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Do new generations of active pharmaceuticals for human use require an adaption of the environmental risk assessment?, Part I: Literature review

Final report

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Do new generations of active pharmaceuticals for human use require an adaption of the environmental risk assessment?, Part I: Literature review

Final report

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Abstract: Do new generations of active pharmaceuticals for human use require an adaption of the environmental risk assessment?, Part I: Literature review

Pharmaceuticals can be a problem for the environment and its inhabitants. Several hundred active pharmaceutical ingredients were already found in the ng/L to µg/L range in surface water, groundwater, or soil. To date, all pharmaceutical which exceed an action limit of 0.01 µg/L in the environment are subjected to an environmental risk assessment. However, there is the risk that some of the pharmaceuticals are effective even at lower concentrations, or that the guideline does not recommend studies with the most sensitive test organism for the respective mode of action. In order to answer this question, the current project focussed on the identification of active pharmaceutical ingredients approved since 2006, as well as on the summary of relevant information for these substances. Based on these data, three substance classes were chosen, which contribute most to the high number of pharmaceuticals in the environment, i.e. 'neurology', 'cardiology', and 'oncology'. The literature review furthermore identified sensitive ecotoxicological test systems, based on data obtained for substanceclass specific model substances. Finally, five substances per group were chosen for testing the alternative test systems. Requirements for the chosen test substance were the availability of an ERA according to the EMA guideline as well as information on the effects of a model substance with similar mode of action in the alternative test systems. Based on experimentally obtained data it should be determine, if a tailored risk assessment is suitable for the selected substance class or if studies according to the EMA guideline are sufficiently predictive for an environmental risk assessment.

Kurzbeschreibung: Erfordern neue Wirkstoffgenerationen bei Humanarzneimitteln eine Anpassung der Umweltbewertung?, Teil I: Literaturstudie

Pharmazeutika können ein Problem für die Umwelt und die darin lebenden Organismen darstellen. Einige hundert Wirkstoffe wurden bereits in unterschiedlichen Umweltkompartimenten wie Oberflächenwasser, Grundwasser oder Boden im ng/L bis µg/L gefunden. Bislang werden alle Pharmazeutika, die eine Aktionsgrenze von 0.01 µg/L in der Umwelt überschreiten, einer Umweltrisikobewertung unterzogen. Es besteht allerdings die Gefahr, dass Substanzen auch in geringeren Konzentrationen Effekte auf Umweltorganismen ausüben, oder dass in der Richtlinie nicht die empfindlichsten Organismen zur Testung empfohlen werden. Um diese Fragestellung zu beantworten, wurde im Rahmen dieses Vorhabens eine Literaturstudie durchgeführt, die zunächst alle ab 2006 neu zugelassenen Wirkstoffe identifiziert und relevante Informationen zusammengefasst hat. Basierend auf diesen Daten wurden zunächst drei Wirkstoffgruppen ausgewählt, für die eine zugeschnittene Bewertungsstrategie definiert werden sollte. Die Auswahl der Gruppen beruhte auf der Anzahl der Substanzen je Wirkstoffklasse. Somit wurden Pharmazeutika der Gruppen ‚Neurologie', ‚Kardiologie' und ‚Onkologie' ausgewählt. Durch eine weitere Literaturrecherche wurden sensitive ökotoxikologische Testsysteme basierend auf Daten von Modellsubstanzen identifiziert. Im Abschluss des Projekts wurden ca. fünf Wirkstoffe pro Substanzklasse ausgewählt, mit denen weitere praktische Studien mit den alternativen Testsystemen durchgeführt werden sollen. Voraussetzung für die Auswahl der Testsubstanzen war, dass sowohl Daten der Umweltrisikobewertung basierend auf der EMA-Richtlinie als auch Daten der alternativen Testmethoden von Modellsubstanzen mit vergleichbaren Wirkmechanismus vorlagen. Basierend auf experimentell erhobenen Daten soll im Anschluss an dieses Projekt ermittelt werden, ob für die ausgewählten Gruppen eine zugeschnittene Risikobewertung sinnvoll ist oder ob mit den Standard-Endpunkten eine ausreichend hohe Aussagekraft über das Umweltrisiko der Wirkstoffe erreicht wird.

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5-FU	5-Fluorouracil
ACh	Acetylcholine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
API	Application programming interface
CAP	Capecitabine
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CisPt	Cisplatin
CP	Cyclophosphamide
CRED	Criteria for reporting and evaluating ecotoxicity data
CRMP-2	Collapsin response mediator protein-2
CYT	Cytarabine
DAC	Deacetylase
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
EC	Effective concentration
ED	Endocrine disruptor
EMA	European medicines agency
EPAR	European public assessment report
ERA	Environmental risk assessment
ET	Etoposide
FDA	Food and drug administration
FET	Fish embryo toxicity test
FKZ	Forschungskennzahl (Project No.)
FPEN	Penetration factor
FSDT	Fish sexual development test
GABA	Gamma-aminobutyric acid
GemC	Gemcitabine
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
IF	Ifosfamide
IM	Imatinib mesylate
Kow	Octanol-water partition coefficient
LC	Lethal concentration
LOEC	Lowest observed effect concentration
logP	Logarithm of the octanol-water partition coefficient
MET	Methotrexate
MoA	Mode of action

mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NME	New molecular entity
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
PEC	Predicted environmental concentration
PMR	Photomotor response
RNA	Ribonucleic acid
SeqAPASS	Sequence alignment to predict across species susceptibility
siRNA	Small interfering RNA
SNRI	Serotonin norepinephrine reuptake inhibitor
SQL	Standardized query language
SSRI	Selective serotonin reuptake inhibitors
SW	Surface water
TAM	Tamoxifen
TG	Test guideline
TRA	Tailored risk assessment
UBA	Umweltbundesamt / German Environment Agency
XML	Extensible markup language

Summary

Introduction

Pharmaceutical can be a problem for the environment and its inhabitants. To date, several hundred active pharmaceutical ingredients were detected in the environment. They were found in aquatic compartments like surface water or groundwater, as well as in terrestrial compartments like soil, and they reach concentrations in the ng/L to the µg/L range. These concentrations could already result in effects in environmental organisms. Based on these premises, a guideline was released for the environmental risk assessment of medicinal products for human use (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2). This guideline requires no testing of the fate and effects on the environment, if the predicted environmental concentration of a substance in surface water is below 0.01 µg/L, unless the drug substances may affect the reproduction of vertebrate or lower animals at concentrations lower than 0.01 µg/L.

This guideline was however developed based on effects of active pharmaceutical ingredients developed and marketed before 2006, but should be applied to those, which were marketed later. Concerns are risen that effects of pharmaceuticals of the new generation were not appropriately assessed by this guideline. The pharmaceuticals can either be effective at even lower concentrations, or the specific mode of action is not covered by the standard tests. For those substances, a tailored risk assessment should be developed. One example for a substance class, for which a tailored risk assessment already exists, is the group of endocrine disruptors. These substances are known to be effective at much lower concentrations and thus, the action limit of 0.01 µg/L is not applicable. Based on the results of this literature research, an analogue tailored risk assessment strategy should be developed for other substance classes.

Work package 1

In work package 1, a strategy was developed to search for new innovative pharmaceuticals on the market since 2006. For this purpose, literature databases as well as online portals should be used to gain knowledge about new active ingredients for human pharmaceuticals. One of the resources used for an overview was the yearly published report on new approved drugs by the FDA (Food and drug adminstration) and EMA (European medicines agency), which were published in the journal Nature Reviews Drug Discovery. Knowing that the research and development process for new drugs can last for more than 10 years, another resource for data acquisition was ClinicalTrials.gov. This allocates additional substances which might come up as New Molecular Entities (NMEs) in future.

A second focus of work package 1 was to add already available data from other online repositories regarding potential ecotoxicological effects. We linked European Public Assessment Reports (EPAR) from EMA website to the datasets and added data from PubChem as well as links to the DrugBank for more detailed information on ecotoxicological effects, if available.

In total, 470 newly approved pharmaceuticals were identified. Substances, which were in the clinical trial phase 3 were recorded, however not classified in order to keep the amount of data concise. Active pharmaceutical ingredients approved since 2006 were grouped into substance classes based on their medical application. Substance classes with the highest number of chemicals were ‘oncology, ‘neurology’ and ‘infections. As antibiotics were already subjected to a specific approach, the substance class ‘cardiology’ was included instead.

As a next step alternative testing strategies for each of the substance groups ‘neurology’, ‘oncology’ and ‘cardiology’ were identified. As no studies were found for the newly approved substances, the literature research included studies with appropriate model substances. A substance was assigned as model substance, if it functioned by the same MoA and if it belonged

to the same pharmacological indication group. The focus of the literature research was on the ecotoxicological risk assessment.

Work package 2

In work package 2, results of work package 1 should be aligned to the current EMA guideline (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2) and specific approaches for a risk assessment should be suggested.

Therefore, it was attempted to identify sensitive organisms based on the structural similarity of the pharmaceutical target to the similar target in non-target organisms. It was assumed that non-target organisms with high homology of the amino acid sequence to the target in humans possess a high relative intrinsic susceptibility to the pharmaceutical. To perform these analyses, the SeqAPASS (Sequence alignment to Predict across species susceptibility) program was used. The programm requires the NCBI accession number of the target protein in order to determine organisms with high structural homology. This analysis was performed with each active pharmaceutical ingredient of the three substance classes. Species with high homology were determined and noted.

The literature was furthermore subjected to a quality assessment. Therefore, the CRED (Criteria for reporting and evaluating ecotoxicity data) system was adopted to the requirements of the study. Information on test species, test and culture conditions, exposure time and schedules, suitable endpoints and statistical analysis, information on source and chemical characteristics of the test compounds and suitable chemical analysis should be available.

Furthermore, initial predicted environmental concentration for surface water (PECsw) calculations (using default-values and a penetration factor Fpen = 0.01) were performed previous to the definition of a risk assessment strategy. These calculations served to identify those substances with PEC values below the action limit and would have been thus exempted from a risk assessment according to the EMA guideline. However, this analysis showed that most of the substances would exceed a PEC of 0.01 µg/L. Thus, the risk would be minimal that a high number of substances would be exempted, provided that no refinement of the PEC would be performed.

The literature research to alternative test strategies led to the following results.

For the substance class ‘neurology’, 50 substance with neuroactive MoA were identified. The therapeutical effects of substances were based on their effects on neurotransmitters and their receptors. The disturbance of electrical and chemical signal transduction is the most relevant ecotoxicological endpoint. Relevant targets were for example the dopamine receptors, serotonin receptors, AMPA receptors, acetyl choline receptors or GABA receptors. The SeqAPASS analysis identified fish and invertebrates to possess structural homologies to the human targets.

The literature research furthermore demonstrated that the studies required by the EMA guideline are most likely not sufficient to predict the risk of neuroactive substances. Literature studies demonstrated that behavioural assays resulted in effects in the µg/L range. The studies were performed with fish, amphibians, and invertebrates, with no preference for one species. As alternative to the studies required by the guideline, behavioural assays like the photomotor response with fish or the determination of the phototactic behaviour in daphnids are suggested.

The class of oncologically active substances was divided into two types, i.e. the cytotoxic and the cytostatic substances. Cytotoxic substances lead to effects on DNA replication and cell growth, while cytostatics act on kinases and thus influence the metabolic processes. The SeqAPASS analysis identified fish, water fleas, and fruit flies with high homology to the human target.

The literature research showed that studies investigating the ecotoxicological potential of cytostatics and cytotoxics were mainly standard studies according to OECD test guidelines. These consisted of acute studies as well as studies assessing the reproduction of test organisms. Furthermore, studies assessing the genotoxicity in ecotoxicologically relevant test systems were performed. It was determined that actively dividing organisms are more susceptible to oncologically active substances. As especially cytotoxics act on DNA rather than a specific target, no specific effects were predicted. Actively dividing organisms are for example algae, the macrophyte *Lemna sp*. Or the water flea *Ceriodaphnia dubia*. Another rapidly dividing organism is the embryo of the zebrafish, which could be used to determine teratogenic effects.

Cardiologically active substances are mainly applied for the treatment of hypertension, as antithrombotic agents and lipid lowering agents. Targets are for example the adrenergic receptor (beta blockers), the angiotensin II receptor (sartans) or the HMG-CoA reductase (statins). The SeqAPASS analysis identified fish as most sensitive group. For the HMG-CoA reductase and calcium channels, invertebrates were also determined to be sensitive.

The literature research showed that no standard endpoints were reported for the model substances. Interestingly, some higher plants express a protein similar to the HMG-CoA reductase. It was indeed shown that the macrophyte *Lemna gibba* is sensitive to statins. For beta blockers, the determination of the glycogen level in zebrafish liver cells seems to be suitable. However, this would be considered as vertebrate study. Furthermore, beta blockers can be tested with bivalves, as these respond by a reduced growth. The determination of the heart rate could be determined with zebrafish embryos as well as with the water flea *Daphnia magna.*

Work package 3

In work package 3, substances of each substance class were chosen to compare an ERA according to the EMA guideline to the suggested alternative test systems. Therefore, two criteria for selection of substances were set:

- ERA data for algae, daphnia and fish are available
- Literature data for model substances with the same MoA are available

For the substance class ‘neurology’, most data were available for model substances acting on sodium channels, serotonin receptors, and dopamine receptors. Based on this information, the substances Vortioxetine, Iloperidone (both serotonin & D2 receptors), Lorcaserin (5HT2C serotonin receptor) and Eslicarbazepine (sodium channels) were chosen. In order to determine if the suggested strategy could also be applied to substances with a different MoA, the substances Dimethyl fumarate (HO-1) and Varenicline (acetyl choline receptors) were chosen.

For the substance class ‘oncology’, mostly data to the group of cytostatics were available. In this case, three substances with identical MoA were identified, namely Palbociclib, Ribociclib, and Abemaciclib (CDK4/CDK6 kinases). Choosing these substance would allow a direct comparison of substances with similar MoA. Substances with effects on other kinases, like Bosutinib and Vandetanib, could be chosen additionally. For the group of cytotoxic substances, Cabazitaxel (beta tubulin) was identified as potential test substance. Another cytotoxic substance, for which however no data of a model substance exists, would be Panobinostat (deacetylase).

For the substance class ‘cardiology’ the selection of substances was reduced, as there were only few for which both criteria were applicable. Tow substances were identified, Pitavastatin (HMG-CoA reductase) and Valsartan (angiotensin receptor). Substances with rather unknown ecotoxicological effects would be Apixaban, Edoxaban, and Dronedarone HCl, which all target the enzyme factor Xa.

Discussion

During the literature research it was determined that only few information on ecotoxicological effects of pharmaceuticals of the new generation is available. Thus, data from model substances were considered in order to be able to define new test strategies.

Furthermore, it was difficult to determine which test strategy results in the most sensitive results, as results and sensitivities varied between the model substances.

The focus of the study was on the assessment of ecotoxicological effects and the development of a test strategy for the assessment of sensitive endpoints on environmental organisms. The most actual version of the EMA guideline describes a very detailed process on how to handly non biodegradable and non-transformable substances of Phase I, depending on the affected compartment (soil or sediment) The results of this process lead to a refinement of the PEC, resulting in a respective labelling of the substance. Thus, it is assumed that the risk to miss potentially persistent pharmaceuticals is small compared to the risk to overlook potential ecotoxicological risks.

Special emphasis should be placed on a tailored risk assessment of biopharmaceuticals, which are substances originating form biological sources. In total, 114 substances belonged to this category. The category was further divided into pharmaceutical antibodies and other proteins/peptides. Antibodies are subjected to a number of instability mechanisms. In the environment, antibodies are more exposed to physical instabilities, so they might persist only for a short time in their native form. Studies assessing the fate of biopharmaceuticals further determined that many were ready degradable and are not classified as persistent.

For the class of siRNAS, the environmental risk is difficult to predict. On the one hand, they are only stable if delivered within a formulation, on the other hand, even this formulation could result in negative effects. This could however not be considered in this project. However, there is also the risk of off-target effects, provoked by an incomplete binding of the siRNA to the mRNA, not resulting in the degradation of the mRNA but to a downregulated expression of the target protein.

The literature review demonstrated that there is still less information to define an effective tailored risk assessment strategy. Alternative approaches are mainly performed with a limited number of model substances, while EPARs only exist for pharmaceuticals approved later than 2006. Thus, to date, alternative approaches are recommended to complement the standard studies, in order to improve the existing data base.

Zusammenfassung

Einleitung

Pharmazeutika stellen ein Problem für die Umwelt und die darin lebenden Organismen dar. Heutzutage können mehrere hundert Wirkstoffe in der Umwelt nachgewiesen werden. Sie kommen sowohl in aquatischen Kompartimenten wie Oberflächengewässern oder dem Grundwasser als auch in terrestrischen Kompartimenten wie dem Boden vor und erreichen dort Konzentrationen im ng/L- bis µg/L-Bereich. In diesen Konzentrationen können bereits Effekte in Umweltorganismen auftreten. Basierend auf diesen Voraussetzungen wurde 2006 eine Richtlinie zur Umweltrisikobewertung von Humanarzeimitteln erlassen. Diese Richtlinie sieht vor, dass Substanzen, deren Umweltkonzentration unter einer Aktionsgrenze von 0.01 µg/L liegt, keiner weiteren Testung auf Verhalten und Wirkung in der Umwelt unterzogen werden, außer es liegen gesicherte Hinweise für eine Wirkung unterhalb der Aktionsgrenze bzw. besonders problematische Eigenschaften (z.B. endokrine Wirkung) vor.

Diese Richtlinie wurde allerdings basierend auf den Wirkungen der Wirkstoffe entwickelt, die vor 2006 entwickelt wurden, soll aber auf die Wirkstoffe angewendet werden, die seitdem auf dem Markt zugelassen wurden. Es besteht die Befürchtung, dass Effekte der Wirkstoffe der neuen Generation durch diese Richtlinie nicht umfassend ermittelt werden können. So können die Wirkstoffe entweder bereits in geringeren Konzentrationen wirksam sein. Eine weitere Gefahr besteht darin, dass die Richtlinie keine Effekte erfasst, die für die jeweilige Wirkweise spezifisch sind. Für solche Gegebenheiten soll eine zugeschnittene Bewertungsstrategie entwickelt werden. Ein Beispiel für eine Substanzgruppe, für die eine angepasste Umweltrisikobewertung bereits vorgesehen ist, sind die endokrinen Disruptoren, für die bekannt ist, dass sie bereits in wesentlich geringeren Konzentrationen wirken und damit die Aktionsgrenze das Risiko unterschätzen würde. Analog dazu sollen zu weiteren Substanzgruppen zugeschnittene Bewertungsstrategien basierend auf einer Literaturrecherche definiert werden.

Arbeitspaket 1

Im Arbeitspaket 1 wurde zunächst eine Strategie entwickelt, um nach neuen innovativen Pharmazeutika zu suchen, die seit 2006 zugelassen wurden. Dazu sollten Literatur-Datenbanken sowie Online-Portale genutzt werden, um Wissen zu neuen Wirkstoffen für Human-Pharmazeutika zu erlangen. Eine der Quellen, die genutzt wurde, war der jährlich publizierte Report zu den neu durch die FDA (Food and drug administration) und die EMA (European medicine agency) zugelassenen Substanzen, der in der Zeitschrift Nature Reviews Drug Discovery erscheint. Da bekannt ist, dass der Forschungs- und Entwicklungsprozess für neue Wirkstoffe länger als 10 Jahre dauern kann, wurde als weitere Quelle das Online-Portal ClinicalTrials.gov genutzt. Diese Quelle lieferte weitere Substanznamen, die in Zukunft als neue Wirkstoffgruppen auf den Markt kommen können.

Ein zweiter Fokus des Arbeitspakets 1 war die Zuordnung von bereits verfügbaren Daten zu potentiellen ökotoxikologischen Effekten aus anderen Datenquellen. So wurden die European Public Assessment Reports (EPAR) der EMA Webseite mit den vorhandenen Daten verknüpft. Daten von PubChem sowie Verknüpfungen zu der DrugBank wurden für detaillierten Informationen zu ökotoxikologischen Effekten, soweit vorhanden, eingefügt.

