready biodegradability was evaluated by an optode-based (Fibox 3, PreSens, Regensburg, Germany) CBT (OECD 301D) (Friedrich et al., 2012; OECD, 1992) and an MRT (OECD 301F) using the OxiTop system (OC110-System, WTW GmbH, Weilheim, Germany) (OECD, 1992). The initial test concentration in the CBT and MRT corresponded to 5 mg L^{-1} and 30 mg L^{-1} theoretical oxygen demand ($ThOD_{NH3}$), respectively, with or without considering nitrification ($ThOD_{NO3-}$) in the MRT. As inoculum source 2 drops L^{-1} (~200 μL L^{-1}) and 80 mL L^{-1} of the secondary effluent derived from a municipal sewage treatment plant (AGL Abwasser, Grün & Lüneburg Services GmbH, Lüneburg, Germany, 325000 eq. inhabitants) were used for the CBT and MRT, respectively. Before using, the inoculum was filtered through a paper filter while the first 200 mL were discharged. The filtrate was maintained aerobic. For some substances, an additional MRT was performed utilizing 30 mg suspended solids L^{-1} of activated sludge from the same sewage treatment plant as mentioned above. The sludge was washed three times with tap water before use to lower the organic background. The mineral media was prepared following the corresponding OECD guidelines 301D and 301F (OECD, 1992). The tests consisted of "blank", "quality control" (sodium acetate), "test series" and "toxicity control". In addition, the MRT comprised an additional "sterile control" containing 320 mg L^{-1} NaN_3 to prevent microbial growth and monitor abiotic degradation. All tests were performed in quadruplets at 20 ± 1 °C in the dark to inhibit photosynthesis and degradation by photolysis. Samples were taken on day 0 and day 28. Samples from MRT were filtered through a 0.45 μm PES (Polyethersulfone) membrane filter (Macherey-Nagel, Düren, Germany) before being stored at −20 °C for further analysis.

2.3. Identification of metabolites by LC-MS^n

Separation of transformation products resulting from incomplete mineralization after 28 days of biodegradation testing was done by ultra-high performance liquid chromatography (HPLC, Ultimate 3000, Thermo Scientific, Dreieich, Germany). Separation was carried out on a NUCLEODUR C18 Gravity (125 × 4 mm, 5 μm, Macherey Nagel, Germany) column equipped with an EC 4/3 NUCLEODUR C18 Gravity guard column (Macherey Nagel, Germany) using gradient mode. The column temperature was maintained at 25 °C and the flow rate was set to 0.5 mL min^{-1}. The gradient consisted of 0.1% formic acid (A) and acetonitrile (B). The injection volume was 25 μL and 15 μL for CBT and MRT samples, respectively. The HPLC was coupled to a high-resolution mass spectrometer (LTQ-Orbitrap-XL, Thermo Scientific, Dreieich, Germany) equipped with a HESI source. Information on the operating parameters of the HESI source and mass analyzer can be found in the SI (Table S1).

2.4. Field-based QSAR model and contour maps

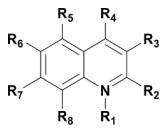
To establish a structure biodegradability relationship, a model has been established with the field-based QSAR module in Maestro, Version 12.8.117 (Release 2021-2) from Schrödinger (Inc., New York). A merged dataset of CBT and MRT results was created, using the highest degradation value of each substance. All negative degradation values were set to zero. Results with a standard deviation above 30% have not been used for model generation. The merged dataset contained 79 substances in total. The 3D structures have been prepared with the LigPrep module (force field method OPLS4), without any ionization or tautomers. In addition, all structures were aligned based on the largest common Bemis-Murcko scaffold to Quinoline (**1**) as a reference. A QSAR model was created using the biodegradation values in percentage and the prepared and aligned structures. A test set with 25% of the entries was randomly selected. Based on the QSAR statistics (Table S5) the optimal number of PLS factors has been selected. The field-based QSAR model was re-calculated with the chosen number of PLS factors and the whole dataset to create the contour maps for the different field fractions.

3. Results and discussion

The OECD 301D and OECD 301F tests differ in the used substance concentration and the bacterial density and diversity of the inoculum. The CBT is the strictest test, with a low bacterial density and diversity (1–100 × 10^4 cells L^{-1}), representing the conditions in surface waters. For the MRT on the other hand the inoculum has a higher α- and β-density of microorganisms (1000–10000 × 10^4 cells L^{-1}) (OECD, 1992). This often leads to higher biodegradation levels, compared to CBT.

The majority of the tests have been valid according to the OECD guidelines (OECD, 1992). However, some of the MRT results did show a high variation between the different test samples (Table 1, 2 and 5). This was usually the case for substances with a long lag phase. For example, for **16** the lag phase was at least 20 days long, which shows that an adaption of the bacteria was necessary first. The duration of this lag phase might vary in the different flasks, resulting in a high variation between the test samples after 28 days (Fig. S1). Substances are classified as readily biodegradable if they reach a biodegradation level of at least 60% in one of the performed OECD 301 tests after 28 days.

Table 1
Structures and biodegradability of quinoline scaffolds in the CBT and MRT (n = 4).



									Biodegradation level [% $ThOD_{NH3}$]	
Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	CBT	MRT
1		H	H	H	H	H	H	H	80 ± 9.6	N.d.
2	OH	H	H	H	H	H	H	H	1 ± 3.7	−8 ± 11.3[c]
3		OH	H	H	H	H	H	H	64 ± 2.1	84 ± 7.4[a]
4		SH	H	H	H	H	H	H	−2 ± 7.1	−14 ± 11.4[c]
5		NH_2	H	H	H	H	H	H	1 ± 1.5	−8 ± 8.0[c]
6		OH	H	H	H	F	H	H	30 ± 18.1	75 ± 10.6[c]
7		H	OH	H	H	H	H	H	1 ± 0.8	−2 ± 3.6
8		H	H	OH	H	H	H	H	69 ± 6.0	67 ± 10.8
9		COOH	H	OH	H	H	H	H	89 ± 4.6	N.d.
10		H	NO_2	OH	H	H	H	H	1 ± 2.5	2 ± 7.2
11		H	H	H	OH	H	H	H	1 ± 1.1	−10 ± 9.0
12		H	H	H	H	OH	H	H	56 ± 5.3	68 ± 4.0
13		H	H	H	H	F	H	H	6 ± 2.5	85 ± 5.3[b]
14		H	H	H	H	H	OH	H	35 ± 2.1	12 ± 4.6
15		H	H	H	H	H	H	OH	−3 ± 5.6	1 ± 15.5
16		H	H	H	H	H	H	F	−3 ± 7.9	33 ± 63.8[a,b]
17		OH	H	OH	H	H	H	H	71 ± 4.8	93 ± 4.2[a]
18		OH	H	H	H	OH	H	H	64 ± 7.1	86 ± 13.5[a]
19		OH	H	H	H	H	OH	H	46 ± 13.7	77 ± 14.9[a]
20		OH	H	H	H	H	H	OH	1 ± 4.1	86 ± 14.1[a]

[a] Based on the $ThOD_{NO3-}$.
[b] Utilizing activated sludge as an inoculum source.
[c] n = 2.