Insgesamt wurden ca. 470 neu zugelassene Pharmazeutika identifiziert. Substanzen, die sich in der Klinischen Phase 3 befinden, wurden zwar erfasst, aber nicht weiter klassifiziert, um die Datenmenge in einem übersichtlichen Rahmen zu halten. Die seit 2006 neu zugelassenen Wirkstoffe wurden anschließend in Substanzklassen basierend auf ihrer medizinischen

Anwendung gruppiert. Die Gruppen, denen die meisten der Substanzen zugeordnet wurden, waren ‚Onkologie', ‚Neurologie' und ‚Infektionen'. Da für Antibiotika allerdings bereits ein spezifisches Vorgehen vorgeschlagen wird, wurde als weitere Gruppe die ‚Kardiologie' für eine Definition einer zugeschnittenen Bewertungsstrategie ausgewählt.

Im nächsten Schritt wurden alternative Teststrategien für die identifizierten Substanzgruppen ‚Neurologie', Onkologie' und ‚Kardiologie' identifiziert. Da für die jeweiligen Substanzen keine Studien in der Literatur zu finden waren, wurde nach Studien mit passenden Modellsubstanzen gesucht. Eine Substanz wurde als Modellsubstanz definiert, wenn sie auf eine identische Zielstruktur wirkt und zur gleichen pharmazeutischen Indikationsgruppe gehört. Der Fokus der Literaturrecherche lag auf der ökotoxikologischen Risikobewertung.

Arbeitspaket 2

Im Arbeitspaket 2 sollten die Ergebnisse des Arbeitspakets 1 mit der existierenden EMA-Richtlinie zur Umweltrisikobewertung von Humanarzneimitteln (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2) abgeglichen werden und spezifische Ansätze für eine Risikobewertung vorgeschlagen werden.

Dazu wurde zunächst versucht, basierend auf der strukturellen Ähnlichkeit der Zielstruktur im Menschen zu der in anderen Nicht-Ziel-Organismen besonders sensitive Organismen zu identifizieren. In diesem Ansatz wird davon ausgegangen, dass Nicht-Ziel-Organismus mit einer hohen Homologie der Aminosäuresequenz zur Zielstruktur beim Menschen eine hohe intrinsische Empfindlichkeit zum Pharmazeutikum besitzen. Dazu wurde das SeqAPASS (Sequence alignment to predict across species susceptibility) Programm genutzt. Dazu wurde die NCBI accession number des Zielmoleküls einer Substanz im Menschen in das Programm eingegeben. Organismen mit hoher Homologie der Struktur wurden erfasst. Diese Analyse wurde mit jedem Wirkstoff der drei Klassen durchgeführt. Die Spezies mit der größten Homologie wurden jeweils ermittelt und notiert.

Die Literatur zu alternativen Teststrategien wurde im Arbeitspaket 2 in einem weiteren Schritt auf ihre Qualität geprüft. Dazu wurden die CRED (Criteria for reporting and evaluating ecotoxicity data) an die Anforderungen unserer Studie angepasst. So mussten Informationen zur Testart, Test- und Kulturbedingungen, Expositionszeit und –Plänen, Endpunkten und entsprechende Statistik, Informationen zur Testsubstanz und zur chemischen Analytik vorliegen.

Weiterhin wurden vor der Definition einer Bewertungsstrategie erste Kalkulationen für die Umweltkonzentrationen in Oberflächengewässern (Predicted Environmenal Concentration in surface water, PECsw) vorgenommen. Diese initialen Kalkulationen (unter Verwendung des Default-Wertes für den Penetrationsfaktor Fpen = 0.01) sollten solche Substanzen identifizieren, die unter dem Aktionsgrenze liegen und somit keiner Risikobewertung nach EMA-Richtlinie unterzogen würden. Allerdings zeigte diese Analyse, dass der Großteil der Wirkstoffe basierend auf dieser Berechnung über einer Umweltkonzentration von 0.01 µg/L liegen würde. Somit würde, wenn es zu keiner Anpassung der PEC-Berechnung kommen würde, nur ein geringes Risiko bestehen, dass Substanzen überhaupt keiner Risikobewertung unterzogen würden.

Die Literaturrecherche zu alternativen Testsystemen führte zu folgenden Ergebnissen.

Für die Substanzklasse ‚Neurologie' wurden 50 Substanzen mit neuroaktiver Wirkweise identifiziert. Die therapeutischen Effekte der Substanzen basierten auf ihrer Wirkweise auf Neurotransmitter und ihrer Rezeptoren. Von ökotoxikologischer Relevanz ist somit die Störung der elektrischen und Inhibition der chemischen Reizweiterleitung. Relevante Zielstrukturen waren z.B. Dopamin-Rezeptoren, Serotonin-Rezeptoren, AMPA-Rezeptoren, Acetylcholin-

Rezeptoren oder GABA-Rezeptoren. Die SeqAPASS-Analyse ergab, dass Fische und Invertebraten größere strukturelle Homologien zu den menschlichen Zielstrukturen aufwiesen.

Die Literaturrecherche ergab weiterhin, dass für Substanzen der Klasse ‚Neurologie' die Standardstudien nach der EMA-Richtlinie wahrscheinlich nicht ausreichend sind, um das Risiko der neuroaktiven Substanzen zu bewerten. Literaturstudien zeigen, dass Verhaltensstudien zu Effekten im µg/L-Bereich führen. Die Studien wurden mit Fischen, Amphibien und Invertebraten durchgeführt, mit keiner klaren Präferenz zu einer spezifischen Art. Als Alternative zu den Richtlinien werden also Verhaltensstudien wie die Photomotor-Response bei Fischen oder die Bestimmung des phototaktischen Verhaltens bei Daphnien vorgeschlagen.

Die Klasse der onkologischen Substanzen wurde in zwei unterschiedliche Typen von Pharmazeutika unterteilt, in zytotoxische und zytostatische Substanzen. Zytotoxische Substanzen wirken hauptsächlich auf DNA Replikation und Zellwachstum, während Zytostatika durch ihre Wirkung auf Kinasen in den Metabolismus eingreifen. Die SeqAPASS-Analyse ergab, dass Fische, Wasserflöhe oder Fruchtfliegen eine hohe Homologie zu den menschlichen Zielstrukturen aufweisen.

Die Literaturrecherche ergab, dass zur Untersuchung des ökotoxikologischen Potentials von Zytostatika und Zytotoxika hauptsächlich Standard-OECD-Studien durchgeführt und publiziert wurden. Diese beinhalteten sowohl Akutstudien als auch Reproduktionsstudien. Weiterhin wurden Genotoxizitätsstudien mit ökotoxikologisch relevanten Testsystemen durchgeführt. Es wurde festgestellt, dass sich schnell teilende Organismen besonders empfindlich gegenüber onkologischen Substanzen sind. Da gerade zytotoxische Substanzen kein Protein, sondern die DNA als Ziel haben, gibt es hier wenig spezifische Effekte, sondern den generellen Mechanismus der Inhibition der Zellteilung. Organismen mit schneller Zellteilung sind zum Beispiel Algen, die Makrophyten-Art *Lemna sp*. oder der Wasserfloh *Ceriodaphnia dubia*. Ein weiterer, sich schnell entwickelnder Organismus ist der Embryo des Zebrafisches, der zur Bestimmung von teratogenen Effekten herangezogen werden kann.

Die kardiologischen Substanzen werden hauptsächlich zur Behandlung von Bluthochdruck und Thrombose oder zur Cholesterinsenkung eingesetzt. Zielstrukturen sind zum Beispiel adrenerge Rezeptoren (Beta-Blocker), der Angiotensin II Rezeptor (Sartane), oder die HMG-CoA-Reduktase (Statine). Die SeqAPASS-Analyse identifizierte Fische als sensitivste Gruppe. Für die HMG-CoA-Reduktase und Kalziumkanäle sind aber auch Invertebraten als sensitiv identifiziert.

Die Literaturrecherche ergab, dass für die Modellsubstanzen keine Daten zu Standard-Endpunkten vorlagen. Interessanterweise besitzen Pflanzen aber eine ähnliche Struktur wie die HMG-CoA-Reduktase. So ist zum Beispiel die Makrophytenart *Lemna gibba* tatsächlich empfindlich gegenüber den Statinen. Für Beta-Blocker bietet sich als Testsystem die Messung des Glykogengehalts in der Leber von z.B. Zebrafischen an. Allerdings würde es sich hierbei um eine Tierversuchsmethode handeln. Weiterhin kann für blutdrucksenkende Mittel die Muschel als Testorganismus dienen, da diese ein verringertes Wachstum durch Beta-Blocker zeigt. Die Erfassung der Herzschlagrate kann sowohl im Zebrafisch-Embryo als auch im Wasserfloh *Daphnia magna* durchgeführt werden.

Arbeitspaket 3

Im Arbeitspaket 3 wurden Substanzen jeder Substanzklasse ausgewählt, um damit die Risikobewertung nach der EMA-Richtlinie mit den vorgeschlagenen alternativen Testsystemen zu vergleichen. Dabei wurden zwei Kriterien zur Auswahl der Substanzen gesetzt:

- ERA Daten zu Alge, Daphnie und Fisch sollten vorliegen

- Zum spezifischen Wirkmechanismus der Substanz sollten Literaturdaten zu Modelsubstanzen in den einzelnen Testsystemen vorliegen

Für die die Substanzgruppe ‚Neurologie' lagen die meisten Daten für Modellsubstanzen mit Wirkungen auf den Natrium-Kanal, den Serotonin-Rezeptor und den Dopamin-Rezeptor vor. Basierend auf diesen Informationen wurden die Substanzen Vortioxetin, Iloperidon (beide Serotonin- und D2-Rezeptor), Lorcaserin (5HT2C Serotonin-Rezeptor) und Eslicarbazepin (Natrium-Kanal) ausgewählt. Um abzuschätzen, ob die vorgeschlagenen Strategien auch für Substanzen mit anderer Wirkweise gültig sind, wurden zusätzlich die Substanzen Dimethylfumarat (HO-1) und Vareniclin (Acetylcholin-Rezeptor) ausgewählt.

Für die Substanzgruppe ‚Onkologie' lagen hauptsächlich Daten zu der Gruppe der Zytostatika vor. Hier konnten drei Substanzen mit identischem Wirkungsort identifiziert werden, nämlich Palbociclib, Ribociclib und Abemaciclib (CDK4-, CDK6-Kinase). Dies ermöglicht einen direkten Vergleich der Substanzen und ermöglicht eine Aussage, ob Substanzen mit identischer Wirkweise ähnlich auf Nicht-Ziel-Organismen wirken. Bosutinib und Vandetanib wirken auf andere Kinasen, könnten also zusätzlich zur Testung heran gezogen werden. Für die Gruppe der zytotoxischen Substanzen wurde die Substanz Cabazitaxel (Beta-Tubulin) als potentielle Testsubstanz identifiziert. Als weitere zytotoxische Substanz, für die allerdings keine Daten einer Modellsubstanz vorlagen, wurde Panobinostat (Deacetylase) ausgewählt.

Für die Substanzgruppe ‚Kardiologie' reduzierte sich die Auswahl der Substanzen, für die beide Kriterien zutrafen, auf zwei Wirkstoffe, nämlich Pitavastatin (HMG-CoA-Reduktase) und Valsartan (Angiotensin-Rezeptor). Als Substanzen mit eher unbekannter Wirkweise wurden Apixaban, Edoxaban und Dronedaron HCl ausgewählt, die alle drei auf den Enzymfaktor Xa wirken.

Diskussion

Während der Literaturrecherche wurde festgestellt, dass nur sehr wenige Informationen zu den ökotoxikologischen Effekten der Pharmazeutika der neuen Generation vorliegen. Aus diesem Grund wurden Daten zu Modellsubstanzen herangezogen, um neue Teststrategien zu definieren.

Weiterhin stellte es sich als schwierig heraus, basierend auf den Daten der Modelsubstanzen vorherzusagen, welcher Testansatz die sensitivsten Ergebnisse liefern wird, da die Ergebnisse und Sensitivitäten stark variierten.

Der Fokus der Studie lag auf der Erfassung der ökotoxikologischen Effekte und der Entwicklung einer Teststrategie zur Erfassung sensitiver Effekte auf Umweltorganismen. Die aktuelle EMA-Richtlinie beschreibt bereits einen sehr detaillierten Prozess, wie nicht abbaubare und nicht transformierbare Substanzen der ersten Phase, je nach betroffenem Kompartiment (Boden oder Sediment) folgend untersucht werden müssen. Die Ergebnisse dieser Studien führen zu einer Anpassung des PEC und einer entsprechenden Kennzeichnung der Substanz. Somit wird postuliert, dass das Risiko, potentiell persistente Wirkstoffe zu übersehen, vergleichsweise klein ist im Vergleich zur Gefahr, potentielle ökotoxikologische Risiken zu übersehen.

Spezielle Beachtung sollten im Rahmen der zugeschnittenen Risikobewertung auch die Biopharmazeutika, also Substanzen, die aus biologischen Quellen stammen, erhalten. Insgesamt fielen 114 Substanzen in diese Kategorie. Dabei wurde noch einmal zwischen pharmazeutischen Antikörpern und anderen Proteinen/Peptiden unterschieden. Antikörper unterliegen einer Vielzahl von Instabilitäts-Mechanismen. In der Umwelt sind Antikörper besonders physikalischen Instabilitäten (z.B durch pH-Änderungen oder Phytolyse) ausgesetzt, sodass sie nur für einen kurzen Zeitraum in ihrer nativen Form vorliegen. Untersuchungen zum

Umweltverhalten von Substanzen haben weiterhin ergeben, dass viele der Biopharmazeutika schnell abgebaut werden und als nicht persistent eingestuft werden.

Für die Klasse der siRNAs kann das Umweltrisiko bislang noch sehr schlecht vorausgesagt werden. Einerseits sind sie nur in Formulierung stabil verabreichbar, aber auch die Formulierungen selber können zu negativen Effekten in der Umwelt führen. Diese sind aber in dieser Fragestellung nicht zu bearbeiten. Allerdings besteht bei der siRNA die Gefahr von Nebenwirkungen, ausgelöst durch unvollständige Bindung der siRNA an die mRNA. Diese Bindung führt somit nicht zum Abbau der mRNA, kann aber zu einer verminderten Expression des Zielmoleküls führen.

Die Literatursuche zeigte, dass für eine effektive Definition von angepassten Bewertungsstrategien noch eine ungenügende Datenbasis vorliegt, da alternative Strategien mit nur wenigen Modellsubstanzen durchgeführt wurden und die EPARs lediglich für die Pharmazeutika nach 2006 vorliegen. Aus diesem Grund wird vorgeschlagen, alternative Strategien als Ergänzung zu den Standard-Studien zu empfehlen, um die Datenlage zu verbessern.

1 Introduction

Since the 1990s pharmaceuticals were identified to exert environmental effects and since then the number of available monitoring and effect studies has increased steadily. Today, several hundred active pharmaceutical ingredients have been found in sewage water, surface water, groundwater, soil, air, or biota in concentrations from ng/L to more than µg/L (Agerstrand, Berg et al. 2015). In this concentration range the active pharmaceutical ingredients can lead to adverse effects in aquatic or terrestrial organisms and their respective population. There are several examples of active pharmaceutical ingredients to cause effects on organisms in the environment, e.g. the estrogenic substance ethinylestradiol causing impaired reproduction in fish (Jobling, Nolan et al. 1998) or the nonsteroidal painkiller diclofenac which caused a collapse of the vulture population in India (Oaks, Gilbert et al. 2004). Thereupon the European Medicines Agency (EMA) released the guideline on the environmental risk assessment of medicinal products for human use in 2006 (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2). The guideline describes a tiered approach. In Phase I of the risk assessment procedure the predicted environmental concentrations for surface water are estimated, while Phase II includes the environmental fate and effects on organisms.

This guideline was designed for pharmaceuticals for human use which were developed before 2006, but is applied for newly developed pharmaceuticals. Additionally, environmental risk assessment (ERA) for vitamins, electrolytes, amino acids, peptides, proteins, carbohydrates, and lipids may consist of a justification for not submitting studies since they are considered unlikely to result in significant risk to the environment. However, the guideline is under review to date in order to address concerns that were raised in the meantime and furthermore, to account for the increasing numbers of new molecular entities.

For pharmaceuticals of the next generation the required tests in the EMA guideline can be obsolete, as the pharmaceuticals become more and more specific in their effectiveness to reduce the risk of unwanted side effects. However, due to the high specificity and potency of the active pharmaceutical ingredients, effects on non-target organisms (non-human) can already occur at very low concentrations.

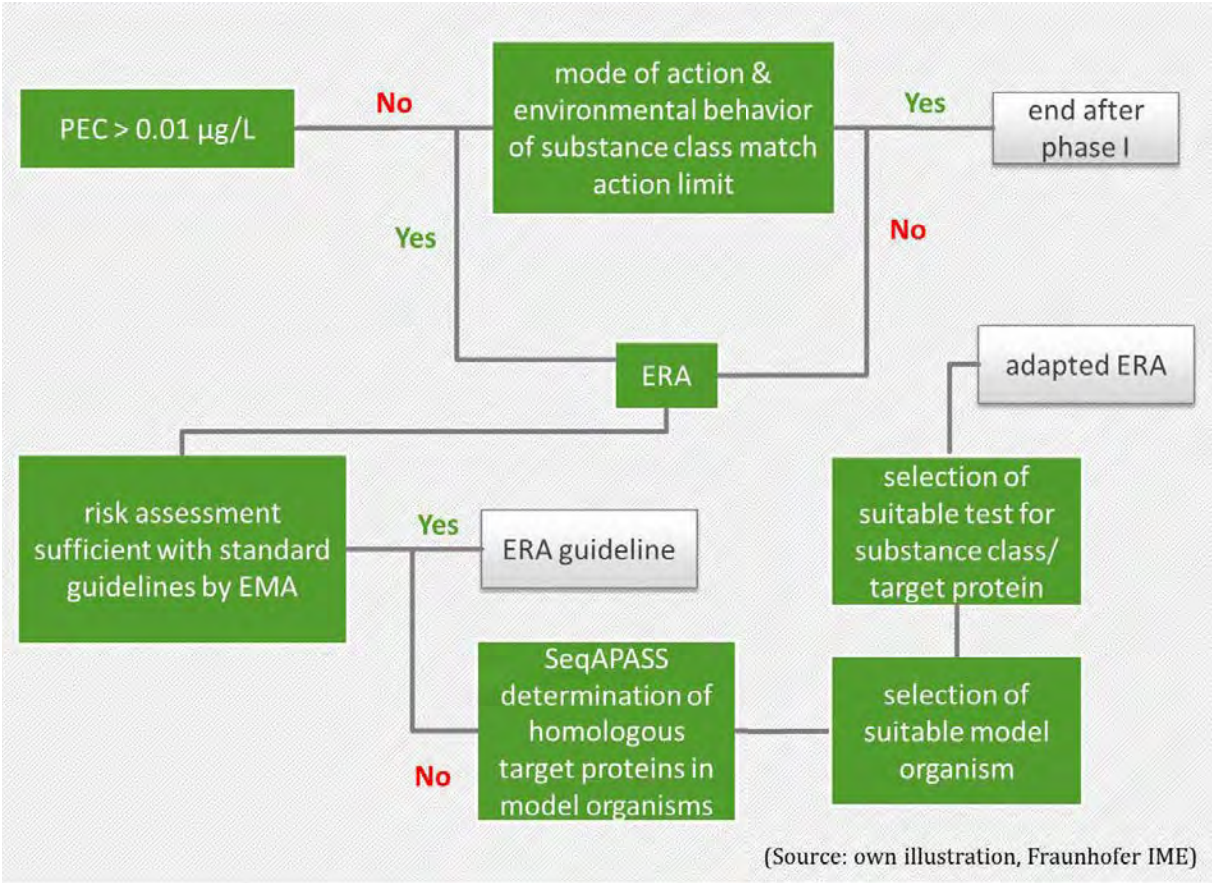
The environmental risk of new molecular entities (NMEs) or biopharmaceuticals (i.e. biologicals) like antibiotics, antidepressants, immunosuppressive drugs and antifungal active pharmaceutical ingredients might not be sufficiently assessed as the guideline does not account for the specific modes of action.