3.1. Ready biodegradability of quinolines

In total 11 out of 20 tested quinolines were readily biodegradable in either CBT or MRT (Table 1). While in the CBT only 6 structures could be classified as readily biodegradable following the OECD guideline, 5 additional quinolines fulfilled the required criteria of more than 60% degradation level after 28 days in the MRT (OECD, 1992).

None of the investigated quinolines were considered toxic to the applied microorganisms according to the guidelines (OECD, 1992). Nevertheless, inhibition of biodegradation of sodium acetate in the toxicity controls by the known bactericidal disinfectant **15** was evident, showing a typical lag phase of 3–5 days in CBT and MRT.

The fluorinated quinolines, **13** and **16**, were only readily biodegradable using activated sludge as an inoculum source in the MRT. However, even then they showed a lag phase between 13 and 23 d whereas incubation with sodium acetate decreased the lag phase to 9 and 15 d respectively, probably because co-metabolism was induced, or the bacteria growth was enhanced due to the sodium acetate. For **16** the results also did highly vary between the different test samples, due to their long lag phase (Table 1, Fig. S1). Hence, in the cases of **13** and **16**, it looks like a critical number of specific microorganisms capable to metabolize these compounds is required, which were not (always) present or only as minor fractions in secondary effluent. However, once adaptation of the inoculum was achieved mineralization proceeded rapidly.

Hydroxylation at C2 of the fluorinated quinoline (**6**) enhanced the biodegradation rate and level already under CBT conditions in comparison to **13**, indicating hydroxylation of the quinoline scaffold at C2 as the rate-limiting step in the degradation. In the MRT **6** could be classified as readily biodegradable.

Like **6**, hydroxylation at C2 enhanced the biodegradation level for various other quinolines in the CBT and MRT (Table 1). In addition, the lag phase of dihydroxylated quinolines (**17**–**20**) was significantly reduced compared to monohydroxylated quinolines, confirming hydroxylation at C2 as a rate-limiting element in the biodegradation pathway of quinolines by the inoculum (data not shown). The analysis of degradation intermediates after 28 days supports this hypothesis. The degradation of monohydroxylated substances like **12** and **14** did undergo a hydroxylation at C2 resulting in dihydroxylated quinolines like **18** and **19**, respectively (Fig. 2, Table S3 and S4). The identified metabolic intermediates of **3**, which is hydroxylated at C2, were the dihydroxylated quinoline **18** and a trihydroxylated intermediate (Fig. 2, Table S2). Moreover, C2 hydroxylation removed the antimicrobial effects of **15** to the inoculum since the resulting substance **20** was readily degradable in the MRT. However, the SH-and NH_2-bioisosteres (**4, 5**) of **3** were persistent in the CBT and MRT.

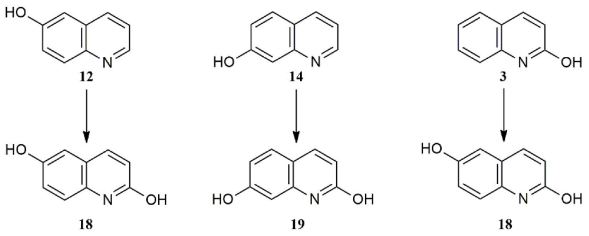


Fig. 2. Via LC-HRMS analysis identified degradation intermediates during CBT or MRT of **12**, **14**, and **3**.

The general enhancement of biodegradability is in accordance with the literature, reporting microbial degradation of quinolines. There are different degradation pathways for quinolines described in the literature (Kaiser et al., 1996; Shukla, 1986; Fetzner et al., 1998). In most cases, 2-oxo-quinoline is the starting point for quinoline degradation. The ubiquitous *Pseudomonas* strains usually start via monohydroxylation at C2 by a 2-oxidoreductase to form **3** and its corresponding keto-tautomer, which are further hydroxylated depending on the microbial pathway (Fig. 3,)Kaiser et al., 1996; Shukla, 1986; Fetzner et al., 1998). In the 8-hydroxycumarin pathway, a unique pathway of unsubstituted **3**, **3** is converted to **20** followed by the formation of 8-hydroxycumarin, which is oxidized to further products (Shukla, 1986). While **3** and 8-hydroxycumarin show rapid microbial oxidation, the metabolization of **20** is reported to occur rather slowly even in pure cultures (Shukla, 1986). Since **20** is readily biodegradable under MRT but not under CBT conditions, the oxidation process to 8-hydroxycumarin is considered a rate-limiting step in the degradation pathway under CBT conditions. According to the LC-HRMS analysis, **18** was found as the intermediate of the degradation of **3** (Fig. 2, Table S2). This suggests that the degradation under CBT and MRT conditions did also follow the 5,6-dihydroxy-1H-2-oxoquinoline pathway (Fig. 3).

Furthermore, **18** has been herein tested to be readily biodegradable in the CBT. The 5,6-dihydroxy-1H-2-oxoquinoline pathway is catalyzed by 2-oxo-1,2-dihydroquinoline 5,6-dioxygenase in many strains using **3** and 3-methylquinolin-2-one as substrates which are converted to the corresponding 6-hydroxy- and 5,6-dihydroxyquinolin-2-ones (Fetzner et al., 1998; Schach et al., 1995). The degradation of **8** by *Pseudomonas putida* has been shown to follow the anthranilate pathway involving hydroxylation at C3 and cleavage to N-formylanthranilic acid and CO (Fetzner et al., 1998). However, no degradation products could be found by LC-HRMS analysis in our study.

Overall, **9** was identified as the most biodegradable quinoline owning a biodegradation kinetic similar to sodium acetate under CBT conditions. **9** is a microbial metabolite in the degradation process of the amino acid L-tryptophan and is further oxidized to known well-biodegradable L-glutamic acid, DL-alanine, and acetic acid as the major products (Stanier et al., 1951; Taniuchi and Hayaishi, 1963; Hayaishi et al., 1961). As a biodegradation pathway, *Pseudomonas fluorescens* has been reported to oxidize **9** to the catechol intermediate 7,8-dihydroxykynurenic acid which undergoes opening of the aromatic ring to additional readily biodegradable metabolites (Taniuchi and Hayaishi, 1963). Besides **9** the 7,8-dihydroxy-1H-2-oxoquinoline pathway has been shown for **8** and 4-methylquinoline by some *Pseudomonas* strains (Fetzner et al., 1998).

3.2. Ready biodegradability of isoquinolines

In contrast to the tested quinolines, none of the isoquinolines were readily biodegradable in either of the biodegradation assays nor were any toxic effects observed (Table 2). Only **24** showed up to 32% $ThOD_{NH3}$ after 28 days in the CBT and is therefore classified as partially biodegradable under CBT conditions. However, the obtained level varied between the CBTs. On the other hand, **23**, which

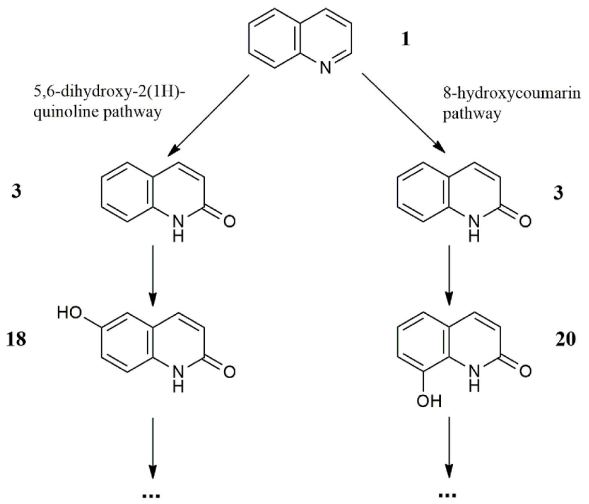
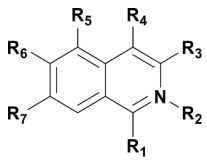


Fig. 3. Two different degradation pathways of quinoline as described in the literature (Kaiser et al., 1996; Shukla, 1986; Fetzner et al., 1998).

Table 2
Structures and biodegradability of isoquinoline scaffolds in the CBT and MRT (n = 4).



								Biodegradation level [% $ThOD_{NH3}$]	
Compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	CBT	MRT
21	H		H	H	H	H	H	0 ± 6.3	2 ± 5.8
22	H	OH	H	H	H	H	H	−1 ± 8.2	−15 ± 5.4[a]
23	OH		H	H	H	H	H	5 ± 3.9	−4 ± 11.5[a]
24	H		OH	H	H	H	H	19 ± 14.6	20 ± 1.9[a]
25	H		H	OH	H	H	H	2 ± 7.1	−18 ± 15.8[a]
26	H		H	H	OH	H	H	−3 ± 3.6	−14 ± 5.8
27	H		H	H	H	OH	H	3 ± 3.7	5 ± 4.5[a]
28	H		H	H	H	H	OH	−4 ± 12.3	25 ± 37.2[a]

[a] n = 2.

has been reported as the primary metabolite in the biodegradation process of **21** by various bacterial strains, was not mineralized by the inoculum (Röger et al., 1995; Aislabie et al., 1989; Sutherland et al., 1998). Boyd et al. reported degradation of **21** by *Pseudomonas putida* following the degradation pathway of PAHs. For **21** they identified *cis*-7,8-dihydroxyisoquinoline and **26** as metabolites (Boyd et al., 1987). Therefore, the degradation of **24** is proposed to be similar to the degradation of 2-naphthol involving additional hydroxylation to a catechol intermediate and further oxidation by a catechol dioxygenase (Zang and Lian, 2009).

3.3. Ready biodegradability of quinolones, naphthyridones, and 4H-pyrido[1,2-a]pyrimidines with a β-keto carboxylic acid pharmacophore

Only 1 out of 27 tested molecules was readily biodegradable in the MRT (Tables 3 and 4). None of the structures were classified as toxic to the inoculum according to the OECD guidelines. However, a lag phase in the toxicity controls has been shown for many halogenated structures tested, especially in the MRT. This is due to the higher test concentrations, indicating general toxic effects on the inoculum.

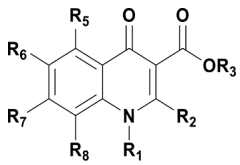
29 showed a lag phase of 6 up to 10 d and a biodegradation phase of 4 up to 8 d in the first plateau phase (≥ 60% $ThOD_{NO3-}$), whereas **9**, an isomer of **29**, was already readily biodegradable in the CBT showing no lag-phase. Moreover, the results suggest a rate-limiting element since the biodegradation phase of **29** proceeds rapidly. This could be assigned either to the required growth of specific organisms or the slow formation of an essential metabolite. After typically 16 d an additional degradation phase was observed attributed to nitrification (Fig. 4). The final biodegradation level corresponds to 82 ± 10.1% $ThOD_{NO3-}$, which confirms the complete mineralization of the heterocycle by the inoculum. Any other derivatives, including the 6-F-, 8-F-, and 3-NO_2-bioisosteres (**33, 47, 10**), interfered with biodegradation processes and compromised them completely. Therefore, it is possible that the electron density is more important than the interaction with the responsible enzyme and the bioisosteres might function as a competitive inhibitor in the biodegradation process. The influence of electron density on biodegradability has also been shown for phenols and anilines. It was demonstrated that the initial attack on the aromatic nucleus has an electrophilic character and is the rate-limiting step (Pitter, 1985). In the case of halogenation, the fluorination of the aromatic heterocycle causes deactivation of the ring towards electrophilic reactions. This impaires the biodegradation of aromatic systems since their degradation usually involves the hydroxylation by an electrophilic oxygen species (such as hydroperoxides). Similar to fluorine but even stronger, the substitution of the carboxylic acid with the nitro bioisostere depletes the electron density of the arene by causing a -I- and intensive -M-effect compared to the carboxylic acid. Due to the similarity of **29** and **9**, it is assumed that the biodegradation pathway of **29** proceeds correspondingly and is initiated by oxidation to the corresponding 7,8-epoxide (Taniuchi and Hayaishi, 1963).

Compound **30** was very unstable, which lead to a breakdown of the structure to 2,4-dihydroxy-quinolone already during shipping and storage. **30** could have been a promising biodegradable quinolone scaffold since hydroxylation at C2 was identified as a rate-limiting step for quinolines. However, the hydroxylation leads to a significant destabilization of the whole molecule. Yet, hydroxylation at C2 did not enhance the biodegradability or decrease the stability of **32**, indicating the important influence of substituents at N1. Nevertheless, if substituents could be found which stabilize the 1,3-diketone-2-carboxy derivative during storage and application but still allow a break-down by environmental bacteria, **30** may be a promising scaffold for greener quinolone derivatives.

3.4. Ready biodegradability of commercial quinolone and fluoroquinolone antibiotics

None of the commercially available quinolone and fluoroquinolone antibiotics were biodegradable in either the CBT or MRT (Table 5). Similar results were reported in the literature investigating the ready and inherent biodegradability of fluoroquinolones (Gartiser et al., 2007; Bergheim et al., 2015). Hence, all tested fluoroquinolones have to be considered to be persistent to biodegrada-

Table 3
Structures and biodegradability of quinolone scaffolds in the CBT and MRT (n = 4).