In general it is described by the EMA guideline, that if the predicted environmental concentration in surface water (PECSW) is below the action limit of 0.01 µg/L and no other environmental concerns are apparent, it is assumed that the medicinal product in unlikely to represent a risk for the environment following its prescribed usage in patients (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2).

An exception of this PEC action limit are e.g. substances which may affect the endocrine system or reproduction of vertebrates or lower animals at concentrations lower than 0.01 µg/L. Endocrine disruptors (ED) are assigned to the category of so-called 'however' substances, which pose a potential risk at concentrations below the action limit. Environmental risk assessment has to be performed even though the environmental concentrations are low. For EDs, a tailored risk assessment (TRA) strategy should be applied. Regardless of the limit, a tailored risk assessment is already required for specific substances with specific properties. For example, antibiotics should be evaluated using the more sensitive blue-green algae instead of green algae.

Different types of newly developed pharmaceuticals can potentially exhibit an effect on the environment at concentrations below the threshold value of 0.01 µg/L and probably the risk assessment for these substances according to the EMA guideline is not sufficient, as non-standard test organisms or –endpoints may be more sensitive compared to the standard test organisms described in the EMA guideline. Also for these substances a tailored risk assessment would be necessary.

Figure 1: Decision tree for the suggested tiered risk assessment of human pharmaceuticals



The decision tree for the TRA for pharmaceuticals shows each step of the evaluation of the pharmaceuticals in this study. The included steps are described in detail in the following sections.

2 Work package 1

In work package 1, a strategy was developed to search for new innovative human pharmaceuticals on the market since 2006 in literature databases as well as online portals to gain knowledge about new active ingredients for human pharmaceuticals. One of the resources used for an overview was the yearly published report on new approved drugs by Food and Drug Administration (FDA) and European Medicine Agency (EMA) in the journal Nature Reviews Drug Discovery. Knowing that the research and development process for new drugs can last for more than 10 years, another resource for data acquisition was ClinicalTrials.gov. This allocates additional substances which might come up as New Molecular Entities (NMEs) in future.

A second focus of work package 1 was to add already available data from other online repositories regarding potential ecotoxicological effects. We linked European Public Assessment Reports (EPAR) from EMA website to the datasets and added data from PubChem as well as links to the DrugBank for more detailed information on ecotoxicological effects. No other resources were used as offered in the proposal as the high number of records created by the procedure was adequate to discuss the results and develop test strategies (work package 2 and 3).

2.1 Identification of pharmaceuticals approved later than 2006

The following data sources were evaluated for the registration of substances currently approved or in the approval process.

2.1.1 Nature Reviews Drug Discovery

All newly approved substances (a total of 448, 69 of which were oncological preparations as the largest group, see Figure 2) from the Nature Reviews in the period from 2006 to 2018 were manually recorded in an Excel table (see example in Figure 4) and, where possible, manually supplemented with information from the DrugBank (https://www.drugbank.ca/). This second manual process is to be automated as far as possible in the future in order to be able to evaluate newly approved substances more quickly (see 2.1).

2.1.2 Clinical Trials

All completed Phase 3 studies, the last clinical trial phase before approval of a drug by an agency, in the period from 2006 to 07.03.2019 were downloaded as ZIP archives from ClinicalTrial.gov (https://clinicaltrials.gov) and automatically unpacked as XML files via a workflow and extracted the essential information about XPath nodes. This information was prepared in such a way (long texts were shortened to 32760 characters, multiple entries in columns were separated by commas and superfluous spaces were removed) that they can be extracted into an Excel table. The resulting file contains 14155 studies (see Figure 4) and was cleaned up in a further step (see 2.2).

2.1.3 European Public Assessment Reports (EPAR)

From the website of the EMA all human relevant EPA reports (https://www.ema.europa.eu/en/medicines/download-medicine-data#european-public-assessment-reports-(epar)-section) were downloaded as Excel files and the contained links were activated as click-able hyperlinks. Different approaches are conceivable for linking the data with the other data collections (see 2.3). In addition, the European PubMed Central searched for 'EPAR' or 'European Public Assessment Reports' and saved the results as an Excel file.

2.1.4 WikiPharma Database

The WikiPharma database (http://www.wikipharma.org/welcome.asp) was downloaded as a complete MS Access database (APIdb.mdb) and the contained data was transferred via SQL states via workflow into a common table and stored as an Excel file. For linking the data with the other data sources see 2.3.

2.2 Proposed future actions

The extensive information from the various sources listed (in particular PubChem and DrugBank) should be reduced to the necessary and thus manageable level.

2.2.1 Supplementing Nature Review data with additional information from Pub-Chem and DrugBank

The manual addition of data from the DrugBank is error-prone and time-consuming. Therefore it is suggested that this manual procedure should be automated by a workflow. The first preliminary work by creating a parser for PubChem has already been done. Unfortunately, the format of the PubChem website changes again and again, so that the parser must be adapted accordingly each time the website is changed.

A key for testing access to the DrugBank via application programming interface (API) has already been provided. This access method does not work yet. A solution will be sought in the near future. A permanent access to the API would also cost 300 €/month. If the license expires, all downloaded data (except the links to the DrugBank) must be deleted.

2.2.2 Cleaning up the ClinicalTrials records

To reduce the size of the data set in order to achieve a better overview for manual review, the following steps are suggested:

- Marking or hiding/deleting studies on
  1. medical devices [841 studies] (no substance applied)
  2. vaccines [759 studies] (prospective application, effect on infectious agent)
  3. substances with endocrine potential [21] (TRA already applied).
- Marking or hiding/deleting studies with multiple substances (Bridging Studies/combination studies) [7134 Drug Studies] (no substance-specific effect)
- Mark or hide/delete other studies (Behavioural, Device, Other, Genetic, Combination Product, Dietary Supplement, Procedure, Radiation, Diagnostic Test) (no substance-specific effect).

Thus still 2202 Drug + 417 Biologicals studies with only one specific substance remain.

2.2.3 Linking the different data sources with each other.

The connection of the different data sources can take place over different mechanisms. On the one hand, a macro-based linking of different Excel files or the implementation of a real database would be conceivable. The proposal would be to use the community version of orientDB (https://orientdb.org/).

Additional tested sources, which might be included in future versions but require extra programing efforts as there are no APIs available

- ► US EPA DSST: https://www.epa.gov/chemical-research/distributed-structure-searchable-toxicity-dsstox-database
- FDA Professional Drug Information: https://www.drugs.com/pro/

2.3 Categorization of Pharmaceuticals

The here given pharmaceuticals were already grouped into 'Substance Classes'. These classes describe their field of application in a medicine context (for example Cardiology, Pain, Urology, and Neurology).

For many substance classes, several classes were combined, e.g. Neurology also includes the classes Neurology – Endocrinology and Psychiatry. Approximately 25 substances could not be grouped, as no clear field of application nor was the substance class given.

In Table 1 the major substance classes are given, as well as the combined classes. The majority of the substances belonged to the categories Oncology (19%), Infections (19%) and Neurology (12%) (Figure 2). The category infections was exempted, as these category mainly involves antibiotics. The revised draft guideline on the environmental risk assessment of medicinal products for human use suggest a tailored risk assessment focusing on the effect on lower trophic levels including bacteria, algae and aquatic invertebrates, as scientific knowledge and empirical data demonstrate that these tests are sufficiently sensitive for antibiotics. Thus, it was decided to put emphasis on a substance class of higher concern. Thus, the category Cardiology was included.

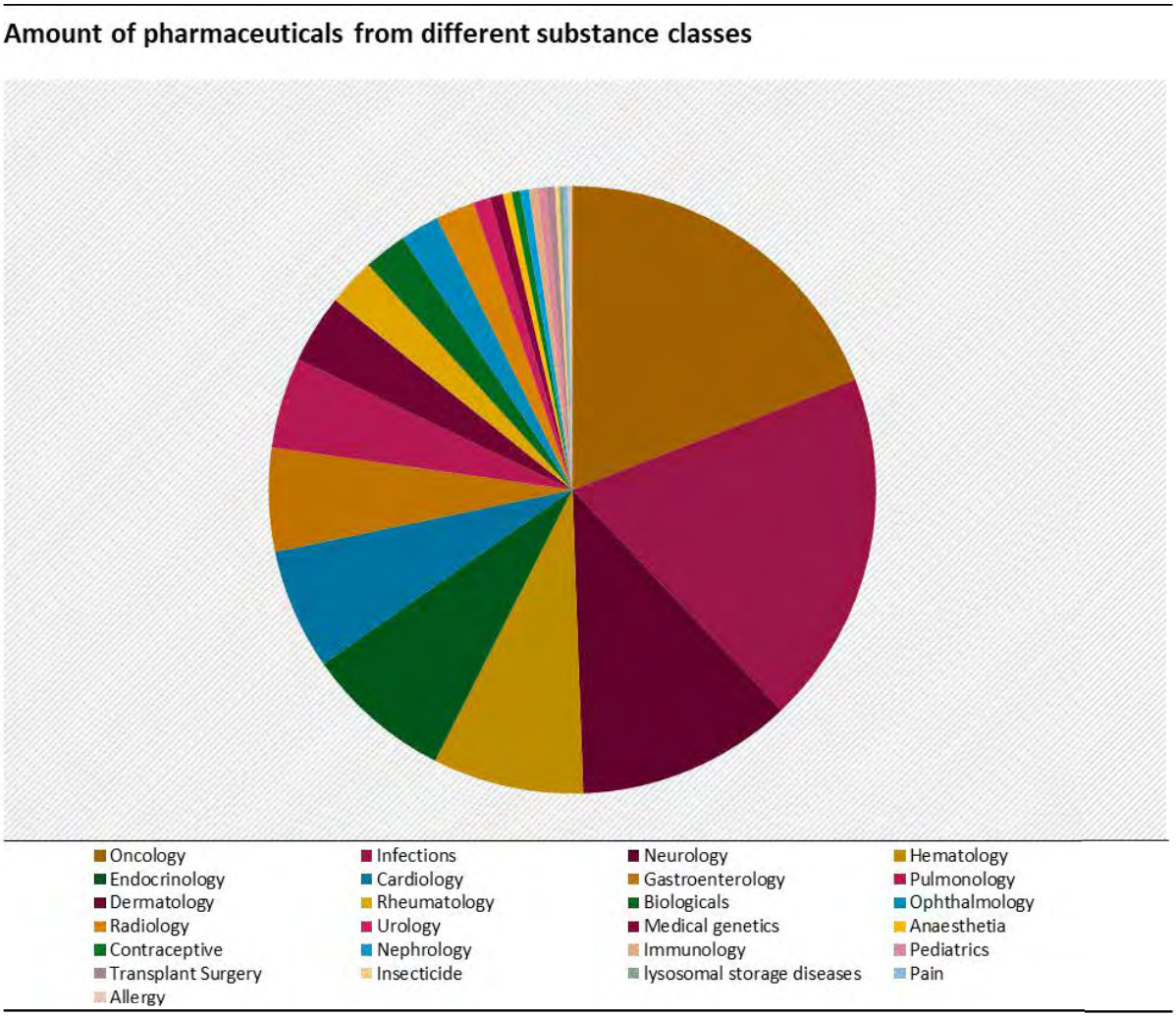
Table 1: Substance classes for the categorization of pharmaceuticals and their number

Substance class	Included terminology	Number of substances
Allergy	Allergy, Immunology, Dermatology	1
Anaesthetia	Anaesthetia Anesthesiology	1 1
Biologicals	Biologicals Biosimilars	7 3
Cardiology	Cardiology	28
Contraceptive	Contraceptive	2
Dermatology	Dermatology Dermatology, Rheumatology Dermatology, Medical Genetics Dermatology, Gastroenterology, Rheumatology Dermatology, Oncology	10 2 1 1 2
Dietary and Nutritional Therapy	Dietary and Nutritional Therapy	1
Emergency medicine	Emergency medicine	1
Endocrinology	Endocrinology	35
Gastroenterology	Gastroenterology, Rheumatology	24
Gene therapy	Gene therapy	1

Substance class	Included terminology	Number of substances
General surgery	General surgery	1
Gynecology	Gynecology	6
Hematology	Hematology Hematology, Oncology Hematology, Immunology, Neurology, Rheumatology, Medical genetics, Endocrinology Internal medicine, Endocrinology, Cardiology, Gastroenterology Hematology, Cardiology	11 21 1 1 1
Immunology	Immunology, Dermatology, Infections (viral), Endocrinology Immunology, Angiology	2
Infections	Infections (viral) Infections (parasitic) Infections (bacterial) Infections (fungal)	42 5 26 6
Inflammation	Inflammation	3
Insecticide	Insecticide	1
Lysosomal storage diseases	Lysosomal storage diseases	1
Medical genetics, Pulmonology	Medical genetics, Pulmonology	3
Nephrology	Nephrology	2
Neurology	Neurology, Endocrinology Psychiatry Psychiatry, Psychology Psychiatry, Gynecology	35 3 11 1
Oncology	Oncology Oncology, Dermatology Oncology, Gastroenterology Oncology, Hematology Oncology, Urology	70 5 3 1 4
Ophthalmology	Ophthalmology	9
Pain	Pain	1
Pediatrics	Pediatrics Pediatrics, Obstetrics	1 1
Pulmonology	Pulmonology Pulmonology, Allergy, Immunology Pulmonology, cardiology Respiratory	12 1 4 1
Radiology	Radiology Radiology, Oncology	8 1

Substance class	Included terminology	Number of substances
Rheumatology	Rheumatology Rheumatology, Endocrinology Rheumatology, Gastroenterology Rheumatology, Neurology Rheumatology, Orthopedics	7 1 1 1 1
Transplant Surgery	Transplant Surgery	2
Urology	Urology	4

Figure 2: Proportional amount of pharmaceuticals from different substance classes of indication



Source: own illustration, Fraunhofer IME

The following description is exemplary on pharmaceuticals of the 'Neurology' substance class, will however also applied to the substances classes 'Cardiology' and 'Oncology'. To cluster the pharmaceuticals in a substance class, the name of the target was determined from the 'mechanism of action' description (compare Figure 4). Most of the pharmaceuticals target human/bacterial/viral or fungal proteins as their main mode of action (MoA). Very common target proteins among the group of neuroactive pharmaceuticals are several different receptors,

transporters, ion channels, or enzymes. To define these proteins as 'target groups' (compare Figure 4), they were clustered into superior descriptive groups. For example sodium, calcium and potassium channels are classified as 'ion channel', whereas different subtypes of dopamine receptors are classified as 'dopaminergic'. The substance class of 'Neurology' and 'Psychiatry' possess the following target groups: acetylcholine receptor, adrenergic, binding protein, cytokine receptor, cytotoxic, dopaminergic, enzyme, GABAergic, glutaminergic, hydroxyl radicals, interferons, ion channel, melatonin receptor, monoamine transporter, motor neuron system, neuropeptide, receptor, regulator, RNA interference, serotonergic.

2.3.1 Orphan drugs

Orphan drug status, defined by FDA and adopted by the EMA in 2007, is assigned to a substance which is used to treat rare diseases, e.g. with a patient number of 5 out of 10.000 individuals. The orphan drugs with the respective target are listed in Table 2 and Table 3. For these pharmaceuticals, the PEC has to be reevaluated. However, even though the refined PEC finally result in a value below the action limit, these drugs may pose a risk due to their individual MoA. Furthermore, risks exist as the orphan drugs can be used to treat multiple conditions once the pharmaceutical is approved, increasing the environmental concentration while circumventing the necessity to perform a full ERA. The status as orphan drug thus does not result in an exemption of these substances for defining a TRA for a specific MoA.

The following table illustrates all found orphan drugs of the substance classes 'Neurology', 'Cardiology' and 'Oncology'.

Table 2: List of orphan drugs and their target proteins

Substance Class	Active Component	Target group	Name of target
Oncology	Vorinostat	DNA	deacetylase (DAC)
Oncology	Pralatrexate	DNA	dihydrofolate reductase
Oncology	Romidepsin	DNA	deacetylase (DAC)
Oncology	Belinostat	DNA	deacetylase (DAC)
Cardiology	Riociguat	enzyme	guanylate cyclase (sGC)
Cardiology	Ivabradine	ion channel	HCN channel
Cardiology	Ambrisentan	receptor	Endothelin receptor
Cardiology	Droxidopa	adrenergic	adrenergic receptors
Cardiology	Macitentan	receptor	endothelin receptor
Cardiology	Idarucizumab	receptor	PAR-1
Cardiology	Evolocumab	enzyme	PCSK9
Cardiology	Selexipag	receptor	prostacyclin (IP, PGI2)
Neurology	Cerliponase alfa	enzyme	tripeptidyl peptidase-1 (TPP1)
Neurology	Stiripentol	GABAergic	GABA receptor
Neurology	Abobotulinum-toxin A	acetylcholin receptor	ACh receptor
Neurology	Amifampridine	ion channel	potassium channel
Neurology	Tasimelteon	melatonin receptor	melatonin receptors MT1 and MT2
Neurology	Gabapentin enacarbil	adrenergic	norepinephrine transporter
Neurology	Clobazam	GABAergic	GABA receptor
Neurology	Patisiran	RNA interference	TRR RNA
Neurology	Edaravone	hydroxyl radicals	hydroxyl radicals
Neurology	Nusinersen	motor neuron system	SMN protein
Neurology	Inotersen	RNA interference	TRR RNA

2.4 Literature of ecotoxicological assays for similar substances

A literature research was applied to find beneficial assays to test the here given pharmaceuticals. The search criteria were on one hand the target proteins of the respective pharmaceutical, on the other hand pharmaceutical indication groups, like anticonvulsants or antidepressants in the substance class of Neurology. Identified literature always focused on ecotoxicological risk assessment. Suitable literature was noted in column M (compare Figure 4) with the mentioned substances. Literature was identified as suitable if the tested substances possessed the same MoA. In order to define a model substance (i.e., a substance with similar MoA or similar biological target, not a substance to be tested in a testing strategy for validation), the indication field of the pharmaceutical of interest was integrated into the literature search. The tested

substances were thus classified as potential model substances for the specific MoA. Search terms, which were combined to find literature, were the type of indication (e.g. anti-depressants, epilepsy, and multiple sclerosis), the target protein type (e.g. dopamine receptor, ACh receptor, and sodium channel), name of the substance, similar substances, ecotoxicology, test assays, and exemplary model organisms (e.g. zebrafish, common water flea, algae).

- Appropriate search engines were
- PubMed (http://www.ncbi.nlm.nih.gov/pubmed)
- European PubMed Central (https://europepmc.org/)
- Scopus (https://www.elsevier.com/solutions/scopus)
- Science Direct (http://www.sciencedirect.com/)
- Web of Science (http://apps.webofknowledge.com)
- BioMedCentral (http://www.biomedcentral.com/)
- Google Scholar (https://scholar.google.de/)

In addition, a reverse search was applied to find similar substances to the pharmaceutical of interest. For this purpose, the target protein was searched on DrugBank (www.drugbank.ca). Related drugs' names were listed on DrugBank and used for further literature search.