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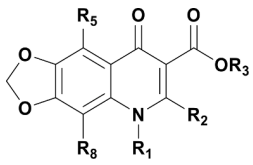

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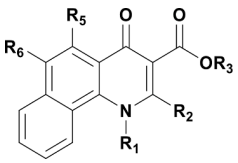
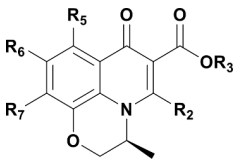


49

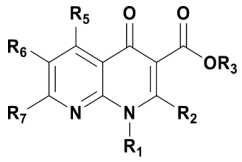


50

								Biodegradation level [% $ThOD_{NH3}$]	
Compound	R_1	R_2	R_3	R_5	R_6	R_7	R_8	CBT	MRT
29	H	H	H	H	H	H	H	6 ± 2.6	82 ± 10.1[a]
30	H	OH	H	H	H	H	H	–	–
31	Ethyl	H	H	H	H	H	H	0 ± 4.8	5 ± 2.1
32	Ethyl	OH	H	H	H	H	H	1 ± 1.6	10 ± 8.0[b]
33	H	H	H	H	F	H	H	−1 ± 6.5	0 ± 6.3
34	H	H	H	H	F	Morpholinyl	H	2 ± 2.1	−8 ± 15.7
35	Ethyl	H	H	H	F	Morpholinyl	H	2 ± 3.9	−10 ± 10.4
36	Cyclopropyl	H	H	H	F	F	OH	0 ± 3.3	−7 ± 11.3
37	H	H	H	OCH_3	H	H	OCH_3	n.d.	−0.3 ± 1.2[b]
38	H	H	H	H	H	OCH_3	H	3 ± 1.8	12 ± 9.3
39	H	H	H	H	OCH_3	OCH_3	H	1 ± 1.0	4 ± 0.5[b]
40	Cyclopropyl	H	H	H	F	F	OCH_3	0 ± 3.1	−37 ± 13.9[b]
41	Cyclopropyl	H	H	H	F	Cl	H	−2 ± 1.0	−28 ± 9.2[b]
42	H	H	H	H	H	H	CH_3	−3 ± 4.8	−1 ± 2.5
43	H	H	Ethyl	H	H	H	CN	−4 ± 7.1	2 ± 1.4[b]
44	H	H	H	H	F	1-piperazinyl	H	−2 ± 5.3	−8 ± 13.6
45	H	H	H	H	H	H	OCH_3	5 ± 3.5	−2 ± 2.4
46	H	H	H	H	H	Cl	H	2 ± 4.1	1 ± 3.1
47	H	H	H	H	H	H	F	5 ± 2.3	−4 ± 5.5
48	H	H	Ethyl	H			H	−4 ± 10.0	1 ± 11.6
49	H	H	H	H	H			3 ± 5.0	−3 ± 1.3
50		H	H	H	F	F		1 ± 5.3	−32 ± 9.9[b]

[a] Based on the $ThOD_{NO3-}$.
[b] n = 2.

Table 4
Structures and biodegradability of naphthyridone and 4H-pyrido[1,2-*a*]pyrimidine scaffolds in the CBT and MRT (n = 4).



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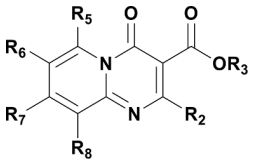


54-55

								Biodegradation level [% $ThOD_{NH3}$]	
Compound	R_1	R_2	R_3	R_5	R_6	R_7	R_8	CBT	MRT
51	H	H	H	H	H	CH_3		−3 ± 7.6	−1 ± 8.7[a]
52	Cyclopropyl	H	H	H	F	Cl		−9 ± 8.5	−4 ± 3.2
53	2,4-Difluorophenyl	H	Ethyl	H	F	Cl		−2 ± 1.7	−2 ± 0.7[a]
54		H	H	H	H	H	H	1 ± 2.8	5 ± 2.5
55		H	H	H	H	H	OH	1 ± 1.6	4 ± 2.1[a]

[a] n = 2.

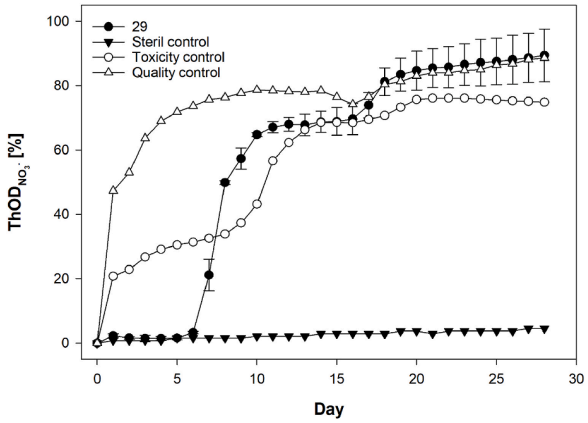
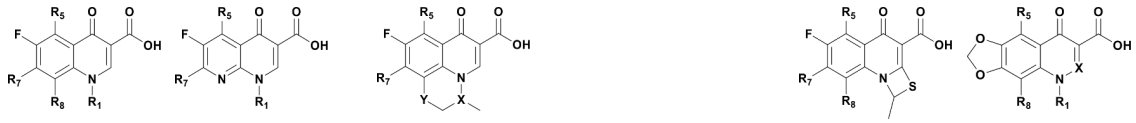


Fig. 4. Biodegradation curve of **29** in the MRT.

Table 5
Structures and biodegradability of commercially available fluoroquinolones in the CBT and MRT (n = 4).



56-64 65-68 69-73 74-75 76-77

							Biodegradation level [% $ThOD_{NH3}$]	
Compound	R_1	R_5	R_7	R_8	X	Y	CBT	MRT
56	Cyclopropyl	H	3-(methylamino)-1-piperidyl	OCH_3			−5 ± 4.2	−37 ± 1.9[a]
57	Cyclopropyl	H	4aS,7aS-octahydro-1H-pyrrolo[3,4-*b*]pyridin-6-yl	OCH_3			−5 ± 2.1	−15 ± 4.0[a]
58	Cyclopropyl	H	1-piperazinyl	H			−1 ± 1.1	−32 ± 0.5[a]
59	Cyclopropyl	NH_2	3R,5S-3,5-dimethylpiperazinyl	F			−4 ± 7.1	−15 ± 6.1[a]
60	4-Fluorophenyl	H	1-piperazinyl	H			−4 ± 3.1	−13 ± 6.6[a]
61	2-Fluorethyl	H	4-methyl-1-piperazinyl	F			−3 ± 10.1	−1 ± 29.0[a]
62	Ethyl	H	1-piperazinyl	H			−3 ± 2.8	−10 ± 20.5[a]
63	Ethyl	H	3-methyl-1-piperazinyl	F			−4 ± 1.5	−11 ± 8.7[a]
64	Cyclopropyl	H	3-methyl-1-piperazinyl	OCH_3			−8 ± 6.8	−2 ± 41.7[a]
65	Ethyl	H	1-piperazinyl				−2 ± 6.8	−10 ± 15.7
66	Cyclopropyl	H	4Z-Aminomethyl-3-methyloxyiminopyrrolidin-1-yl				2 ± 1.9	−20 ± 2.1[a]
67	2,4-Difluorophenyl	H	3-Aminopyrrolidinyl				−2 ± 3.1	−11 ± 0.7[a]
68	Ethyl	H	CH_3				−6 ± 6.0	8 ± 8.0[a]
69		H	1-aminocyclopropyl		C	O	3 ± 3.3	−30 ± 2.1[a]
70		H	4-methyl-1-piperazinyl		N	O	−6 ± 5.9	−4 ± 27.1[a]
71		H	4-methyl-1-piperazinyl		C	O	−9 ± 4.8	−23 ± 5.4[a]
72		H	4-hydroxypiperidinyl		C	C	−2 ± 2.1	−17 ± 1.4[a]
73		H	H		C	C	−2 ± 3.0	−14 ± 11.8[a]
74			4-((5-methyl-2-oxo-1,3-dioxol-4-yl)methyl)piperazin-1-yl	H			3 ± 7.2	−5 ± 0.7[a]
75			1-piperazinyl	H			−7 ± 4.1	1 ± 24.5[a]
76	Ethyl	H		H	C		1 ± 7.4	−8 ± 18.6[a]
77	Ethyl	H		H	N		6 ± 3.6	15 ± 24.5[a]