3 Work package 2

3.1 Homologous action targets (proteins) in test organisms in ecotoxicology (with SeqAPASS)

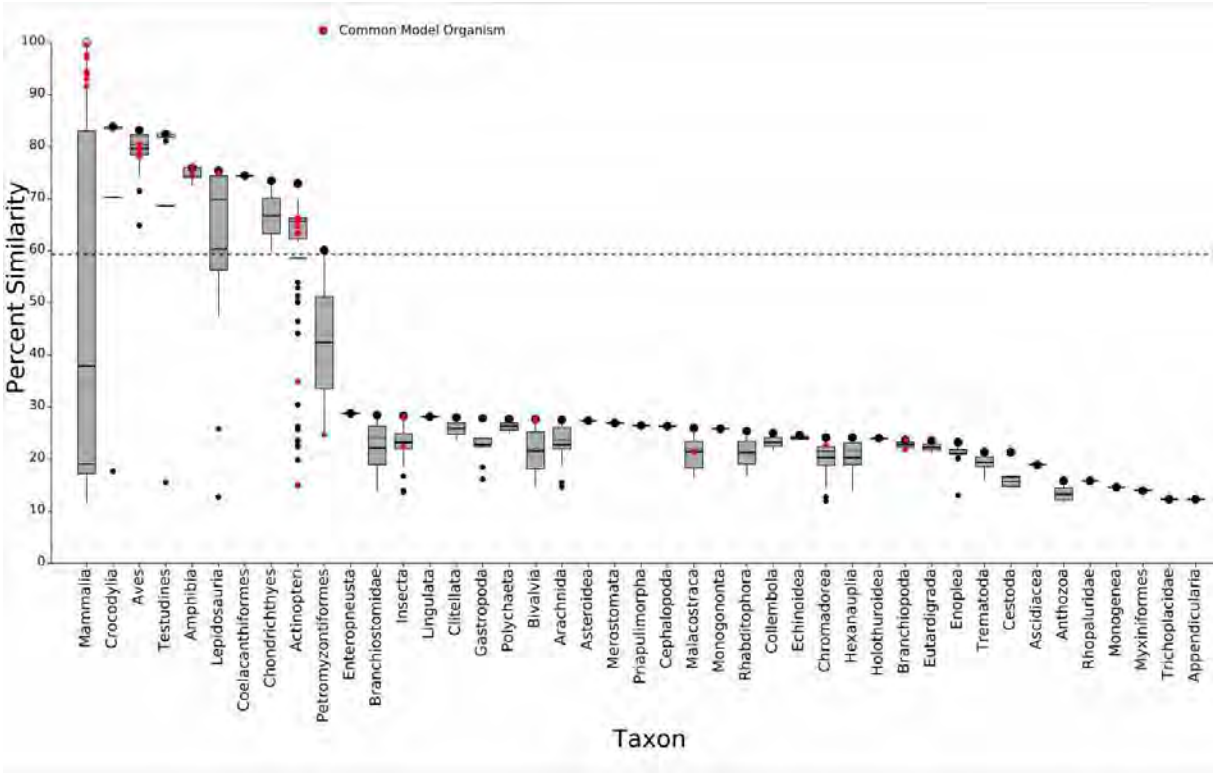
In order to find suitable model organisms for ecotoxicological studies, it was attempted to determine those organisms which show a strong homology in their amino acid sequence code and therefore possess comparable proteins, which is called a relative intrinsic susceptibility to the pharmaceutical. The susceptibility is 'relative', because only the molecular target similarity is considered for the evaluation, for example no further physical or chemical characterizations, or information on the health condition of the organism is considered. The SeqAPASS (Sequence Alignment to Predict Across Species Susceptibility) program compares the sequence of the protein of interest with listed proteins of a multitude of organisms in the NCBI protein database. SeqAPASS is an open access online screening tool, provided by the U.S. Environmental Protection Agency (developed by Dr. Carlie LaLone and the Environmental Modeling and Visualization Laboratory) and can be accessed free of charge. It has been developed to predict across species relative intrinsic susceptibility to chemicals with known molecular targets. It however recognizes that the similarity of the molecular target is only one consideration. Considerations on the life cycle of a chemical are not recognized by SeaAPASS. SeqAPASS allows the comparison of primary amino acid sequence, functional domains and individual amino acid residue positions across species.

The first step was to search the target protein on the protein database (https://www.ncbi.nlm.nih.gov/protein/), in order to receive the 'NCBI accession' number (compare Figure 4). In this database, the amino acid code of proteins (of different organisms) are listed from several research sources.

To compare the target protein with all listed proteins in the database, a SeqAPASS analysis is run. For this, the NCBI accession number is inserted into the field NCBI Protein Accession of a SeqAPASS Submission in the 'Request SeqAPASS Run' tab, where a search 'by Accession' is selected. The 'SeqAPASS Run Status' can be tracked in the correspondent tab. Each SeqAPASS run is given a SeqAPASS Run ID (compare Figure 4). After a successful run, the output is given as a report, which can be accessed. The output data can be visualized in boxplot diagrams (compare Figure 3). For each class of organisms (for example: Actinopterygii, Insecta, Branchiopoda) a boxplot shows the percentage of similarity of proteins, that were found in these organisms to the target protein of the analysis. In the visualization, it is possible to add a highlighting of common model organisms in the figure. Model organisms of each class are marked as a red dot and can be pointed at to read the names. It is also possible to set a Susceptibility Cut- off based on orthologue candidates into the figure (illustrated as a line), which is representing a certain percent similarity of local maxima. Three different cut offs can be chosen from a certain local maximum.

From suitable model organisms for ecotoxicological studies, three organisms with the most conformity were chosen and noted in column L for each SeqAPASS run ID (compare Figure 4). These organisms are recommended for testing the pharmaceutical in assays that are recommended in column N (compare Figure 4; explanation in chapter 3.2).

Figure 3: Exemplary SeqAPASS boxplot of the human Dopamine receptor D2, compared to proteins of the NCBI protein database. Model organisms in taxa are marked in red.



3.2 Recommendations for assays to test pharmaceuticals with given data from literature

The assays and tests with different model organisms, which are beneficial to be applied for environmental risk assessment for the respective pharmaceutical, were noted (compare Figure 4). To choose a suitable assay plus test organism, it is required to take the information on organisms that showed a high homology in their proteins with the target protein of the pharmaceutical and information on the assays for the identification of a matching assay. Several beneficial assays from different literature are described. For example, if a fish embryo test is recommended, a fish species has to be chosen. The description only contains a short name for the assays, further details have to be read in the mentioned literature belonging to the assay.

Literature was reviewed by selected CRED (Criteria for Reporting and Evaluating ecotoxicity Data) (Kase, Korkaric et al. 2016, Moermond, Kase et al. 2016). These criteria include assessments about reliability, relevance and reporting. Selected criteria for the review of literature included available information on the test species, test and culture conditions, exposure time and schedules, suitable endpoints and statistical analysis, information on source and chemical characteristics of the test compounds and suitable chemical analysis. The applied criteria are listed in Table 11. Literature was evaluated by these criteria and final scores from 1 to 4 (1 = reliable and relevant and 4 = not assignable) were given. Only sufficiently detailed literature was used for further evaluation. A summary on the overall evaluation for reliability and relevance is given in Table 12.

3.3 Data on the maximum daily dose per inhabitant

To calculate a PEC for each pharmaceutical, the first step was to collect data on its application dosage on human patients. For this purpose, data from www.rote-liste.de and official brand's

websites on the maximum daily dose per inhabitant was gathered. Maximum daily dose per inhabitant means the maximum recommended dose of the respective pharmaceutical taken by one patient on one day. Some of the pharmaceuticals were taken daily, and some pharmaceuticals are applied by a depot medication, however, the single daily intake was noted here. For those pharmaceuticals, which weren't available on www.rote-liste.de, data on the maximum dosage was received from the respective brands websites. The collected values are given as mg (compare Figure 4).

3.4 Calculation of the PEC values using the maximum daily doses

The PEC values were calculated from following formula:

$$PEC_{SW} = \frac{DOSE_{AS} \times F_{PEN}}{WASTEW_{INHAB} \times DILUTION}$$

DOSEAS ≙ substance specific, maximum daily dose of the active substance consumed per inhabitant [mg/inhabitant/day]

FPEN ≙ fraction of a population receiving the active substance, here: 0.01

WASTEWINHAB ≙ amount of wastewater per inhabitant per day [L/inhabitant/d], here: 200

DILUTION ≙ dilution factor, here: 10

The maximum daily dose values (see 3.3) are inserted for DOSEAS. The remaining values that are part of the PEC calculation are constant factors, which are given in the EMA guideline (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2). The PECs for each pharmaceutical is stated in mg/L and µg/L. In Table 3 the calculated PECs for each pharmaceutical of the Neurology and Psychiatry, Cardiology and Oncology substance classes are noted. Furthermore, the table provides information on the type of chemical, i.e. if the substance belongs to the type of biologicals (e.g. peptides, enzymes, hormones, anti-bodies) or to the type of small synthetic molecules and other available information e.g. log Kow.

Table 3: PEC calculation for the substance class of Neurology, Cardiology and Oncology

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Neurology							
Abobotulinum-toxin A	x	acetylcholin receptor	ACh receptor	0.00000975 mg	4.875E-08	b	
Amifampridine	x	ion channel	potassium channel	60 mg	0.3	s	
Aripiprazole lauroxil		serotonergic & dopaminergic	5HT2A (serotonin) & D2 (dopamine) receptors	1064 mg	5.32	s	
Asenapine		serotonergic & adrenergic	5HT2A (serotonin) & D2 (dopamine) receptors	20 mg	0.1	s	log Kow = 4.77 (est)
Brexpiprazole		serotonergic & dopaminergic	5HT2A (serotonin) & D2 (dopamine) receptors	4 mg	0.02	s	
Brivaracetam		ion channel & binding protein	sodium channel & synaptic vesicle glycoprotein 2A (SV2A)	200 mg	1	s	
Canakinumab						b	
Cariprazine		serotonergic & dopaminergic	dopamine D2 and serotonin 5-HT1A receptors and antagonist activity at serotonin 5-HT2A receptors	6 mg	0.03	s	
Cerliponase alfa	x	enzyme	tripeptidyl peptidase-1 (TPP1)	300 mg	1.5	b	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Clobazam	x	GABAergic	GABA receptor	40 mg	0.2	s	
Daclizumab		cytokine receptor	interleukin-2 receptor	150 mg	0.75	b	
Dalfampridine		ion channel	potassium channel	20 mg	0.1	s	
Desvelafaxine		serotonergic & adrenergic	serotonin & noradrenaline	100 mg	0.5	s	
Deutetrabenazine		monoamine transporter	vesicular monoamine transporter 2 (VMAT2)	12 mg	0.06	s	
Dimethyl fumarate		enzyme	heme oxygenase 1 (HO-1)	480 mg	2.4	s	
Edaravone	x	hydroxyl radicals	hydroxyl radicals	60 mg	0.3	s	
Eslicarbazepine		ion channel	sodium channel	1600 mg	8	s	
Ezogabine		ion channel	potassium channel	1200 mg	6	s	log Kow = 3.57 (est)
Fingolimod		receptor	sphingosine 1-phosphate receptors 1, 3, 4, and 5	0.5 mg	0.0025	s	
Flibanserin		serotonergic	5HT2A (serotonin)	100 mg	0.5	s	log Kow = 3.41 (est)
Fremanezumab		neuropeptide	Calcitonin Gene-Related Peptide (CGRP)	225 mg	1.125	b	
Gabapentin enacarbil	x	adrenergic	norepinephrine transporter	1800 mg	9	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Galcanezumab		neuropeptide	Calcitonin Gene-Related Peptide (CGRP)	240 mg	1.2	b	
Iloperidone		serotonergic & dopaminergic	5HT2A (serotonin) & D2 (dopamine) receptors	24 mg	0.12	s	
IncobotulinumtoxinA		acetylcholin receptor	ACh receptor	0.00000088 mg	4.4E-09	b	
Inotersen	x	RNA interference	TRR RNA	284 mg	1.42	b	
Lacosamide		ion channel & regulator	sodium channel & collapsin response mediator protein-2 (CRMP-2)	600 mg	3	s	0.728 (LogP)
Lisdexamfetamine		adrenergic	norepinephrine and dopamine transporters	70 mg	0.35	s	
Lorcaserin		serotonergic	5HT2C (serotonin)	20 mg	0.1	s	
Lurasidone		serotonergic & dopaminergic, andrenergic	Dopamine-2 (D2), 5-HT2A, 5HT1A, 5-HT7 receptors, alpha-2C adrenergic receptor	160 mg	0.8	s	
Milnacipran HCl						s	
Nitoman		monoamine transporter	monoamine transporter type 2	200 mg	1	s	log Kow = 2.66 (est)
Nusinersen	x	motor neuron system	SMN protein	12 mg	0.06	b	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Ocrelizumab		cytotoxic	CD20	600 mg	3	b	
Paliperidone		serotonergic & dopaminergic	5HT2A (serotonin) & D2 (dopamine) receptors	525 mg	2.625	s	
Patisiran	x	RNA interference	TRR RNA	30 mg	0.15	b	
Peginterferon beta-1A		interferons	beta interferon	0.125 mg	0.000625	b	
Perampanel		glutaminergic	AMPA receptor	12 mg	0.06	s	
Pimavanserin		serotonergic	5HT2A (serotonin)	34 mg	0.17	s	
Rasagiline mesylate		enzyme	MAO-B	1 mg	0.005	s	
Rotigotine		serotonergic & dopaminergic	Dopamine receptor	3 mg	0.015	s	log Kow = 5.39 (est), 4.7 (LogP)
Rufinamide		ion channel	sodium channel	3200 mg	16	s	0.835 (LogP)
Safinamide		enzyme & ion channel	MAO-B & sodium channel & calcium channel	100 mg	0.5	s	
Stiripentol	x	GABAergic	GABA receptor	3500 mg	17.5	s	
Suvorexant		receptor	orexin receptors OX1R and OX2R	20 mg	0.1	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Tasimelteon	x	melatonin receptor	melatonin receptors MT1 and MT2	20 mg	0.1	s	2.43 (LogP)
Teriflunomide		enzyme	dihydroorotate dehydrogenase	14 mg	0.07	s	
Valbenazine		monoamine transporter	vesicular monoamine transporter 2 (VMAT2)	80 mg	0.4	s	
Varenicline		acetylcholin receptor	ACh receptor	2 mg	0.01	s	
Vigabatrin		GABAergic	GABA (gamma-aminobutyric acid transaminase)	3000 mg	15	s	
Vilazodone		serotonergic	5HT1A (serotonin)	40 mg	0.2	s	
Vortioxetine		serotonergic & dopaminergic	serotonin (5-HT)(3A) receptor, h5-HT(7) receptor, h5-HT(1B) receptor, h5-HT(1A) receptor, 5-HT transporter	20 mg	0.1	s	log Kow = 4.94 (est)
Cardiology							
Alirocumab		enzyme	PCSK9	300 mg	1.5	b	
Aliskiren		enzyme	Renin	300 mg	1.5	s	log Kow = 2.45 (pH 7.4), 3.3 (LogP)

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Ambrisentan	x	receptor	Endothelin receptor	10 mg	0.05	s	
Angiotensin II		receptor	angiotensin II receptor	2.5 mg	0.0125	b	
Apixaban		enzyme	factor Xa	20 mg	0.1	s	
Azilsartan		receptor	angiotensin II receptor	80 mg	0.4	s	
Betrixaban		enzyme	factor Xa	160 mg	0.8	s	
Cangrelor		purinergic	P2Y12	144 mg	0.72	s	
Clevidipine butyrate		ion channel	calcium channel	384 mg	1.92	s	
Dabigatran		receptor	PAR-1	300 mg	1.5	s	log Kow = -2.4 (n-octanol buffer, pH 7.4)
Dronedarone HCl		ion channel	sodium, potassim & calcium channels	800 mg	4	s	
Droxidopa	x	adrenergic	adrenergic receptors	1800 mg	9	s	
Edoxaban		enzyme	factor Xa	60 mg	0.3	s	
Evolocumab	x	enzyme	PCSK9	420 mg	2.1	b	
Idarucizumab	x	receptor	PAR-1	5000 mg	25	b	
Ivabradine	x	ion channel	HCN channel	15 mg	0.075	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Macitentan	x	receptor	endothelin receptor	10 mg	0.05	s	
Nebivolol		adrenergic	beta-1 adrenergic receptor	40 mg	0.2	s	4.04 (LogP)
Pitavastatin		enzyme	3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase	4 mg	0.02	s	log Kow = 4.82 (est)
Prasugrel		purinergic	P2Y12	60 mg	0.3	s	
Ranolazine		ion channel	sodium and potassium ion channel	1500 mg	7.5	s	log Kow = 0.51 (est), 1.6 (LogP)
Riociguat	x	enzyme	guanylate cyclase (sGC)	7.5 mg	0.0375	s	
Rivaroxaban		enzyme	factor Xa	30 mg	0.15	s	log Kow = 2.18 (est)
Sacubitril		enzyme	neprilysin	97.2 mg	0.486	s	
Selexipag	x	receptor	prostacyclin (IP, PGI2)	3200 mg	16	s	
Sulfur hexafluoride lipid-type A microspheres				85.7 mg	0.4285	s	
Ticagrelor		purinergic	P2Y12	180 mg	0.9	s	
Tolvaptan						s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Valsartan		receptor	angiotensin II receptor	103 mg	0.515	s	log Kow = 4.00 (average value), 5.8 (LogP)
Vorapaxar		receptor	PAR-1	2.08 mg	0.0104	s	
Oncology							
Abemaciclib		kinase	CDK4, CDK6	300 mg	1.5	s	
Abiraterone		enzyme	CYP17	1000 mg	5	s	
Acalabrutinib		kinase	BTK	200 mg	1	s	
Ado-trastuzumab emtansine		kinase	HER2 (ErbB2)	540 mg	2.7	b	
Afatinib		kinase	EGFR (ErbB1), HER2 (ErbB2), ErbB3 and ErbB4 [L2937]	40 mg	0.2	s	
Alectinib		kinase	ALK	1200 mg	6	s	
Asparaginase Erwinia chrysanthemi		enzyme	asparaginase			b	
Atezolizumab		immune checkpoint	PDL1	1200 mg	6	b	
Avelumab		immune checkpoint	PDL1	1500 mg	7.5	b	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Axitinib		kinase	VEGFR1, VEGFR2, VEGFR3	10 mg	0.05	s	
Belinostat	x	DNA	deacetylase (DAC)	3000 mg	15	s	
Bendamustine hydrochloride		DNA	alkylation	300 mg	1.5	s	
Binimetinib		kinase	MEK1, MEK2	90 mg	0.45	s	
Blinatumomab		antigen	CD19, CD3	0.028 mg	0.00014	b	
Bosutinib monohydrate		kinase	tyrosine kinase BCR-ABL	500 mg	2.5	s	
Brentuximab vedotin		antigen	CD30	180 mg	0.9	b	
Brigatinib		kinase	ALK	180 mg	0.9	s	
Cabazitaxel		tubuli	beta tubulin	75 mg	0.375	s	
Cabozantinib		kinase	VEGFR1, VEGFR2, VEGFR3	140 mg	0.7	s	
Calaspargase pegol		enzyme	asparaginase			b	
Carfilzomib		DNA	20S proteasome	123 mg	0.615	s	
Cemiplimab		immune checkpoint	PD1	350 mg	1.75	b	
Ceritinib		kinase	ALK	450 mg	2.25	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Cobimetinib		kinase	MEK1, MEK2	60 mg	0.3	s	
Copanlisib dihydrochloride		kinase	PI3K	60 mg	0.3	s	
Crizotinib		kinase	ALK	500 mg	2.5	s	
Dabrafenib		kinase	BRAF-serine-threonine kinase	300 mg	1.5	s	
Dacomitinib		kinase	EGFR (ErbB1)	45 mg	0.225	s	
Daratumumab		antigen	CD38	2400 mg	12	b	
Dasatinib		kinase	tyrosine kinase BCR-ABL	140 mg	0.7	s	
Decitabine		DNA	DNA methyltransferase	60 mg	0.3	s	-1.89 (LogP)
Degarelix		receptor	GnRH receptors	240 mg	1.2	s	
Dinutuximab		ganglioside	GD2	52.5 mg	0.2625	b	
Durvalumab		immune checkpoint	PDL1	1500 mg	7.5	b	
Duvelisib		kinase	PI3K	25 mg	0.125	s	
Elotuzumab		antigen	SLAMF7	1500 mg	7.5	b	
Enasidenib mesylate		enzyme	IDH2	100 mg	0.5	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Encorafenib		kinase	BRAF-serine-threonine kinase	450 mg	2.25	s	
Enzalutamide		receptor	androgen receptor	160 mg	0.8	s	
Eribulin		tubuli	beta tubulin	3.69 mg	0.01845	s	
Fluciclovine f-18		/	/			s	
Gallium Ga 68 dotatate		/	/			b	
Gilteritinib		kinase	FLT3	120 mg	0.6	s	
Glasdegib				100 mg	0.5	s	
Glucarpidase		enzyme	methotrexate			b	
Ibrutinib		kinase	BTK	560 mg	2.8	s	
Idelalisib		kinase	P110δ	300 mg	1.5	s	log Koc = 3.88 (est)
Inotuzumab ozogamicin		antigen	CD22	5.4 mg	0.027	b	
Iobenguane I-123		adrenergic	noradrenaline transporter			s	
Ipilimumab		antigen	CTLA4	480 mg	2.4	b	
Ivosidenib		enzyme	IDH1	500 mg	2.5	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Ixabepilone		tubuli	beta tubulin	88 mg	0.44	s	
Ixazomib				40 mg	0.2	s	
Lapatinib		kinase	HER2 (ErbB2)	1500 mg	7.5	s	
Larotrectinib		kinase	TRKA, TRKB, TRKC	100 mg	0.5	s	
Lenvatinib		kinase	VEGFR1, VEGFR2, VEGFR3	18 mg	0.09	s	
Lorlatinib		kinase	ALK	100 mg	0.5	s	
Midostaurin		kinase	FLT3	200 mg	1	s	
Mogamulizumab		receptor	CXCR4	150 mg	0.75	b	
Moxetumomab pasudotox		antigen	CD22	6 mg	0.03	b	
Necitumumab		kinase	EGFR (ErbB1)	800 mg	4	b	
Neratinib		kinase	HER2 (ErbB2)	240 mg	1.2	s	
Netupitant		receptor	NK1, HT3	300 mg	1.5	s	
Nilotinib		kinase	tyrosine kinase BCR-ABL	800 mg	4	s	log Kow = 5.01 (est)
Niraparib		enzyme	PARP	300 mg	1.5	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Nivolumab		immune checkpoint	PD1	480 mg	2.4	b	
Obinutuzumab		antigen	CD20	1000 mg	5	b	
Ofatumumab		antigen	CD20	1000 mg	5	b	
Olaparib		enzyme	PARP	600 mg	3	s	
Olaratumab		receptor	PDGFRα	2250 mg	11.25	b	
Omacetaxine mepesuccinate				3.75 mg	0.01875	s	
Osimertinib		kinase	EGFR (ErbB1)	80 mg	0.4	s	
Palbociclib		kinase	CDK4, CDK6	125 mg	0.625	s	
Palonosetron		receptor	NK1, HT3	0.5 mg	0.0025	s	
Panitumumab		kinase	EGFR (ErbB1)	900 mg	4.5	b	
Panobinostat		DNA	deacetylase (DAC)	20 mg	0.1	s	
Pazopanib HCl		kinase	membrane bound kinases (RET, VEGFR1, VEGFR2, VEGFR3, KIT, PDGFR-alpha, PDGFR-beta, FGFR1, FGFR2, TIE2, DDR2, TrkA, Eph2A, RAF-1, BRAF, BRAFV600E	42 mg	0.21	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
			, SAPK2, PTK5, and Abl) (serine-threonine-kinases)				
Pembrolizumab		immune checkpoint	PD1	400 mg	2	b	
Pertuzumab		kinase	HER2 (ErbB2)	840 mg	4.2	b	
Plerixafor		receptor	CXCR4	40 mg	0.2	s	
Pomalidomide		regulator	cereblon	4 mg	0.02	s	log Kow = -1.16 (est)
Ponatinib		kinase	membrane bound kinases (RET, VEGFR1, VEGFR2, VEGFR3, KIT, PDGFR-alpha, PDGFR-beta, FGFR1, FGFR2, TIE2, DDR2, TrkA, Eph2A, RAF-1, BRAF, BRAFV600E , SAPK2, PTK5, and Abl) (serine-threonine-kinases)	45 mg	0.225	s	
Pralatrexate	x	DNA	dihydrofolate reductase	90 mg	0.45	s	
Ramucirumab		kinase	VEGFR1, VEGFR2, VEGFR3	1500 mg	7.5	b	
Regorafenib		kinase	membrane bound kinases (RET, VEGFR1,	160 mg	0.8	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
			VEGFR2, VEGFR3, KIT, PDGFR-alpha, PDGFR-beta, FGFR1, FGFR2, TIE2, DDR2, TrkA, Eph2A, RAF-1, BRAF, BRAFV600E, SAPK2, PTK5, and Abl) (serine-threonine-kinases)				
Ribociclib		kinase	CDK4, CDK6	600 mg	3	s	
Rolapitant		receptor	NK1	180 mg	0.9	s	
Romidepsin	x	DNA	deacetylase (DAC)	42 mg	0.21	s	
Rucaparib		enzyme	PARP	1200 mg	6	s	
Ruxoltinib		kinase	JAK1, JAK2	50 mg	0.25	s	
Sonidegib				200 mg	1	s	
Sunitinib malate		kinase	multiple kinases	50 mg	0.25	s	
Tagraxofusp		mast cell growth factor	IL3			b	
Talazoparib		enzyme	PARP	1 mg	0.005	s	
Tbo-Filgrastim		hormone	G-CSF	0.75 mg	0.00375	b	
Temsirolimus		kinase	mTOR	175 mg	0.875	s	

Substance	Orphan drug status (x)	Target group	Precise target	Maximum daily dose per inhabitant	PEC_{SW} [µg/L]	Biological (b)/ synthetical (s)	$LogK_{OW}$
Tipiracil		enzyme	thymidine phosphorylase	160 mg	0.8	s	
Trabectedin		DNA	alkylation	4.5 mg	0.0225	s	
Trametinib		kinase	MEK1, MEK2	2 mg	0.01	s	
Trifluridine		enzyme	thymidine phosphorylase	65.5 mg	0.3275	s	
Vandetanib		kinase	RET, VEGFR1, VEGFR2, VEGFR3, EGFR	300 mg	1.5	s	
Vemurafenib		kinase	BRAF-serine-threonine kinase	1920 mg	9.6	s	log Kow = 5.17 (est), 5.1 (LogP)
Venetoclax		regulator	BCL-2	400 mg	2	s	
Vismodegib				150 mg	0.75	s	
Vorinostat	x	DNA	deacetylase (DAC)	400 mg	2	s	
Ziv-aflibercept		kinase	VEGFR1, VEGFR2, VEGFR3	600 mg	3	b	

3.5 Action limit and logKow as criteria for further investigations

The action limit of PECSW is 0.01 µg/L. If the value of a substance is below this limit, is it assumed that the medicinal product is unlikely to represent a risk for the environment (EMEA 2006). Generally, all substances that exceed the action limit are subjected to a Phase II environmental risk assessment. According to the PECs presented in Table 3, most of the substances would exceed the action limit and would have been urged to perform an ERA. However, if a drug possesses orphan drug status, a refined PEC is calculated, by reducing the Fpen. Further refinement of the Fpen could be based on the treatment regime. The treatment regime considers the worst-case treatment period and the worst-case number of treatment repetitions per year. This is e.g. done for substances which are intended for single use, e.g. after surgeries.

If a pharmaceutical is highly lipophilic (logKow > 4.5) the drug substances should be screened, in a stepwise procedure, for persistence, bioaccumulation and toxicity, irrespective of the calculated PEC. These substances are likely to persist in the environment or bioaccumulate. Their cumulative potential leads to an uncertainty when calculating the PEC, and chronic effects might occur even at low market penetration.

The logKow values were only available for a low number of substances (Table 3). For the substances Asenapine, Rotigotine, Vortioxetine, Pitavastatin, Nilotinib and Vemurafenib, a logKow > 4.5 was reported. Thus, these substances are highly lipophilic and are thus likely to bioaccumulate. Thus, these substances would have been subject to a PBT-Screening in Phase I in the ERA irrespective of their action limit.

However, also substances with a PEC below the action limit might pose a risk as the specific MoA of the pharmaceuticals might not be covered by the standard test. These substances are indicated as 'however' substances, which have effects at very low concentrations in the environment and thus might pose a risk even though they do not reach the action limit. For these substances, an ERA has to be performed, either a TRA, if the MoA requires an adaption, or a standard ERA at low concentrations, if this is sufficient to adequately assess potential effects. Furthermore, the standard tests might not represent the most sensitive endpoint, as these tests mainly assess systemic toxicity rather than specific MoAs. Especially neuroactive substances could result in behavioural changes which could lead to negative effects at the population level, while these are not determined by default e.g. in a fish early life stage toxicity test according to the OECD TG 210.

Figure 4: Exemplary part of the excel sheet for pharmaceuticals of the substance classes 'Neurology' & 'Psychiatry'

Active Component	Orphan drug status	Year of Approval	Indication	Target group	Name of target	SeqAPASS	NCBI accession	maximum daily dose per inhabitant (www.rote-liste.de) [daily/ depot]
Tasimelteon	orphan drug	2014	Non-24-hour sleep–wake disorder	melatonin receptor	melatonin receptors MT1 and MT2	1319	AAH20757.1	20 mg
Gabapentin enacarbil	orphan drug	2011	Moderate-to-severe restless legs syndrome	adrenergic	norepinephrine transporter	1312	NP_001165975.1	1800 mg
Valbenazine		2017	Tardive dyskinesia	monoamine transporter	vesicular monoamine transporter 2 (VMAT2)	1321	NP_003045.2	80 mg
Paliperidone		2006	Schizophrenia	serotonergic & dopaminergic	5HT2A (serotonin) & D2 (dopamine) receptors	1310/1311	AAA66493.1 & AAH21195.1	525 mg
Lurasidone		2010	Schizophrenia	serotonergic & dopaminergic, andrenergic	Dopamine-2 (D2), 5-HT2A, 5HT1A, 5-HT7 receptors, alpha-2C adrenergic receptor	1310/1311	AAA66493.1 & AAH21195.1 & NP_000674.2	160 mg

Active Component	PEC_SW (µg/L)	Mechanism of Action	Pharmacokinetics	Substance Class	Bioactivity	Type (drugbank.ca)	SMILES (drugbank.ca)
Tasimelteon	0.10	Melatonin-receptor agonist		Neurology	bioactive compound	Small molecule	[H][C@@]1(CN=C(O)CC)C[C@@]1([H])C1=C2CCOC2=CC=C1
Gabapentin enacarbil	9.00	Voltage-activated calcium channel inhibitor		Neurology	bioactive compound	Small molecule	NCC1(CC(O)=O)CCCCC1
Valbenazine	0.40	VMAT2 inhibitor		Neurology	bioactive compound	Small molecule	COC1=C(OC)C=C2[C@H]3C[C@@H](OC(=O)[C@@H](N)C(C)C)[C@H](CC(C)C)CN3CCC2=C1
Paliperidone	2.63	Atypical antipsychotic		Psychiatry, Psychology	bioactive compound	Small molecule	CC1=C(CCN2CCC(CC2)C2=NOC3=C2C=CC(F)=C3)C(=O)N2CCCC(O)C2=N1
Lurasidone	0.80	Atypical antipsychotic agent		Psychiatry, Psychology	bioactive compound	Small molecule	[H][C@@]12[C@H]3CC[C@H](C3)[C@]1([H])C(=O)N(C[C@@H]1CCCC[C@H]1CN1CCN(CC1)C1=NSC3=CC=CC=C13)C2=O

Remark: In this study the main focus lies on pharmaceuticals which fall below the action limit, PEC ≥ 0.01 µg/L, because their risk could be underrated if tests are lacking. All pharmaceuticals whose PEC value is below 0.01 µg/L are marked in red in the excel sheet. These substances have priority in further description (Source: own illustration, Fraun-hofer IME)

3.6 Neuroactive substances

3.6.1 Results of the literature review on ecotoxicological effects of neuroactive substances

For the neuroactive MoAs 50 substances were found, belonging to the substance class of neurology and psychiatry. The substances are mainly used for the treatment of e.g. depressions, epilepsy, Schizophrenia, bipolar disorder and multiple sclerosis. The therapeutic effects of antidepressants and neuro active substances are related to their effects on neurotransmitters. One MoA relevant for econeurotoxicity is related to disturbances in electric signal transduction and inhibition of chemical signal transduction, mainly through interference with different types of neurotransmitters (Legradi, Di Paolo et al. 2018).

The pharmaceuticals are designed to target specific metabolic and molecular pathways in humans. The relevant targets for the neuroactive substances are the e.g. dopamine receptors, serotonin receptors, AMPA receptors, acetylcholine receptors and GABA receptors. The substances were sorted by their specific target (Table 4).

The SeqAPASS cross-species extrapolation was performed for the distinct target sites of the neuroactive substances. Mainly the model organisms common carp, rainbow trout, japanese medaka, channel catfish, sheepshead minnow, zebrafish were predicted to be susceptible to neuroactive substances.

Table 4: Model organisms with high conformity to neuroactive targets found by the SeqAPASS analysis with the corresponding NCBI accession number

Target protein	NCBI accession	SeqAPASS
Dopamine receptor	AAA66493.1 & AAH21195.1	zebrafish, common carp, japanese medaka
Serotonin receptor	AAA66493.1 & AAH21195.1	zebrafish, common carp, japanese medaka
AMPA receptor	Q9P003.1	channel catfish, common carp, zebrafish
acetylcholin receptor	CAA26344.1	atlantic salmon, common carp, rainbow trout
GABA receptor	AAB38510.1	japanese medaka, rainbow trout, common water flea
interleukin-2 receptor	AAB46883.1	/
melatonin receptors MT1 and MT2	AAH20757.1	/
orexin receptors	AAC39601.1 & AAC39602.1	japanese medaka, guppy, zebrafish/ zebrafish, common carp, rainbow trout
beta interferon	AAC33300.1	channel catfish, zebrafish, common carp
Calcitonin gene-related peptide (CGRP)	CAA34070.1	japanese medaka, zebrafish, common carp
B-Lymphocyte-antigen CD20	NP_068769.2	/

Target protein	NCBI accession	SeqAPASS
dihydroorotate dehydrogenase	AAH65245.1	japanese medaka, sheepshead minnow, rainbow trout
heme oxygenase 1 (HO-1)	NP_002124.1	zebrafish, guppy, channel catfish
Monoaminoxidase B (MAO-B)	AAB27229.1	common carp, rainbow trout, japanese medaka
vesicular monoamine transporter 2 (VMAT2)	NP_003045.2	zebrafish, channel catfish, rainbow trout
monoamine transporter type 2	CAA63824.1	common carp, atlantic salmon, rainbow trout
norepinephrine transporter	NP_001165975.1	zebrafish, common carp, japanese medaka/ atlantic salmon, common carp, zebrafish (fruit fly, honey bee)
potassium channel	AAB97315.1	channel catfish, zebrafish, rainbow trout
sodium channel	BAA78033.1	channel catfish, sheepshead minnow, common carp
SMN protein (survival motor neuron)	AAC52048.1	japanese medaka, guppy, rainbow trout
sphingosine 1-phosphate receptors 1, 3, 4, and 5	NP_110387.1	common carp, japanese medaka, zebrafish
tripeptidyl peptidase-1 (TPP1)	AAH14863.1	common carp, zebrafish, rainbow trout

To a lesser extent the invertebrate model organisms were predicted to be susceptible. The common waterflea showed a low homologous to the sodium channel & collapsin response mediator protein-2 (CRMP-2), which is the target protein of e.g. Lacosamide. For norepinephrine and dopamine transporters, the fruit fly and honey bee showed a homology. Also for the GABA (gammaaminobutyric acid transaminase) and the monoamine transporter the common water flea showed a homology.

Many of the substances with the target 'serotonin receptor' are selective serotonin reuptake inhibitors. E.g. Desvenlafaxine (O-desmethylvenlafaxine), the major active metabolite of venlafaxine, is an antidepressant from the serotonin norepinephrine reuptake inhibitor (SNRI) class (www.drugbank.ca). SNRI have been found to effect behaviour, reproduction, and development in both invertebrates and vertebrates.

With SeqAPASS the strongest homology with the serotonin receptor was found in the model organisms zebrafish, common carp and japanese medaka. Furthermore Gould et al. (2007) described that fish serotonin transporters have a high affinity to the selective serotonin reuptake inhibitors (SSRIs) used in human therapy (Gould, Brooks et al. 2007).

For most of the pharmaceuticals of the neuroactive substances data on ecotoxicological effects are missing in literature, therefore data were obtained for model substances (Table 5), which act on similar targets. The ecotoxicological data found for the model substances acting on the serotonin receptor indicate fish as the sensitive test organisms but also tadpoles and invertebrates, e.g. *Daphnia magna*, the amphipod marine *Echinogammarus marinus*, *Gammarus pulex* and the freshwater snails *Stagnicola elodes*.

Exposure of *Gammarus pulex* to fluoxetine caused a reduced consumption and increased swimming velocity with a LOEC of 0.1 µg/L (De Castro-Catala, Munoz et al. 2017). Also the marine amphipod, *Echinogammarus marinus* showed an altered swimming behaviour at 0.001 µg/L with effect on velocity after 1 day exposure to fluoxetine (Bossus, Guler et al. 2014). Behavioural effects were also detected for *Daphnia magna*, with an increased positive phototactic behaviour, at 0.1 µg/L (Rivetti, Campos et al. 2016). The most sensitive test organism was the freshwater snail Stagnicola elodes with a LOEC of 313 pg/L for detachment from the substrate (Fong and Hoy 2012) after exposure to the serotonin-norepinephrine reuptake inhibitor venlafaxine. From the vertebrates, the tadpoles of Rana pipiens were sensitive, with a delayed development compared to the controls at concentrations of 0.029 µg/L of fluoxetine. For the zebrafish embryos exposed to amitriptyline (Yang, Qiu et al. 2014) a time-dependent lethal concentrations was determined at the mg/L range. At sublethal levels, the exposure to amitripty-line reduced the hatching time and body length of embryos, decreased the adrenocorticotropic hormone level (LOEC 10 ng/L), increased oxidative stress and antioxidant parameters (LOEC 100 ng/L), as well as nitric oxide production and total nitric oxidesynthase activity (LOEC 1 ng/L) (Yang, Qiu et al. 2014).

For fathead minnows (*P. promelas*) a reduced survival was shown for adult male after 21 days exposure to venlafaxine with a LOEC of 0.3 µg/L and a LOEC of 0.0052 µg/l for sertaline (Schultz, Painter et al. 2011).

For some other molecular targets of the neuroactive substances, such as the dopamine receptor, AMPA receptor, GABA receptor, and sodium channel, ecotoxicological relevant data for model substances acting on these targets was found.

For the dopamine receptor ecotoxicological data was found for the model substances sertraline, haloperidol and apomorphine. Sertraline inhibited the growth of two algae taxa with an EC50 of 67µg/L for the marine algae Skeletonema marinoi and 105 µg/L for P. subcapitata (Minguez, Pedelucq et al. 2016). For haloperidol the most sensitive endpoint was the increase of gonadotropin-releasing hormone transcripts in the male brain of fathead minnow in a 21 d reproduction assay with a LOEC of 20 µg/L (Villeneuve 2010). The behavioural endpoints on the swimming track density, speed and degree of turning angles of daphnids gave a LOEC of 3 mg/L (Bownik et al 2018).

For substances targeting the GABA receptor, data on the model substance diazepam was found. Here the behavioural endpoint of *Daphnia magna* was very sensitive with a LOEC of 100 ng/L for an increased positive phototactic behaviour and also an effect on reproduction with a LOEC of 1 µg/L (Rivetti et al., 2015). Also the pumpkinseed sunfish (Lepomis gibbosus) was affected in the behaviour, the fish spend more time swimming and less time in a refuge area (black compartment of an aquarium) at concentrations of 533 µg/L (Brandao et al 2013).

The anticonvulsant and analgesic drug Carbamazepine is acting on the voltagegated sodium channel. For this target the most sensitive endpoint were the response of D. magna to light. An increased positive phototactic behaviour was found by Rivetti et al. (2015) with a LOEC 100 ng/L and also by Simao et al. (2019) with a LOEC of 100 ng/L.