[a] n = 2.

tion under environmental conditions and are only partially removed by adsorption on activated sludge particles (Gartiser et al., 2007). Nevertheless, some fluoroquinolones are known to be degraded by specialized fungi and bacteria in pure culture experiments (Amorim et al., 2014; Wetzstein et al., 1997, 1999, 2012). However, although the microorganisms were able to reduce the antimicrobial activity by primary elimination, they did not achieve complete mineralization (Wetzstein et al., 1997, 1999). Moreover, the uti-

lization of single strains is not suitable to represent the biodegradation process in the environment since these organisms typically represent only a small fraction of the microorganisms. At MRT conditions, all antibiotics showed negative degradation values, which has also been reported in other biodegradation tests using this type of antibiotics (Gartiser et al., 2007). Negative degradation values imply that the inoculum was more active in the blank than in the test flasks. Furthermore, general toxic effects for all quinolones and fluoroquinolones on the inoculum were evident especially in the MRT due to the use of higher test concentrations in comparison to the CBT. Especially the 4th and 5th generation fluoroquinolones show low activity thresholds and a broader activity spectrum. Yet, none of them were classified as toxic according to the corresponding OECD guidelines (OECD, 1992). However, the bactericidal fluoroquinolones have been reported to induce changes in the microbial community, which cannot be detected by the OECD toxicity control since it detects only general toxic effects, and therefore, might interfere with a potential biodegradation process by inhibiting the growth of non-resistant strains (Alexandrino et al., 2017). Overall, the results confirmed the persistence of the group of fluoroquinolone antibiotics to biodegradation.

3.5. Ready biodegradability of quinazolinones

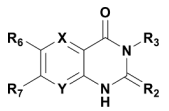
While no quinazolinone was biodegradable under the conditions of the CBT, **79** and its bioisostere **82** were readily biodegradable and fully mineralizable according to OECD guideline 301F (Table 6). However, the pyrido-, 6-F-, and 2-S-bioisosteres of **79** were not, and the substitution with an amine at R_3 inhibited biodegradation completely. The same effects were also shown for the quinolones, where small modifications in electron density and structure interfered with the degradation process. Of note, both, **79** and **82**, already showed co-metabolism with sodium acetate in the CBT resulting in complete mineralization, which renders them attractive as biodegradable lead scaffolds. Moreover, no general toxic effects were observed in both biodegradation assays for all quinazolinones. It is assumed that the biodegradation pathway of quinazolinones is similar to the pathway of pterin by *Pseudomonas fluorescens* and proceeds via the degradation of the pyrimidine system (Soini and Backman, 1975). In the case of **82**, *Pseudomonas fluorescens* has been reported to metabolize **82** to pyrazine-2-carboxylic acid and pyrazine-2-carboxamide (Soini and Backman, 1975).

3.6. Field-based QSAR model and contour maps

Steric groups seem to have a negative influence on the substances' biodegradability in most positions (Fig. 5a). Only at position 2, and in a non-planar orientation at position 7, do steric groups seem to have a positive influence on the degradability. However, since the dataset contains mostly structures with hydroxyl groups at position 2, those results cannot be generalized for many other substituents beyond the investigated ones. The presented contour maps also back up the hypothesis that substituents at the N1 do inhibit a potential degradation by the present microorganisms as already discussed. The hydrophobic field (Fig. 5c) does correlate with the results, especially from Table 1, showing that no hydrophobic groups should be present at positions 2, 4, and 7, which is in accordance with the biodegradability of the different (di)-hydroxyquinolines (Table 1). Furthermore, hydrophobic groups at position 3 and in the heterocyclic ring at positions 1, 2, and 3 seem to have a positive influence on biodegradability. Hydrogen bond acceptors present at positions 1 and 2 have a positive influence on biodegradability, while they hinder biodegradation if they are present at positions 3 and 5.

Those contour maps could be further used to derive rules for the design of better environmentally degradable N-heterocycles (Fig. 5). The QSAR statistics (Table S5) show a relatively bad performance of the underlying field-based QSAR model. Therefore, the present contour maps should only be used as additional input to explain the underlying structure biodegradability relationships. Reasons for its lag in performance may be the skewed dataset (only 14 out of 79 substances are biodegradable), and the different underlying degradation mechanisms and responsible enzymes, which makes it difficult to evaluate them all in the same 3D model. pKs values of

Table 6
Structures and biodegradability of quinazolinone scaffolds the CBT and MRT (n = 4).



							Biodegradation level [% $ThOD_{NH3}$]	
Compound	R_2	R_3	R_6	R_7	X	Y	CBT	MRT
78	O	NH_2	H	H	C	C	3 ± 2.1	7 ± 5.1
79	O	H	H	H	C	C	1 ± 4.1	86 ± 16.6
80	O	H	F	H	C	C	1 ± 3.7	−2 ± 0.7
81	O	H	H	H	C	N	6 ± 3.0	−8 ± 1.9
82	O	H	H	H	N	N	−1 ± 12.4	67 ± 0.7[a]
83	S	H	H	H	C	C	−9 ± 10.4	−31 ± 8.6[b]
84	O	H	OCH_3	OCH_3	C	C	−9 ± 5.9	−12 ± 6.1[b]

[a] Based on the $ThOD_{NO3-}$.
[b] n = 2.

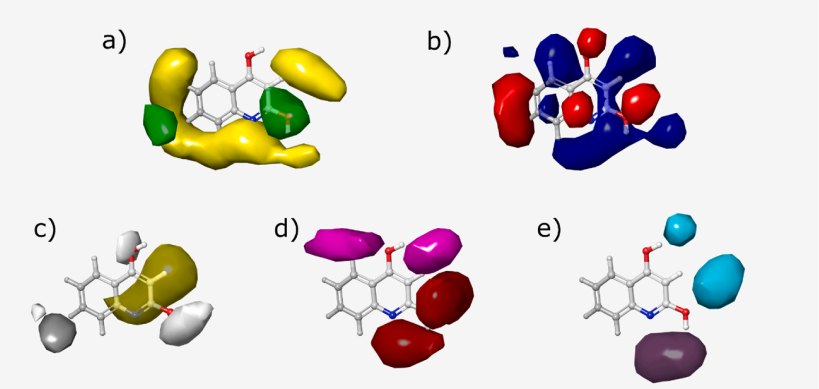


Fig. 5. Contour maps based on the 3D field-based QSAR model created in Maestro (Schrödinger) visualized with the example of **17**. a) steric field (green = positive effect, yellow = negative effect), b) electrostatic field (blue = positive effect, red = negative effect), c) hydrophobic field (yellow = positive effect, white = negative effect), d) H-bond acceptor field (red = positive effect, magenta = negative effect) and e) H-bond donor (blue-violet = positive effect, cyan = negative effect). The field fractions are summarized in Table S6. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

the investigated substances and their potential states at a pH value of 7 ± 1 were not considered. However, resulting charges may also be important to understand the interaction of the substances and degrading enzymes.