For the neuroactive substances the standard acute test like the Daphnia immobilization test, and the algae growth inhibition test often gave results with EC50 >100 mg/L (Minguez 2016). The most sensitive endpoints for testing the effect of neuroactive substances, which were found in literature, are test on behaviour. A variety of behavioural endpoints is used to screen for the effects of neuroactive substances. In comparison to the standard acute or chronic tests the test on behaviour were more sensitive for example avoidance, phototactic behaviour and velocity.

Substances of interest with a PEC below the action limit of .0.01 µg/L are Fingolimod, IncobotulinumtoxinA, Peginterferon beta-1A, Rasagiline mesylate, Abobotulinum-toxin A.

Table 5: Overview of literature on ecotoxicological tests with neuroactive model substances

Target	Substance	Ecotox test	LOEC or EC_{50}	Reference
dopamine receptor	rotigotine	*Daphnia magna*: swimming activity	LOEC > 10 µM	(Barrozo, Fowler et al. 2015)
dopamine receptor	sertraline	*Daphnia magna* 48 h; *Pseuokirchneriella. subcapitata* 72h; *Artemia salina* 48h; *Skeletonema marinoi* 72h	EC_{50} 1.15 mg/L EC_{50} 0.15 mg/L EC_{50} 4.08 mg/L EC_{50} 0.067 mg/L	(Minguez, Pedelucq et al. 2016)
dopamine receptor	haloperidol	fathead minnow 21 d reproduction assay increase of gonadotropin-releasing hormone (cGnRH) transcripts in the male brain, no effects on fish reproduction	LOEC 20 µg/L	(Villeneuve, Garcia-Reyero et al. 2010)
dopamine receptor	apomorphine	Daphnia: decrease swimming track density, speed and degree of turning angles of daphnids exposed for 4 h	LOEC 3 mg/L	(Bownik, Sokolowska et al. 2018)
serotonin receptor	duloxetine	*Daphnia magna,* 48h *Pseuokirchneriella subcapitata,* 72h *Artemia salina,* 48h *Skeletonema marinoi* ,72h	EC_{50} 3.35 mg/L EC_{50} 0.37 mg/L EC_{50} 20.59 mg/L EC_{50} 1.94 mg/L	(Minguez, Pedelucq et al. 2016)
serotonin receptor	clomipramine	*Daphnia magna,* 48h *Pseuokirchneriella subcapitata,* 72h *Artemia salina,* 48h *Skeletonema marinoi* ,72h	EC_{50} 2.74 mg/L EC_{50} 0.46 mg/L EC_{50} >100 mg/L EC_{50} 4.7 mg/L	(Minguez, Pedelucq et al. 2016)
serotonin receptor	paroxetine	*Daphnia magna,* 48h *Pseuokirchneriella subcapitata,* 72h *Artemia salina,* 48h *Skeletonema marinoi* ,72h	EC_{50} 6.24 mg/L EC_{50} 0.63 mg/L EC_{50} 55.45 mg/L EC_{50} 0.12 mg/L	(Minguez, Pedelucq et al. 2016)
serotonin receptor	amitriptyline	*Daphnia magna,* 48h *Pseuokirchneriella subcapitata,* 72h *Artemia salina,* 48h	EC_{50} 4.82 mg/L EC_{50} 0.72 mg/L EC_{50} 16.93 mg/L	(Minguez, Pedelucq et al. 2016)

Target	Substance	Ecotox test	LOEC or EC_{50}	Reference
		Skeletonema marinoi ,72h	EC_{50} 0.041 mg/L	
serotonin receptor	fluoxetine	*Daphnia magna,* 48h *Pseudokirchneriella subcapitata,* 72h *Artemia salina,* 48h *Skeletonema marinoi* ,72h	EC_{50} 5.91 mg/L EC_{50} 0.2 mg/L EC_{50} 13.81 mg/L EC_{50} 0.043 mg/L	(Minguez, Pedelucq et al. 2016)
serotonin receptor	fluoxetine	*Gammarus pulex*; reduced consumption and increased swimming velocity	LOEC **0.1 µg/L**	(De Castro-Catala, Munoz et al. 2017)
serotonin receptor	fluoxetine	*Echinogammarus marinus*: altered swimming behaviour, effect on velocity after 1 d	LOEC **0.001 µg/L**	(Bossus, Guler et al. 2014)
serotonin receptor	fluoxetine	*Echinogammarus marinus*: geotaxis, behavioural changes, 3 weeks	LOEC **0.1 µg/L**	(Guler and Ford 2010)
serotonin receptor	fluoxetine	*Daphnia magna*: reproduction, increased offspring ,	LOEC 10 µg/L	(Campos, Pina et al. 2012)
serotonin receptor	fluoxetine	*Daphnia magna*: increased positive phototactic behaviour	LOEC **0.1 µg/L**	(Rivetti, Campos et al. 2016)
serotonin receptor	fluoxetine	*Lampsilis fasciola*: increase in movement, decreased times to movement, 67 days	LOEC 22.3 µg/L	(Hazelton, Du et al. 2014)
serotonin receptor	fluoxetine	*Daphnia magna*: multi-generational study, length was impacted *Hyalella azteca*: effect on growth, *Potamopyrgus antipodarum*: decrease in reproduction	LOEC 8.9 µg/L LOEC < 33 µg/L LOEC 10 µg/L	(Péry, Gust et al. 2008)
serotonin receptor	fluoxetine	*Ceriodaphnia dubia*: decrease in reproduction	LOEC 112 µg/L	(Brooks, Foran et al. 2003)
serotonin receptor	fluoxetine	*Pimephales promelas*: impact on mating behaviour, e.g. nest building and defensive behaviour, in males	LOEC 1 µg/L	(Weinberger and Klaper 2014)
serotonin receptor	fluoxetine	Gulf toadfish (*Opsanus beta)*: increase in the number of aggressive behaviours in dominant individuals	LOEC 25 µg/L	(McDonald, Gonzalez et al. 2011)

Target	Substance	Ecotox test	LOEC or EC_{50}	Reference
serotonin receptor	fluoxetine	*Rana pipiens*: Tadpoles in stages 21/22: delayed development	LOEC 0.029 µg/L	(Foster, Burton et al. 2010)
serotonin receptor	fluoxetine	*Pimephales promelas*: feeding rate	LOEC 3.7 µg/L	(Stanley, Ramirez et al. 2007)
serotonin receptor	fluoxetine	*Daphnia magna*: response to light: increased this positive geotaxis	LOEC 1 µg/L	(Simão, Martínez-Jerónimo et al. 2019)
serotonin receptor	fluvoxamine	*Daphnia magna*: increased offspring	4 µg/L	(Campos, Pina et al. 2012)
serotonin receptor	venlafaxine	freshwater snails *Stagnicola elodes* and *Leptoxis carinata*: foot detachment from the substrate	LOEC 313 pg/L LOEC 31.3 ng/L	(Fong and Hoy 2012)
serotonin receptor	venlafaxine	*Pimephales promelas*: reduced survival in adult male, 21 days	LOEC 0.3 µg/L	(Schultz, Painter et al. 2011)
serotonin receptor	venlafaxine	*Danio rerio*: 6 weeks exposure, reduced embryo production	LOEC 10 µg/L	(Galus, Kirischian et al. 2013)
serotonin receptor	venlafaxine	*Pimephales promelas*: escape responses were slowed in larvae, 12 days	LOEC 5 µg/L	(Painter, Buerkley et al. 2009)
serotonin receptor	venlafaxine	hybrid striped bass (*Morone saxatilis × Morone chrysops*): Time to capture prey was increased, exposure 6 days	LOEC 50 µg/L	(Bisesi, Bridges et al. 2014)
serotonin receptor	venlafaxine	mosquitofish (*Gambusia holbrooki*): disturbed circadian rhythm with decreased locomotion during the day was seen in adult, exposure for 7 days,	LOEC 100 µg/L	(Melvin 2017)
serotonin receptor	amitriptyline	*Danio rerio*: body length reduced in embryos at 100 ng/L nitric oxide content and total nitric oxide synthase activity reduced at 10 ng/L	LOEC 0.01 µg/L	(Yang, Qiu et al. 2014)
serotonin receptor	citalopram	freshwater snails *Stagnicola elodes, Leptoxis carinata*: foot detachment from the substrate	LOEC 4.05 µg/L; LOEC 405 pg/L	(Fong and Hoy 2012)

Target	Substance	Ecotox test	LOEC or EC_{50}	Reference
serotonin receptor	sertraline	crayfish (*Procambarus virginalis*): spent significantly more time outside the shelters and moved greater distances when shelter was available, spawned more frequently and showed higher mortality.	LOEC 1 µg/L	(Hossain, Kubec et al. 2019)
serotonin receptor	sertraline	*Pimephales promelas*: less time spent in dark shelter when exposed to sertaline	LOEC 3 µg/L	(Valenti, Gould et al. 2012)
serotonin receptor	sertraline	*Pimephales promelas*: reduced survival in adult male, 21 days,	LOEC 0.0052 µg/L	(Schultz, Painter et al. 2011)
GABA receptor	diazepam	*Daphnia magna*: increased positive phototactic behaviour, enhanced reproduction at 1 µg/L	LOEC 0.10 µg/L	(Rivetti, Campos et al. 2016)
GABA receptor	diazepam	pumpkinseed sunfish (Lepomis gibbosus): increase in GST activities in the gills and an inhibition of GRed in the digestive tract, suggesting an antioxidant response. Fish spend more time swimming and less time in a refuge area (black compartment of an aquarium)	LOEC 533 µg/L	(Brandão, Rodrigues et al. 2013)
sodium channel	carbamazepine	*Daphnia magna*, 48h *Pseuokirchneriella subcapitata*, 72h *Artemia salina*, 48h *Skeletonema marinoi* ,72h	EC_{50} >100 mg/L EC_{50} >100 mg/L EC_{50} >100 mg/L EC_{50} >100 mg/L	(Minguez, Pedelucq et al. 2016)
sodium channel	carbamazepine	marine amphipod *Echinogammarus marinus*: geotaxis, 3 weeks, behavioural changes at 10 µg/L (LOEC)	LOEC 10 µg/L	(Guler and Ford 2010)
sodium channel	carbamazepine	*Daphnia magna*: increased positive phototactic behaviour, LOEC 100 ng/L; enhance reproduction 1 µg/L of carbamazepine,	LOEC 100 ng/L	(Rivetti, Campos et al. 2016)
sodium channel	carbamazepine	Sunfish: no effects in any of the behavioural parameters evaluated	no effect	(Brandão, Rodrigues et al. 2013)
sodium channel	carbamazepine	*O. latipes*: activity, feeding rate	LOEC 6.15 mg/L	(Nassef, Matsumoto et al. 2010)

Target	Substance	Ecotox test	LOEC or EC_{50}	Reference
sodium channel	carbamazepine	*Daphnia magna*: response to light, increased positive geotaxis	LOEC 0.1 µg/L	(Simão, Martínez-Jerónimo et al. 2019)

3.6.2 Potential strategies to assess the specific risk of neuroactive substances

The literature review indicates that the standard test approaches, which are applied for ERA, might not be sufficient to assess the risk of neuroactive substances. Few data are available which assess the endpoints of the OECD TGs 201, 211, and 210, which are the aquatic effect studies applied for ERA. However, acute studies with *Daphnia magna* are reported (Minguez et al., 2016), which result in effects in the mg/L range. This might be due to the fact that neuroactive substances initially affect the behaviour of aquatic organisms rather than acute toxicity.

Thus, studies on behaviour (e.g. Barrozo et al., 2015, Castro-Catala, 2017; Guler and Ford, 2010, Simao et al., 2019; Hazelton et al., 2014, Rivetti et al., 2015) resulted in effects at the µg/L range, which is considerably lower than the effects on acute toxicity. Few studies assessed the reproductive success of the test species (Campos et al., 2016; Pery et al., 2008), which might however be due to an altered mating behaviour rather than an endocrine effect. The test species for behavioural assays include fish, amphibians, and invertebrates, with no clear indication which group was more sensitive compared to the other group. No MoA-specific studies on bacteria, algae or macrophytes were found in the literature. For some of the substances, the results of the studies carried out for the authorization procedure are published in the EPAR. Even though these studies are not MoA-specific, they could serve as references in order to determine if tests on behaviour are more sensitive than standard acute and chronic studies on algae, daphnids or fish.

Target molecules include the dopamine receptor, the serotonin receptor, the GABA receptor, and the sodium channel. Kokel et al., 2010 introduced the photomotor response (PMR) in 24 hpf old zebrafish embryos and identified specific action patterns after a light stimulus depending on the mode of action of the applied neuroactive substance. It was possible to assign unknown substances to their specific chemical group depending on these patterns. The PMR was further developed to assess the locomotor activity of 96 hpf old larvae with a specific tracking system (e.g. Klüver et al., 2015). Also the mating behaviour can be assessed, e.g. in *Pimephales promelas*, as performed by Weinberger and Klaper, 2014. The most sensitive test species can be determined prior to testing by a SeqAPASS analysis.

Based on the results on other test organisms, further behavioural assays can be applied, e.g. determination of phototactic behaviour in *Daphnia magna* (Rivetti et al., 2015, Simao et al., 2019) or foot detachment in freshwater snails (Fong and Hoy, 2012). Thus, we suggest the following test pipeline:

a) Check target of the test substance

b) Perform SeqAPASS analysis in order to define the most sensitive test species

c) Perform a behavioural tests on at least two taxonomic classes (e.g. fish and invertebrates)

A test battery of behavioural assays should be defined and validated, of which at least two appropriate assays could be chosen.

3.7 Oncologically active substances

3.7.1 Results of the literature review on ecotoxicological effects of oncologically active substances

The substance class of 'Oncology' consisted mainly of two different types of pharmaceuticals: cytotoxics and cytostatics. Cytotoxics affect DNA replication and cell growth, targets of cytostatics are mainly protein kinase inhibitors and monoclonal antibodies (Besse et al., 2012).

Most of the pharmaceuticals in this study are cytostatics, while most of the targets described in Table 6 are kinases. The second largest target protein group is antigens, namely CD (cluster of differentiation) antigens, which are targets of cytotoxics. Cytostatics present the newer class of pharmaceuticals. For this reason, less literature data on similar substances to cytostatics can be found.

As antigens are very specific for the human organism, the similarity of respective target proteins in model organisms found via SeqAPASS was lower compared to the similarity of kinases in different organisms. The substances were sorted by their specific target.

The most common found model organisms were different fish species (zebrafish, common carp, channel catfish, Japanese Medaka, rainbow trout, guppy, sheepshead minnow, three-spined stickleback). Additional other found organisms from different taxa, which are used in ecotoxicological studies, are oysters, common water flea, honey bee, fruit fly and roundworm.

Table 6: Model organisms with high conformity found to oncologically relevant targets by the SeqAPASS analysis with the corresponding NCBI accession number

Target protein	NCBI accession	SeqAPASS
20S proteasome	/	/
ALK	CRL66424.1	sheepshead minnow, guppy, channel catfish
'alkylation of DNA'	/	all
Androgen receptor		
asparaginase	WP_063087735.1	all
BCL-2	NP_000624.2	common carp, zebrafish, rainbow trout
Beta tubulin	AAB59507.1	japanese medaka, channel catfish, honey bee, fruit fly, common water flea, roundworm
BRAF	AAC23448.1	sheepshead minnow, guppy, japanese medaka, common water flea, , honey bee, fruit fly
BTK	NP_000052.1	sheepshead minnow, japanese medaka, zebrafish, common waterflea, honey bee, fruit fly
CD19, CD3	AAA69966.1, NP_000723.1	(sheepshead minnow, salmons)
CD20	NP_068769.2	rainbow trout, zebrafish, common carp
CD22	BAA36576.1	/
CD30	AAA51947.1	common carp, zebrafish, rainbow trout
CD38	BAA18966.1	guppy, sheepshead minnow, rainbow trout
CDK4, CDK6	CAG47043.1, NP_001138778.1	rainbow trout, channel catfish, guppy, oysters, honey bee/ zebrafish, rainbow trout, guppy, oysters, honey bee

Target protein	NCBI accession	SeqAPASS
cereblon	AAH67811.1	rainbow trout, sheepshead minnow, japanese medaka
CTLA4	AAL07473.1	common carp, zebrafish, japanese medaka
CXCR4	CAA12166.1	zebrafish, common carp, rainbow trout
CYP17	ABF93457.1	sheepshead minnow, japanese medaka, three-spined stickleback
Deacetylase (DAC)	NP_001308829.1	guppy, sheepshead minnow, channel catfish
dihydrofolate reductase	AAA58485.1	rainbow trout, common carp, zebrafish
DNA methyltransferase	CAA11272.1	Common carp, zebrafish
EGFR (ErbB1)	AAI18666.1	zebrafish, common carp, japanese medaka, (honey bee, fruit fly)
EGFR (ErbB1), HER2 (ErbB2), ErbB3 and ErbB4 [L2937]	AAI18666.1; NP_001276866.1; AAH02706.1; AAI43750.1	zebrafish, common carp, japanese medaka, (honey bee, fruit fly)/ channel catfish, rainbow trout, common carp/ sheepshead minnow, guppy, common carp/ sheepshead minnow, channel catfish, japanese medaka
FLT3	CAA81393.1	common carp, zebrafish, rainbow trout
G-CSF	CAA27290.1	/
GD2	/	/
GnRH receptors	AAL89821.1	three-spined stickleback, rainbow trout, japanese medaka
HER2 (ErbB2)	NP_001276866.1	channel catfish, rainbow trout, common carp
IDH1	CAG38738.1	sheepshead minnow, guppy, channel catfish, oysters, common waterflea, roundworm, honey bee, fruit fly
IDH2	AAH71828.1	guppy, sheepshead minnow, japanese medaka, eastern oyster, common waterflea, fruit fly, honey bee, roundworm
IL3	AAH69472.1	/
JAK1, JAK2	BAE02826.1; AAY22962.1	rainbow trout, zebrafish, common carp/ rainbow trout, channel catfish, common carp
MEK1, MEK2	AAI37460.1, AAH18645.1	channel catfish, sheepshead minnow, guppy, common waterflea, honey bee, eastern oyster, fruit fly/ sheepshead minnow, channel catfish, japanese medaka, honey bee, common

Target protein	NCBI accession	SeqAPASS
		waterflea, oysters, fruit fly, roundworm
membrane bound kinases (RET, VEGFR1, VEGFR2, VEGFR3, KIT, PDGFR-alpha, PDGFR-beta, FGFR1, FGFR2, TIE2, DDR2, TrkA, Eph2A, RAF-1, BRAF, BRAFV600E, SAPK2, PTK5, and Abl) (serine-threonine-kinases)	P07949.3; P17948.2; P35968.2; P35916.3; AAC50969.1; P26618.3; P09619.1; AAH15035.1; CAA96492.1; ACF47627.1; NP_001341912.1; BAA34355.1; AAH37166.1; NP_001341618.1; AAA35609.2; AAB60393.1	channel catfish, common carp, japanese medaka/ zebrafish/ rainbow trout/ guppy/ sheepshead minnow/ (honey bee, fruit fly, common waterflea)
methotrexate	/	/
mTOR	NP_004949.1	channel catfish, zebrafish, japanese medaka, (honey bee, common water flea)
multiple kinases	AAA35594.1, NP_001337164.1, AAI18666.1; NP_001276866.1; AAH02706.1; AAI43750.1	zebrafish, rainbow trout, japanese medaka/channel catfish, zebrafish, common carp, (honey bee, common water flea) / zebrafish, common carp, japanese medaka, (honey bee, fruit fly)/ channel catfish, rainbow trout, common carp/ sheepshead minnow, guppy, common carp/ sheepshead minnow, channel catfish, japanese medaka
NK1	AAA59936.1	three-spined stickleback, zebrafish, common carp, fruit fly, oysters, common waterflea
noradrenaline transporter	AAA59943.1	rainbow trout, common carp, zebrafish, fruit fly, honey bee
P110δ	NP_001337164.1	channel catfish, zebrafish, common carp, (honey bee, common water flea)
PARP	NP_001609.2	japanese medaka, guppy, zebrafish, common waterflea, eastern oyster, honey bee
PD1	NP_005009.2	/
PDGFRα	NP_006197.1	rainbow trout, common carp, japanese medaka
PDL1	AAI13735.1	sheepshead minnow, rainbow trout, japanese medaka
PI3K	CAA73797.1	rainbow trout, zebrafish, japanese medaka

Target protein	NCBI accession	SeqAPASS
RET, VEGFR1, VEGFR2, VEGFR3, EGFR	P07949.3; P17948.2; P35968.2; P35916.3; AAI18666.1	channel catfish, common carp, japanese medaka/ zebrafish/ rainbow trout
SLAMF7	AAH27867.1	common carp, sheepshead minnow, zebrafish
thymidine phosphorylase	AAH18160.1	channel catfish, zebrafish, sheepshead minnow, oysters, common waterflea
TRKA, TRKB, TRKC	BAA34355.1; AAB33109.1; CAA12029.1	rainbow trout, guppy, channel catfish, (oysters, common waterflea)/ rainbow trout, japanese medaka, channel catfish, oysters/ zebrafish, sheepshead minnow, channel catfish, (oysters, common waterflea)
BCR-ABL	AAA35594.1	zebrafish, rainbow trout, japanese medaka
VEGFR1, VEGFR2, VEGFR3	VEGFR1, VEGFR2, VEGFR3	channel catfish, common carp, japanese medaka/ zebrafish/ rainbow trout

The main groups of cytotoxic substances involve alkylating agents, platinum complexes or intercalating agents (Besse et al., 2012). These substances directly interact with the DNA of the organisms by inhibition of DNA transcription (alkylating agents), DNA replication (platinum complexes) or by breaking single-stranded DNA (intercalating agents). Furthermore, other cytotoxics interact indirectly with DNA. These include antimetabolites, cytotoxic antibiotics, mitotic spindle inhibitors and topoisomerase inhibitors. They act by blocking enzyme activity and disrupting DNA synthesis (antimetabolites), intercalate between DNA base pairs (cytotoxic antibiotics) or halt chromosome segregation by inhibiting mitotic spindle formation (mitotic spindle inhibitors).