3.7. Biodegradability structure relationships

While most N-heterocycles were persistent to biodegradation under CBT or MRT conditions, 14 out of 84 were classified as readily biodegradable. However, the results also demonstrated that most bioisosteres of the biodegradable scaffolds interfered with the biodegradation process presumably by competitive inhibition.

The quinoline scaffold itself is readily biodegradable, esp. with hydroxyl groups at various positions (2, 4, and 6). Even a fluorine atom or a carboxyl group at position 2 did not hinder the biodegradation of the quinoline derivatives. Isoquinolines on the other hand were not biodegradable at all. This leads to the conclusion that N1 seems to play an important role in the biodegradation process. Since substituents at N1 also decreased the potential biodegradability of the whole molecule, it seems to be important for the protein-ligand interaction with degrading enzymes. 2-Oxoquinoline-8-Monooxygenase has been described in the literature as an enzyme important in the degradation of quinoline (Martins et al., 2005). A look at its binding site (PDB ID: 1Z03) and 2-Oxoquinoline as a co-crystalized ligand does show why bulky groups at position N1 might be a problem for further degradation (Fig. S2 and **S3**). Steric groups at N1, and other positions too, would probably prevent the molecule to fit into the binding site of this specific degrading enzyme. This might be an explanation for why most of the investigated substances with substituents bigger than a hydroxyl group could not be degraded in our tests.

The quinolone scaffold without any substituents was degradable. Adding a hydroxyl group at C2 made it very unstable, which did not allow any further experiments. However, any other substituent at the quinolone scaffold led to a not readily biodegradable molecule. In this case, a fluorine atom was not tolerated anymore, as it was the case for the quinoline scaffold. An oxygen substituent at position 2 enhanced the biodegradability of quinazoline, but this does not seem to be true in any case. Other substituents, like a fluorine atom, already hindered potential biodegradation in those cases.

Based on the obtained biodegradation results the following rules can be stated:

- Use **29** as a biodegradable lead scaffold. However, small modifications to the molecule might interfere with the biodegradation process.
- The biodegradability of the tested quinolines is enhanced by the hydroxylation at C2. While even fluorine was tolerated in combination with the hydroxylation, substitution with alternative bioisosteric H-donators (SH, NH_2) compromised the biodegradation process completely and should therefore be avoided.
- Isoquinolines, even hydroxylated, were not biodegradable in CBT except for **24**, which was partially biodegradable. In the MRT, the biodegradation level did not increase either. Overall, the tested isoquinolines should not be used to design biodegradable molecules.
- **79** and **82** can be used as alternative lead scaffolds i.e., for the design of novel topoisomerase II and topoisomerase IV inhibitors.

Since it seems to be important to avoid substituents at the N1 of the quinoline scaffold it might also be a possible solution to combine the quinoline scaffold with degradable linkers at this specific position. Ciprofloxacin for example was coupled with a hemiaminal linker at the N1 position, resulting in a still-active component (Leder et al., 2021). However, the hemiaminal linker is cleaved at slightly acidic pH conditions, resulting in a molecule without any substituent at N1. The combination of this approach with the herein-presented insights on biodegradable scaffolds could be used to design new degradable APIs.

4. Conclusion

The ready biodegradability of several N-heterocycles has been evaluated to identify completely mineralizable scaffolds for the design of greener chemicals.

Based on the biodegradation results, the important bioactive scaffold **29** could be used to design new potentially biodegradable pharmaceuticals with a broad spectrum of activities. Our previously designed molecule already demonstrated the feasibility of more environmentally friendly fluoroquinolone antibiotics. Yet, even these new improved antibiotics lack complete mineralization but are inactive at least (Leder et al., 2021). Therefore, an important field could be the design of fully mineralizable quinolone antibiotics to slow down antibiotic resistance. However, to avoid interference with the biodegradation process of possible derivatives the biodegradation pathway of **29** and other lead molecules should be examined in future work to gain additional information about possible growth vectors, which can increase either activity or other required properties.

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CRediT author statement

Morten Suk: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – Original Draft, Visualization **Stefanie Lorenz:** Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization **Klaus Kümmerer:** Conceptualization, Resources, Supervision.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2022.100947.

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Electronic Supplementary

Identification of environmentally biodegradable scaffolds for the benign design of quinolones and related substances

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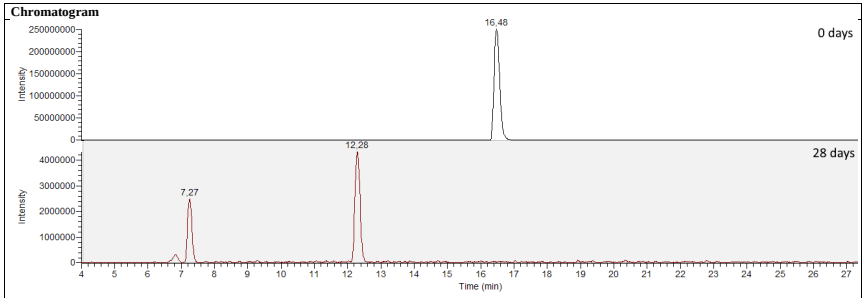
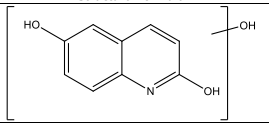
# Both authors contributed equally.

Table S1: HESI source and ion trap mass analyzer parameters.

HESI source	
Heater Temp.	350 °C
Sheath gas flow rate	40 arb
Aux gas flow rate	15 arb
Sweep gas flow rate	0 arb
Spray Voltage	3 kV
Capillary Temp.	275 °C
Capillary Voltage	10 V
Tube Lens	69 V
Ion optics	
Multipole 00 Offset	-5 V
Lens 0 Voltage	- 5.4 V
Multipole 0 Offset	- 6.9 V
Lens 1 Voltage	- 9 V
Gate Lens Voltage	- 46 V
Multipole 1 Offset	- 9 V
Multipole RF Amplitude	660 V p-p
Front Lens	- 8 V

Table S2: LC-MS results of 2-Hydroxyquinoline (**3**) before and after CBT.



RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
7.27	180.0654	162.0548 (-H_2O)	$C_9H_{10}O_3N$	-3.099	

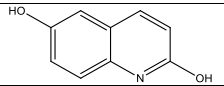
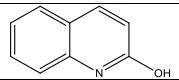
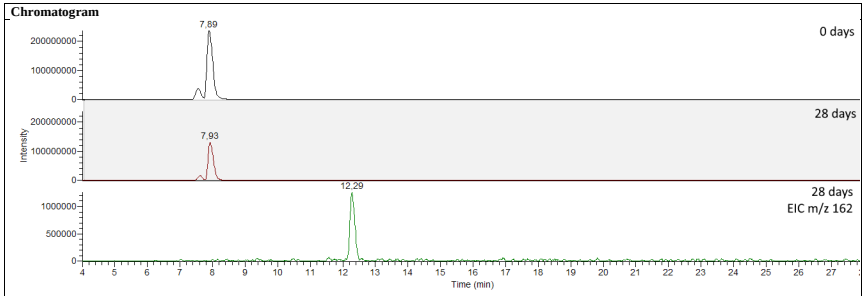
12.28	162.0550	144.0441 (-H_2O)	$C_9H_8O_2N$	-3.168	
16.48	146.0601	128.0492 (-H_2O)	C_9H_8ON	-3.347	

Table S3: LC-MS results of 6-Hydroxyquinoline (**12**) before and after CBT.



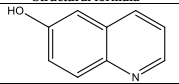
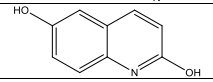
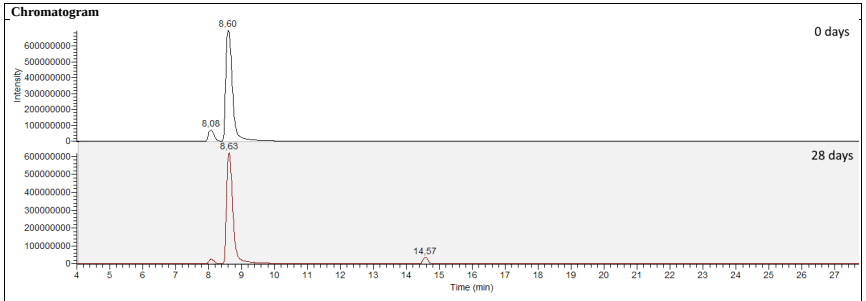
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
7.89	146.0601		C_9H_8ON	-3.552	
12.29	162.0548	144.0441 (-H_2O)	$C_9H_8O_2N$	-4.094	

Table S4: LC-MS results of 7-Hydroxyquinoline (**14**) before and after MRT.



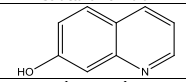
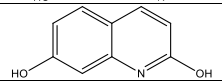
RT (min)	$[M+H]^+$	MS^2	Sum formula	Delta (ppm)	Structural formula
8.60	146.0601		C_9H_8ON	-3.621	
14.57	162.0552	144.0478 (-H_2O)	$C_9H_8O_2N$	-2.119	

Table S5: QSAR statics of the 3D field-based QSAR module from Maestro, Schrödinger

# Factors	SD	R^2	R^2 CV	R^2 Scramble	Stability	F	P	RMSE	Q^2	Pearson-r
1	26.2992	0.2740	0.1891	0.2041	0.99	20.4	3.49e-05	27.48	0.1759	0.4196
2	23.3083	0.4403	0.1917	0.3525	0.916	20.8	2.1e-07	28.54	0.1105	0.4060
3	21.7729	0.5208	0.0871	0.4826	0.812	18.8	2.11e-08	29.52	0.0487	0.3945
4	19.7445	0.6135	-0.1429	0.5666	0.476	20.2	4.94e-10	29.87	0.0261	0.3693
5	17.6378	0.6976	-0.3420	0.6405	0.0893	23.1	6.23e-12	31.63	-0.0920	0.3009

Table S6: Field fractions of the 3D field-based QSAR module from Maestro, Schrödinger

Gaussian steric field	Gaussian electrostatic field	Gaussian hydrophobic field	Gaussian Hbond Acceptor	Gaussian Hbond Donor
0.225	0.065	0.216	0.274	0.22

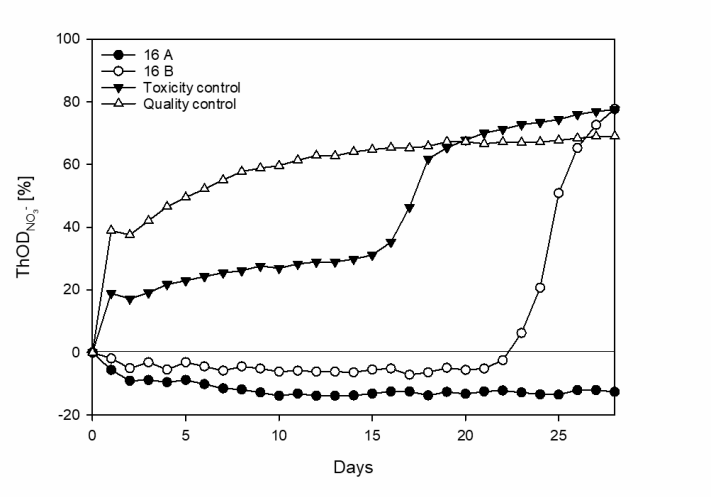


Figure S1: Biodegradation curve of **16** sample A and B in the MRT.

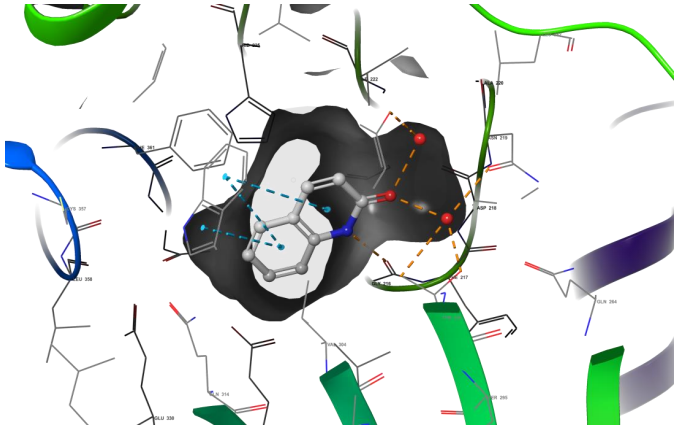


Figure S2: 3D representation of the binding site surface of PDB ID 1Z03 and the co-crystalized ligand.

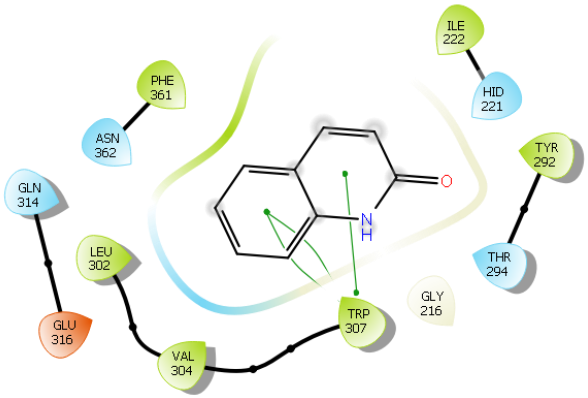
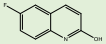
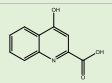
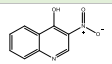
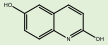
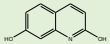
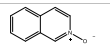
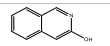
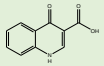
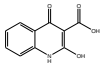
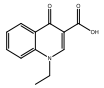
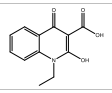
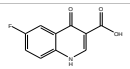
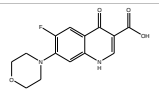
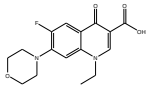
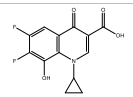
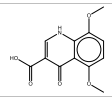
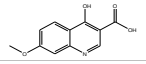
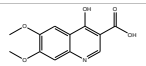
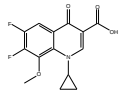
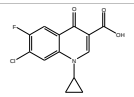
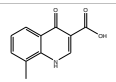
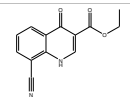
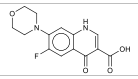
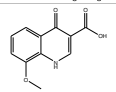
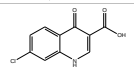
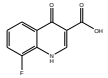
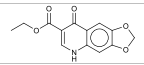
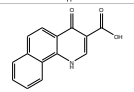
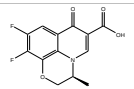
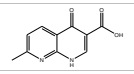
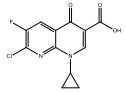


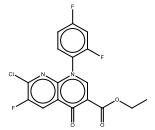
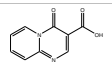
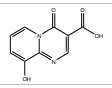
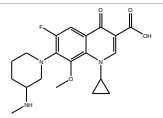
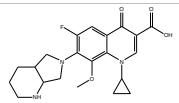
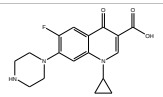
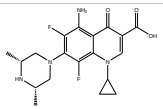
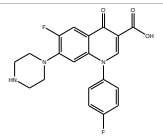
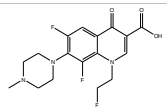
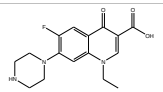
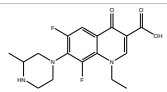
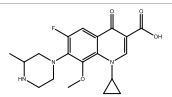
Figure S3: Ligand interaction diagram of the binding site of PDB ID 1Z03 and the co-crystalized ligand.

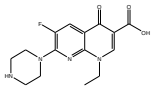
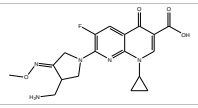
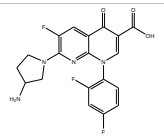
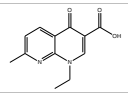
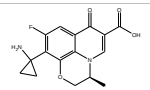
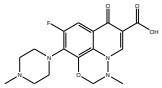
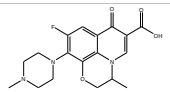
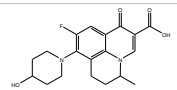
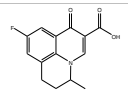
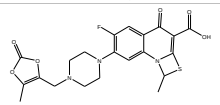
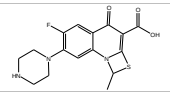
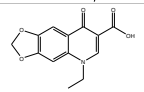
Table S7: Structure and molecular formula of all investigated compounds (environmental biodegradable ones are colored green)

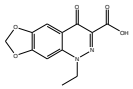
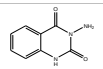
Compound	Structure	Molecular formula
1		C_9H_7N
2		C_9H_7NO
3		C_9H_7NO
4		C_9H_7NS
5		$C_9H_8N_2$
6		C_9H_6FNO
7		C_9H_7NO
8		C_9H_7NO
9		$C_{10}H_7NO_3$
10		$C_9H_6N_2O_3$
11		C_9H_7NO
12		C_9H_7NO
13		C_9H_6FN
14		C_9H_7NO
15		C_9H_7NO
16		C_9H_6FN
17		C9H7NO2
18		$C_9H_7NO_2$
19		$C_9H_7NO_2$
20		$C_9H_7NO_2$

21		C_9H_7N
22		C_9H_7NO
23		C_9H_7NO
24		C_9H_7NO
25		C_9H_7NO
26		C_9H_7NO
27		C_9H_7NO
28		C_9H_7N
29		$C_{10}H_7NO_3$
30		$C_{10}H_7NO_4$
31		$C_{12}H_{11}NO_3$
32		$C_{12}H_{11}NO_4$
33		$C_{10}H_6FNO_3$
34		$C_{14}H_{13}FN_2O_4$
35		$C_{16}H_{17}FN_2O_4$
36		$C_{13}H_9F_2NO_4$
37		$C_{12}H_{11}NO_5$

38		$C_{11}H_9NO_4$
39		$C_{12}H_{11}NO_5$
40		$C_{14}H_{11}F_2NO_4$
41		$C_{13}H_{10}ClFNO_3^+$
42		$C_{11}H_9NO_3$
43		$C_{13}H_{10}N_2O_3$
44		$C_{14}H_{13}FN_2O_4$
45		$C_{11}H_9NO_4$
46		$C_{10}H_6ClNO_3$
47		$C_{10}H_6FNO_3$
48		$C_{13}H_{11}NO_5$
49		$C_{14}H_9NO_3$
50		$C_{13}H_{10}F_2NO_4^+$
51		$C_{10}H_8N_2O_3$
52		$C_{12}H_8ClFN_2O_3$

53		$C_{17}H_{10}ClF_3N_2O_3$
54		$C_9H_6N_2O_3$
55		$C_9H_6N_2O_4$
56		$C_{20}H_{24}FN_3O_4$
57		$C_{21}H_{24}FN_3O_4$
58		$C_{17}H_{18}FN_3O_3$
59		$C_{19}H_{22}F_2N_4O_3$
60		$C_{20}H_{17}F_2N_3O_3$
61		$C_{17}H_{18}F_3N_3O_3$
62		$C_{16}H_{18}FN_3O_3$
63		$C_{17}H_{19}F_2N_3O_3$
64		$C_{19}H_{22}FN_3O_4$

65		$C_{15}H_{17}FN_4O_3$
66		$C_{18}H_{20}FN_5O_4$
67		$C_{19}H_{15}F_3N_4O_3$
68		$C_{12}H_{12}N_2O_3$
69		$C_{16}H_{15}FN_2O_4$
70		$C_{17}H_{19}FN_4O_4$
71		$C_{18}H_{20}FN_3O_4$
72		$C_{19}H_{21}FN_2O_4$
73		$C_{14}H_{12}FNO_3$
74		$C_{21}H_{20}FN_3O_6S$
75		$C_{16}H_{16}FN_3O_3S$
76		$C_{13}H_{11}NO_5$

77		$C_{12}H_{10}N_2O_5$
78		$C_8H_7N_3O_2$
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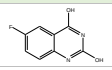
80		$C_8H_5FN_2O_2$
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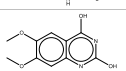
82		$C_6H_4N_4O_2$
83		$C_8H_6N_2OS$
84		$C_{10}H_{10}N_2O_4$