Cytostatics do not interact with DNA and are mainly protein kinase inhibitors with a diverse range of targets, which are involved in a number of biological processes. Furthermore, monoclonal antibodies are designed to block tumoral cells extracellular receptors.

Anticancer drugs which act on endocrine targets (e.g. Abiraterone) are not considered, as these substances, like endocrine receptor antagonists or modulators, which are known endocrine disruptors, are already tested by a panel of in vitro and in vivo tests and follow a proposed tiered hazard assessment strategy.

As for most of the pharmaceuticals of the new generation or of orphan drugs data on ecotoxicological effects are missing in literature, data were obtained for model substances (Table 7), which act by similar modes of action.

For example, Parella et al., 2014 investigated the acute and chronic toxicity of six anticancer drugs on rotifers and crustaceans. These substances include 5-fluorouracil (5-FU) cisplatin (CisPt), doxorubicin (DOX), etoposide (ET), imatinib mesylate (IM), and capecitabine (CAP). These substances encompass drugs which are commonly applied (5-FU, CisPt), drugs which occur at lower concentrations (DOX and ET), and new compounds (IM and CAP). 5-FU and CAP (prodrug of active 5-FU) are pyrimidine analogues, which inhibit DNA polymerase and induce cell cycle arrest and apoptosis. CisPt belongs to the class of platinum complexes. ET is a topoisomerase II inhibitor acting on mitosis. DOX is an anthracycline which intercalates into two

base pairs to block DNA replication, also by inhibiting topoisomerase activity. IM is a tyrosine kinase inhibitor and acts by modulating growth factor signaling. Thus, 5 of the substances interact with DNA and are defined as cytotoxic drugs, while one (IM) is defined as cytostatic drug.

Test organisms include *Daphnia magna*, *Ceriodaphnia dubia*, *Brachionus calyciflorus*, and *Thamnocephalus platyurus*. In acute toxicity tests, *D. magna* and *T. platyurus* were shown to be most sensitive. The cytostatics CisPT and DOX were shown to be most acute toxic. However, even the cytotoxic substances display chronic toxicity. The most sensitive species were again *D. magna*, and *C. dubia*, which were affected in the µg/L range. The lowest EC50 values were determined for CisPt and 5FU. Thus, these cytostatics also pose a risk at low concentrations during long-term exposure.

Zounkova et al., 2010 tested three anticancer drugs (5-FU, cytarabine (CYT) and gemcitabine (GemC)) on their toxicity to *Daphnia magna*, the alga *Desmodesmus subspicatus*, and the bacteria Pseudomonas putida. Furthermore, they performed genotoxicity studies with the bacterial genotoxicity test (umu-test). They conclude that the three antineoplastic drugs were toxic at relatively high concentration (mg/L-range). Genotoxicity was observed at even higher concentrations.

Novak et al., 2017 investigated the cytotoxicity of anticancer drugs in zebrafish liver cells. The tested substances include 5-FU, CisPt, cyclophosphamide (CP), and ifosfamide (IF), which both belong to the group of alkylating agents. They report that the cell viability was reduced by less than 30 % at 300 mg/L with each substance tested. A performed genotoxicity assay with the liver cells (comet assay) resulted in a LOEC of 37.5 mg/L for CP and IF. The effects were assumed to be at environmentally not relevant concentrations. However, a mixture of all four tested substances resulted in DNA strand breaks at lower, environmentally relevant concentrations. They assume that also additive effects, especially for substances with similar targets, should also be considered in risk assessment.

Another study performed by Russo et al., 2018 investigated four different anticancer drugs, 5-FU, CisPt, ET, and IM, based on their different properties and modes of action, on their effects on reproduction and on DNA damage of *Ceriodaphnia dubia*, in a binary mixture with benzalkonium chloride. They chose *C. dubia* as test species due to its short life span, high reproductive capability and genetic uniformity. Furthermore, effects were already described for the tested anticancer drugs. Thus, they assume that this test specie is an appropriate choice for determination of effects of anticancer drugs. Also the comet assay was performed with *C. dubia* due to the abovementioned properties.

Kovács et al., 2016, investigated the effects of 5-FU, Cispt, ET, and IM on the acute and chronic toxicity on zebrafish (*Danio rerio*), assessed by OECD 203, the FET according to OECD 236, and the fish early life stage test according to OECD 210. They observed acute toxicity in the higher mg/L range, while effects on growth were observed at 1 mg/L for 5-FU. The results of the FET were observed in the same range as the results of the fish acute toxicity test according to OECD TG 203. However, they also observed subacute effects in the fish embryo, e.g. on eye development, hatching, and body curvature, at lower concentrations than those resulting in mortality. Thus, the FET might be more appropriate to determine chronic effects exerted by cytotoxic drugs than for example the fish acute toxicity test and the early life stage test, as the FET couls provide a more detailed picture of specific effects like edema, malformations of organs or teratogenicity by the possibility to assess sublethal effect. However, as no EC values were reported, final evidence on an increased sensitivity of the FET is lacking to date.

Bialk-Bielinska et al., 2017 tested six anti-cancer drugs and one metabolite in a biotest battery in order to determine the biological effects of the substances. They used 6 of the most commonly applied anti-cancer drugs, i.e. CP, IF, 5-FU, IM, tamoxifen (TAM) and methotrexate (MET). As tamoxifen is a known endocrine active substance, the results are not shown in this report. The test organisms include the bacterium *Vibrio fischeri*, algae *Raphidocelis subcapitata*, crustaceans *Daphnia magna* and duckweed *Lemna minor*. They observed that *Lemna minor* was the most sensitive organism. The most toxic substances were 5-FU (highly toxic to algae, EC50 = 0.075 mg/L) and MET (very toxic to highly toxic to duckweed depending on the test conditions; EC50 = 0.08 - 0.16 mg/L). It was suspected that the increased sensitivity to algae and duckweed was due to the increased exposure time, as this allowed the specific mode of action (inhibition of DNA replication) of the two substances to cause a specific effect. They concluded that actively dividing cells are susceptible to substances which act on DNA replication.

Ribas et al., 2017, treated zebrafish with decitabine, a drug blocking DNA methylation. They observed mortality at 25 µM and teratogenic effects during early development until 5 dpf. Furthermore, they observed a skewed sex ratio towards females at 75 µM at 90 dpf, if embryos were treated between 0 – 2 dpf. Thus, they concluded that larvae are very sensitive to alteration in DNA methylation, and either respond by decreased survival, teratogenic effects, and altered sex ratio.

In total, ecotoxicological data of 16 different anticancer drugs were found. However, most of the data were generated for the cytotoxic substances 5-FU, CisPt, cyclophasphamide, ifosfamide, and etoposide, as well as for the cytostatic substance imatinib mesylate.

Table 7: Overview of literature on ecotoxicological tests with oncologically active model substances

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
platinum complex	cisplatin	*Ceriodaphnia dubia* reproductive toxicity	Determination of mixture effects	(Russo, Kundi et al. 2018)
platinum complex	cisplatin	*Genotoxicity*	Determination of mixture effects	(Russo, Kundi et al. 2018)
platinum complex	cisplatin	*Danio rerio* liver cells, DNA damage, cytotoxicity assay	Viability reduced in a dose-dependent manner; > 70% at 300 mg/L	(Novak, Zegura et al. 2017)
platinum complex	cisplatin	*Danio rerio* (OECD 203) *Danio rerio* (FET, 120 h)	LC_{50} > 64.5 mg/L LC_{50} > 81.3 mg/L	(Kovacs, Bakos et al. 2016)
platinum complex	cisplatin	*Daphnia magna* acute toxicity test 48 h chronic toxicity test 21 d	LC_{50} = 0.94 mg/L EC_{50} = 1.63 µg/L	(Parrella, Lavorgna et al. 2014)
platinum complex	cisplatin	*Ceriodaphnia dubia* acute toxicity test 24 h chronic toxicity test 7 d	LC_{50} = 2.5 mg/L EC_{50} = 16.83 µg/L	(Parrella, Lavorgna et al. 2014)
platinum complex	cisplatin	*Brachionus calyciflorus* acute toxicity test 24 h chronic toxicity test 48 h	LC_{50} = 6.52 mg/L EC_{50} = 440 µg/L	(Parrella, Lavorgna et al. 2014)
platinum complex	cisplatin	*Thamnocephalus platyurus* acute toxicity test 48 h	LC_{50} = 8.44 mg/L	(Parrella, Lavorgna et al. 2014)
DNA alkylation	cyclophosphamide	Algae	EC_{50} = 11 mg/L	Booker et al., 2014; QSAR
DNA alkylation	cyclophosphamide	*Daphnia magna*	EC_{50} >100 mg/L	Booker et al., 2014; QSAR
DNA alkylation	cyclophosphamide	*Danio rerio*	EC_{50} >100 mg/L	(Booker, Halsall et al. 2014); (Sanderson, Brain et al. 2004)

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
DNA alkylation	cyclophosphamide	*Danio rerio* liver cells, cytotoxicity assay	Viability reduced in a dose-dependent manner; > 70% at 300 mg/L	(Novak, Zegura et al. 2017)
DNA alkylation	cyclophosphamide	*Danio rerio* liver cells, DNA damage	LOEC = 37.5 mg/L	(Novak, Zegura et al. 2017)
DNA alkylation	cyclophosphamide	*Vibrio fischeri* luminescent inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
DNA alkylation	cyclophosphamide	*Raphidocelis subcapitata* growth inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
DNA alkylation	cyclophosphamide	*Daphnia magna* immobilization assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
DNA alkylation	cyclophosphamide	*Lemna minor* growth inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
DNA alkylation	ifosfamide	*Danio rerio* liver cells, cytotoxicity assay	Viability reduced in a dose-dependent manner; > 70% at 300 mg/L	(Novak, Zegura et al. 2017)
DNA alkylation	ifosfamide	*Danio rerio* liver cells, DNA damage	LOEC = 37.5 mg/L	(Novak, Zegura et al. 2017)
DNA alkylation	ifosfamide	*Vibrio fischeri* luminescent inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
DNA alkylation	ifosfamide	*Raphidocelis subcapitata* growth inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
DNA alkylation	ifosfamide	*Daphnia magna* immobilization assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
DNA alkylation	ifosfamide	*Lemna minor* growth inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
DNA alkylation	thiotepa	*Daphnia magna*; immobilization 48 h	EC_{50} = 546 mg/L	(Besse, Latour et al. 2012); FDA-CDER, 1996
Anti-metabolite	cytarabine	UMUC biological assay (genotoxicity test)	EC_{50} >100 mg/L	(Booker, Halsall et al. 2014)
pyrimidine analogue	5-fluorouracil	*Ceriodaphnia dubia* reproductive toxicity	Determination of mixture effects	(Russo, Kundi et al. 2018)
pyrimidine analogue	5-fluorouracil	*Genotoxicity*	Determination of mixture effects	(Russo, Kundi et al. 2018)
pyrimidine analogue	5-fluorouracil	*Danio rerio* liver cells, DNA damage, cytotoxicity assay	Viability reduced in a dose-dependent manner; > 70% at 300 mg/L	(Novak, Zegura et al. 2017)
pyrimidine analogue	5-fluorouracil	*Danio rerio* (OECD 203) *Danio rerio* (FET, 120 h) *Danio rerio* (OECD 210)	LC_{50} > 100 mg/L LC_{50} > 2222 mg/L LOEC = 1 mg/L (growth)	(Kovacs, Bakos et al. 2016)
pyrimidine analogue	5-fluorouracil	*Daphnia magna* acute toxicity test 48 h chronic toxicity test 21 d	EC_{50} = 20.84 mg/L EC_{50} = 26.4 µg/L	(Parrella, Lavorgna et al. 2014)
pyrimidine analogue	5-fluorouracil	*Ceriodaphnia dubia* acute toxicity test 24 h chronic toxicity test 7 d	EC_{50} = 501 mg/L EC_{50} = 3.35 µg/L	(Parrella, Lavorgna et al. 2014)
pyrimidine analogue	5-fluorouracil	*Brachionus calyciflorus* acute toxicity test 24 h chronic toxicity test 48 h	No effect up to 200 mg/L EC_{50} = 322 µg/L	(Parrella, Lavorgna et al. 2014)
pyrimidine analogue	5-fluorouracil	*Thamnocephalus platyurus* acute toxicity test 48 h	EC_{50} = 0.28 mg/L	(Parrella, Lavorgna et al. 2014)

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
pyrimidine analogue	5-fluorouracil	*Pseudomonas putida*	EC_{50} = 0.044 mg/L	(Zounková, Odráška et al. 2007, Zounkova, Kovalova et al. 2010, Booker, Halsall et al. 2014)
pyrimidine analogue	5-fluorouracil	*Desmodesmus subspicatus*	EC_{50} = 48 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	5-fluorouracil	*Daphnia magna acute*	LC_{50} = 15 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	5-fluorouracil	*Daphnia magna reproduction*	LOEC = 0.05 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	5-fluorouracil	*Vibrio fischeri*	EC_{50} = 0.122 mg/L	Booker et al., 2014; Backhaus & Grimme, 1999
pyrimidine analogue	5-fluorouracil	*Pseudokirchneriella subcapitata*	EC_{50} = 0.11 mg/L	Booker et al., 2014; Zounkova et al., 2009
pyrimidine analogue	5-fluorouracil	*Vibrio fischeri* luminescent inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
pyrimidine analogue	5-fluorouracil	*Raphidocelis subcapitata* growth inhibition assay	EC_{50} = 0.075 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
pyrimidine analogue	5-fluorouracil	*Daphnia magna* immobilization assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
pyrimidine analogue	5-fluorouracil	*Lemna minor* growth inhibition assay	EC_{50} = 2.75 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
pyrimidine analogue	capecitabine	*Daphnia magna* acute toxicity test 48 h chronic toxicity test 21 d	EC_{50} = 224 mg/L EC_{50} = 20.5 µg/L	(Parrella, Lavorgna et al. 2014)
pyrimidine analogue	capecitabine	*Ceriodaphnia dubia* acute toxicity test 24 h chronic toxicity test 7 d	EC_{50} = 1230 mg/L EC_{50} = 2400 µg/L	(Parrella, Lavorgna et al. 2014)

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
pyrimidine analogue	capecitabine	*Brachionus calyciflorus* acute toxicity test 24 h chronic toxicity test 48 h	No effect up to 500 mg/L EC_{50} = 15.4 mg/L	(Parrella, Lavorgna et al. 2014)
pyrimidine analogue	capecitabine	*Thamnocephalus platyurus* acute toxicity test 48 h	EC_{50} = 197.7 mg/L	(Parrella, Lavorgna et al. 2014)
pyrimidine analogue	gemcitabine	*Pseudomonas putida*	EC_{50} = 100 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	gemcitabine	*Desmodesmus subspicatus*	EC_{50} = 45 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	gemcitabine	*Daphnia magna* acute	LC_{50} = 110 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	gemcitabine	*Daphnia magna* reproduction	LOEC > 1 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	gemcitabine	UMUC biological assay (genotoxicity test)	EC_{50} > 100 mg/L	(Booker, Halsall et al. 2014)
pyrimidine analogue	cytarabine	*Pseudomonas putida*	EC_{50} = 17 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	cytarabine	*Desmodesmus subspicatus*	EC_{50} = 53 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	cytarabine	*Daphnia magna* acute	LC_{50} = 200 mg/L	(Zounkova, Kovalova et al. 2010)
pyrimidine analogue	cytarabine	*Daphnia magna* reproduction	LOEC = 3.7 mg/L	(Zounkova, Kovalova et al. 2010)
topoisomerase II inhibitor	doxorubicin	*Daphnia magna* acute toxicity test 48 h chronic toxicity test 21 d	EC_{50} = 2.14 mg/L -	(Parrella, Lavorgna et al. 2014)

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
topoisomerase II inhibitor	doxorubicin	*Ceriodaphnia dubia* acute toxicity test 24 h chronic toxicity test 7 d	EC_{50} = 5.18 mg/L -	(Parrella, Lavorgna et al. 2014)
topoisomerase II inhibitor	doxorubicin	*Brachionus calyciflorus* acute toxicity test 24 h chronic toxicity test 48 h	EC_{50} = 612.69 mg/L EC_{50} = 7.7 mg/L	(Parrella, Lavorgna et al. 2014)
topoisomerase II inhibitor	doxorubicin	*Thamnocephalus platyurus* acute toxicity test 48 h	EC_{50} = 0.31 mg/L	(Parrella, Lavorgna et al. 2014)
topoisomerase II inhibitor	etoposide	*Ceriodaphnia dubia* reproductive toxicity	Determination of mixture effects	(Russo, Kundi et al. 2018)
topoisomerase II inhibitor	etoposide	Genotoxicity	Determination of mixture effects	(Russo, Kundi et al. 2018)
topoisomerase II inhibitor	etoposide	*Danio rerio* (OECD 203) *Danio rerio* (FET, 120 h)	LC_{50} > 100 mg/L LC_{50} > 300 mg/L	(Kovacs, Bakos et al. 2016)
topoisomerase II inhibitor	etoposide	*Daphnia magna* acute toxicity test 48 h chronic toxicity test 21 d	25% at 120 mg/L EC_{50} = 239 µg/L	(Parrella, Lavorgna et al. 2014)
topoisomerase II inhibitor	etoposide	*Ceriodaphnia dubia* acute toxicity test 24 h chronic toxicity test 7 d	16% at 120 mg/L EC_{50} = 204 µg/L	(Parrella, Lavorgna et al. 2014)
topoisomerase II inhibitor	etoposide	*Brachionus calyciflorus* acute toxicity test 24 h chronic toxicity test 48 h	No effect up to 120 mg/L EC_{50} = 3.7 mg/L	(Parrella, Lavorgna et al. 2014)
topoisomerase II inhibitor	etoposide	*Thamnocephalus platyurus* acute toxicity test 48 h	EC_{50} = 74.85 mg/L	(Parrella, Lavorgna et al. 2014)
dihydrofolate reductase	methotrexate	*Vibrio fischeri* luminescent inhibition assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
dihydrofolate reductase	methotrexate	*Raphidocelis subcapitata* growth inhibition assay	EC_{50} = 9.51 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
dihydrofolate reductase	methotrexate	*Daphnia magna* immobilization assay	EC_{50} = 72.43 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
dihydrofolate reductase	methotrexate	*Lemna minor* growth inhibition assay	EC_{50} = 61.05 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
dihydrofolate reductase	methotrexate	*V. fischeri*; luminescence	EC_{50} = 1220 mg/L	(Besse, Latour et al. 2012)
dihydrofolate reductase	methotrexate	*S. subspicatus*; growth 72 h	EC_{50} = 260 mg/L	(Besse, Latour et al. 2012)
dihydrofolate reductase	methotrexate	*T. pyrioformis*, growth 48 h	EC_{50} = 45 mg/L	(Besse, Latour et al. 2012)
dihydrofolate reductase	methotrexate	*D. magna*; immobilization 48 h	EC_{50} > 1000 mg/L	(Besse, Latour et al. 2012)
dihydrofolate reductase	methotrexate	*D. rerio*; survival 96 h	EC_{50} = 85 mg/L	(Besse, Latour et al. 2012)
dihydrofolate reductase	methotrexate	*X. laevis;* growth 96 h	EC_{50} = 0.015 mg/L	(Besse, Latour et al. 2012)
DNA methyltransferase	decitabine	*Danio rerio* embryonic development *Danio rerio* sex ratio	Teratogenic effects and mortality up to 66% at 25 µM Skewed sex ratio to females at 75 µM (treatment from 0 – 2 dpf)	(Ribas, Vanezis et al. 2017)
tubulin	paclitaxel	*Daphnia magna*	EC_{50} > 0.74 mg/L	(Zounková, Odráška et al. 2007, Zounkova, Kovalova et al. 2010, Booker, Halsall et al. 2014)
tubulin	paclitaxel	*Daphnia magna*; immobilization 48 h	EC_{50} > 0.74 mg/L	(Besse, Latour et al. 2012); FDA-CDER, 1996

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
tubulin	paclitaxel	*Lemna minor*		Besse, Latour et al. 2012; FDA-CDER, 1996
kinases	imatinib mesylate	*Ceriodaphnia dubia* reproductive toxicity	Determination of mixture effects	(Russo, Kundi et al. 2018)
kinases	imatinib mesylate	*Genotoxicity*	Determination of mixture effects	(Russo, Kundi et al. 2018)
kinases	imatinib mesylate	*Danio rerio* (OECD 203) *Danio rerio* (FET, 120 h) *Danio rerio* (OECD 210)	LC_{50} > 70.8 mg/L LC_{50} > 65.9 mg/L LOEC = 10 mg/L (mortality)	(Kovacs, Bakos et al. 2016)
kinases	imatinib mesylate	*Daphnia magna* acute toxicity test 48 h chronic toxicity test 21 d	EC_{50} = 11.97 mg/L EC_{50} = 308 µg/L	(Parrella, Lavorgna et al. 2014)
kinases	imatinib mesylate	*Ceriodaphnia dubia* acute toxicity test 24 h chronic toxicity test 7 d	EC_{50} = 31.92 mg/L EC_{50} = 115 µg/L	(Parrella, Lavorgna et al. 2014)
kinases	imatinib mesylate	*Brachionus calyciflorus* acute toxicity test 24 h chronic toxicity test 48 h	EC_{50} = 3.82 mg/L EC_{50} = 740 µg/L	(Parrella, Lavorgna et al. 2014)
kinases	imatinib mesylate	*Thamnocephalus platyurus* acute toxicity test 48 h	EC_{50} = 43.27 mg/L	(Parrella, Lavorgna et al. 2014)
kinases	imatinib mesylate	*P. promelas*; overall/ F1 larvae growth/ F1 growth/ increase in VTG, F1 males	NOEC (284 d) = 5 µg/L NOEC (28 d) = 0.08 µg/L NOEC (112 d) = 0.01 µg/L NOEC (112 d) = 0.01 µg/L	(Besse, Latour et al. 2012)
kinases	imatinib mesylate	*torisa*; larval development 5 d	NOEC = 49 mg/L	(Besse, Latour et al. 2012)
kinases	imatinib mesylate	*Vibrio fischeri* luminescent inhibition assay	EC_{50} = 23.06 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)

Target	Substance	Ecotox test	LOEC, NOEC or EC_{50}	Reference
kinases	imatinib mesylate	*Raphidocelis subcapitata* growth inhibition assay	EC_{50} = 5.08 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
kinases	imatinib mesylate	*Daphnia magna* immobilization assay	EC_{50} > 100 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
kinases	imatinib mesylate	*Lemna minor* growth inhibition assay	EC_{50} = 0.08 mg/L	(Bialk-Bielinska, Mulkiewicz et al. 2017)
kinases	erlotinib	*S. capricornutum*; growth 72 h	NOEC = 0.14 mg/L	(Besse, Latour et al. 2012); FASS, 2011
kinases	erlotinib	*D. magna*; reproduction 48 h	NOEC = 0.7 mg/L	(Besse, Latour et al. 2012); FASS, 2011
kinases	erlotinib	*O. mykiss*; survival 14 d	NOEC = 0.02 mg/L	(Besse, Latour et al. 2012); FASS, 2011
cereblon	pomalidomide	*Danio rerio* embryos in vitro: angiogenesis, teratogenesis, neurite outgrowth	Less effective than thalidomide and lenalidomide	(Mahony, Erskine et al. 2013)

3.7.2 Potential strategies to assess the specific risk of oncologically active substances

The literature review identified that studies mainly applied standard tests according to OECD TGs. These include tests on acute toxicity as well as on reproduction. Furthermore, tests on genotoxicity were applied, i.e. the umu-assay as well as the comet assay in different test systems. The comet assay in different test system for the assessment of DNA damage seems to be an appropriate approach to assess effects. The literature furthermore indicates that there is only limited data available, and most of the data focus on cytotoxic substances rather than on cytostatic. Cytotoxic substances rather interact either directly or indirectly with the DNA, while cytostatic substances interact with biological targets involved in growth and survival. The main goal of all anticancer drugs is the inhibition of cell growth and reproduction. Thus, the application of acute toxicity is questionable (Besse et al., 2012). However, the EMA guideline requires chronic studies on water flea and zebrafish, as well as algae growth inhibition test. Generally, there is the opinion that the duration of the test is a critical step during the assessment of anticancer drugs, as effects on DNA modifications might only become evident in multigeneration studies (Besse et al., 2012; Bialk-Bielinska et al., 2017). For example, imatinib mesylate was shown to result in more sensitive NOEC values in the F1 generation in fathead minnow compared to the effects of the parental generation (Williams et al., 2007). This is either due to epigenetic effects or teratogenic effects on embryos and larvae, as at this stage cell replication is forced due to the early developmental stage of the individuals. Teratogenic effects were for example determined during a fish embryo test with the drug decitabine, a DNA methylation blocker. Thus, determination of teratogenic effects in the fish embryo test seems to be suitable for assessment of effects of anticancer drugs.

A test species identified to be sensitive to anticancer drugs was the duckweed *Lemna minor*, which is exposed for 7 days and which responded to a treatment to cytotoxics and cytostatics in the µg/L to low mg/L range. Furthermore, algae were identified to be sensitive to anticancer drugs, as they were also fast dividing and thus susceptible to substances interacting with DNA. The interaction with DNA is furthermore less dependent on analogies with the target organism (human), as the interaction is not dependent on a specific motive rather than on actively dividing cells, i.e., the presence of single-stranded DNA.

This aspect however only accounts for cytotoxics and not for cytostatics, which act on specific target proteins. Thus, an assessment of sequence homologies is only necessary for cytostatics. However, the majority of NMEs is assigned to the class of cytostatics.

For cytostatics, the SeqAPASS analysis seems to be appropriate, as the performed analysis indicated that not only vertebrates but also invertebrates like the water flea, round worm, honey bee or mussels displayed homologies in the targets and seem to be susceptible for interaction with the drug. This is likely due to the ubiquitous presence of kinases and growth factors across the taxonomic classes.

Thus, the following strategy could be appropriate in order to assess the effects of anticancer drugs:

a) Determination of the MoA of the substances. Does it interact with DNA (i.e. cytotoxic) or does it act on kinases or by the inhibition of growth factors, e.g. by antibodies (i.e. cytostatics)?

b) If the substance is a cytostatic or interacts indirectly with the DNA: performance of SeqAPASS analysis in order to determine the most sensitive test organisms across taxonomic classes.

c) Performance of comet assay in an appropriate test system. Appropriate test systems could be for example a zebrafish liver cell line (Novak et al., 2017) or individuals of *Ceriodaphnia dubia* (Russo et al., 2018). These test systems are more appropriate to determine genotoxic effects of anticancer drugs on the environment than human or other mammalian cell culture systems.

d) Performance of chronic test approaches in actively dividing organisms. Appropriate test approaches are for example.

- Algae tests according to OECD TG 201 (included in the ERA according to the EMA guideline)
- Growth inhibition test with the test species *Lemna sp*. according to OECD TG 221
- Reproduction assay with *Daphnia magna* (OECD TG 211, 21 days, EMA guideline) or with *Ceriodaphnia dubia* (7 days)
- Fish embryo test according to OECD TG 236, including the assessment of teratogenic effect. This test would complement the early life stage test according to the OECD TG 210. A combination of these tests would provide the possibility to assess further endpoints like inclusion of an apoptosis assay, in addition to chronic effects on survival and growth.

3.8 Cardiologically active substances

3.8.1 Results of the literature review on ecotoxicological effects of cardiologically active substances

The drugs under the substance class cardiology are mainly for the treatment of hypertension, antithrombotic agents and lipid lowering agents. The relevant targets for the cardiovascular pharmaceuticals were identified and summarized in Table 8, e.g. the adrenergic receptors, angiotensin II receptor or the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase. The SeqAPASS cross-species extrapolation was performed for the distinct target sites and here the model organisms with the high homology were mainly fish species, e.g. common carp, zebrafish and Japanese medaka.

For some target sites e.g. the HMG-CoA reductase and calcium channel, also invertebrate model organisms showed homology to a lesser extent and can therefore be potentially susceptible.

Table 8: Model organisms with high conformity to targets of cardiologically active pharmaceuticals found by the SeqAPASS analysis with the corresponding NCBI accession number.

Target protein	NCBI accession	SeqAPASS
sodium and potassium ion channel	BAA78033.1 & AAB97315.1	channel catfish, rainbow trout, japanese medaka, zebrafish
3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase	AAA52679.1	zebrafish, channel catfish, japanese medaka (honey bee, fruit fly)
adrenergic receptors	NP_000675.1; NP_000016.1	japanese medaka, guppy, zebrafish, common carp, sheepshead minnow
endothelin receptor	AAA58465.1	three-spined stickleback, common carp, japanese medaka

Target protein	NCBI accession	SeqAPASS
factor Xa (Coagulation factor X)	AAH46125.1	common carp, guppy, sheepshead minnow
guanylate cyclase (sGC)	CAA75738.1	sheepshead minnow, guppy, rainbow trout
renin	AAA60363.1	japanese medaka, zebrafish, common carp
endothelin receptor	AAA58465.1	three-spined stickleback, common carp, japanese medaka
prostacyclin (IP, PGI2)	AAI10343.1	zebrafish, channel catfish, japanese medaka
angiotensin II receptor	CAA46621.1	rainbow trout, japanese medaka, common carp
PCSK9 (proprotein convertase subtilisin/kexin type 9)	NP_777596.2	common carp, zebrafish, channel catfish
P2Y12 receptor	NP_795345.1	common carp, zebrafish, guppy
protease-activated receptor 1 (PAR-1)	NP_001298242.1	common carp, zebrafish, guppy
hyperpolarization-activated cyclic nucleotide-gated channel (HCN, funny channels)	NP_001185.3	zebrafish, channel catfish, common carp, honey bee, common water flea
neprilysin	NP_001341571.1	channel catfish, japanese medaka, guppy
calcium channel	NP_000711.1	zebrafish, channel catfish, rainbow trout (honey bee, fruit fly)

For most of the pharmaceuticals of the cardiovascular drugs not much data on ecotoxicological effects were found in literature, also here data were obtained for model substances (Table 9), which act on similar targets.

One major class of pharmaceuticals within the cardiovascular drugs are the lipid lowering drugs, which can be divided into statins which lower the cholesterol, and the group of fibrates which are known to take care of fatty acids and triglycerides.

Statins are competitive inhibitors of HMG-CoA reductase. In plants, these compounds also inhibit HMGR, which regulates cytosolic isoprenoid biosynthesis in the mevalonic acid pathway (Brain, Reitsma et al. 2006). The statins atorvastatin and lovastatin were shown to be phytotoxic to *Lemna gibba* after 7 days exposure. Brain et al (Brain, Reitsma et al. 2006) found decreased concentrations of both stigmasterol and β-sitosterol which are critical components of plant membranes and regulate morphogenesis and development, with EC10 values of 26.1 µg/L for atorvastatin and 32.8 µg/L for lovastatin. For statin pharmaceuticals, sterol concentrations proved to be 2-3 times more sensitive than fresh weight of *Lemna gibba*, based on a comparison of ECx values (Brain, Reitsma et al. 2006).

For the copepod *Nitocra spinipes* the sensitive endpoints development time, RNA content, body length, growth rate (LOEC 0.16 µg/L), were about 5000 time more sensitive than the acute toxicity with an LC50 of 810 µg/L (Dahl, Gorokhova et al. 2006). For the amphipod *Gammarus locusta* reproduction was the most sensitive endpoint with LOEC of 320 ng/L of simvastatin

(Neuparth, Martins et al. 2014). Simvastatin and atorvastatin were tested on primary rainbow trout hepatocytes and where shown to be cytotoxic at concentrations < 200 mg/L (Ellestat 2010). Upon exposure of juvenile rainbow trout to atorvastatin for 7 days, an upregulated gene expression in gill was demonstrated at concentrations < 200 ng/L (Ellestat 2012). Zebrafish embryos were shown to be rather sensitive to simvastatin and showed abnormalities in development at 500 µg/L (Riberio 2015).

Angiotensin II receptor antagonists, also called sartans, modulate the renin–angiotensin system. Their main uses are in the treatment of hypertension (high blood pressure), diabetic nephropathy (kidney damage due to diabetes) and congestive heart failure.

For teleost fish a type-1 angiotensin II receptor was described, but experiments with the specific type-1 angiotensin II receptor antagonist losartan gave inconsistent results in fish, often acting as a partial agonist or as an inhibitor at high concentrations (Russell, Klemmer et al. 2001). In literature not much is found on ecotoxicological tests. The exposure of *Oncorhynchus mykiss*, *Daphnia magna* and *Desmodesmus subspicatus* to the sartans olmesartan and valsartan gave effect concentrations in the mg/L range (Bayer 2014).

Pharmaceuticals acting on the adrenergic receptors, also called beta-blockers, bind to the beta-adrenergic -receptor and block thus its activation or stimulation by natural ligands. Beta-blockers differ in specificity to the different receptor subtypes; some are non-specific like propranolol (i.e. exhibit similar affinity for beta1- and beta2-receptors), while others, e.g. atenolol and metoprolol are specific for the beta1-receptor subtype. Beta-receptors are located in many peripheral regions including lungs and heart, in the heart beta-receptor blockade causes a decrease in heart rate and contractile strength (Sanderson, Brain et al. 2004, Dzialowski, Turner et al. 2006).

Triebskorn et al. found for metoprolol-exposed trout a clear concentration-related effects in the livers (reduction of glycogen stores) with a LOEC of 1 µg/L. Since trout has been shown to contain β2-receptors in the heart and liver, which are structurally very similar to other vertebrate homologues, it is likely that β2- receptor antagonists, like metoprolol, also exert their specific action in fish (Triebskorn, Casper et al. 2007).

Adrenoceptors of the beta-type also exist in bivalves, showing gross pharmacological properties similar to their mammalian counterparts (Franzellitti, Buratti et al. 2011). Franzellitti et al. showed that pro-pranolol can inhibit the cAMP-signaling pathway in mussels at very low concentrations of 0.3 ng/L (Franzellitti, Buratti et al. 2011), furthermore propranolol was reported to lower the scope for growth, byssus strength and abundance with a LOEC of 1000 µg/L (Ericson, Thorsén et al. 2010), and feeding rate of mussels with a LOEC of 147 µg/L (Solé, Shaw et al. 2010). Contardo-Jara et al. report that metallothionein mRNA was immediately up-regulated in the freshwater mussel *Dreissena polymorpha* upon exposure to 0.5 µg/L metoprolol.

Dzialowski et al. report the physiological biomarker, heart rate, was the most sensitive endpoint to subchronic propranolol and metoprolol exposure in *D. magna*. Reproduction and growth of *D. magna* were less sensitive to chronic exposure to propranolol and metoprolol (Dzialowski, Turner et al. 2006). Also Dietrich et al. report on a rather low NOEC of 1.2 µg/L for *Daphnia magna* in a multigeneration experiment, daphnia exposed to metoprolol matured faster and showed a lower number of offspring.

Table 9: Overview of literature on ecotoxicological tests with cardiologically active model substances.

Target	Substance or model substance	Ecotox test	LOEC or EC_x/LC_x	Reference
HMG-CoA reductase	simvastatin	*Oncorhynchus mykiss* hepatocytes: exposure up to 72h, measurement of membrane integrity	LOEC < 200 mg/L	(Ellesat, Tollefsen et al. 2010)
HMG-CoA reductase	simvastatin	*Gammarus locusta*: survival, growth, reproduction, histopathological biomarkers in hepatopancreas and gonads	LOEC 320 ng/L on reproduction	(Neuparth, Martins et al. 2014)
HMG-CoA reductase	simvastatin	*Nitocra spinipes*: RNA content and body length increased significantly at 0.16 µg/L; Development time decreased 0.16 µg/L, growth rate increased at 0.16 µg/L; acute toxicity 96 h-LC50: 810 µg/L	LOEC 0.16 mg/L	(Dahl, Gorokhova et al. 2006)
HMG-CoA reductase	simvastatin	Danio rerio: embryo, 80h exposure, morphological abnormalities	LOEC 500 µg/L	(Ribeiro, Torres et al. 2015)
HMG-CoA reductase	iovastatin	*Lemna gibba*: 7 day exposure, concentrations of sterols and ubiquinone and plastoquinone	EC_{10} 32.8 µg/L stigmasterol decrease	(Brain, Reitsma et al. 2006)
HMG-CoA reductase	lovastatin	*Xenopus laevis*: Frog Embryo Teratogenesis Assay–Xenopus (FETAX), exposure for 96h.Detection of teratogenic effects like gut coiling	EC_{10} 14.2 mg/L	(Richards and Cole 2006)
HMG-CoA reductase	atorvastatin	*Lemna gibba*: 7 day exposure, concentrations of sterols and ubiquinone and plastoquinone	EC_{10} 26.1 µg/L stigmasterol decrease	(Brain, Reitsma et al. 2006)
HMG-CoA reductase	atorvastatin	*Oncorhynchus mykiss* gill cells: 7 day exposure, upregulated gene expression for genes involved in membrane transport (pgp, mrp1), oxidative stress response (sod, mt), apoptosis (bax) and biotransformation (sult2b) in gills at 200ng/L	LOEC 200 ng/L	(Ellesat, Holth et al. 2012)
HMG-CoA reductase	atorvastatin	*Oncorhynchus mykiss* hepatocytes: exposure up to 72h, measurement of membrane integrity	LOEC < 200 mg/L	(Ellesat, Tollefsen et al. 2010)
HMG-CoA reductase	atorvastatin	*Xenopus laevis*: Frog Embryo Teratogenesis Assay–Xenopus (FETAX), exposure for 96h.Detection of teratogenic effects like gut coiling	EC_{10} 17.8 mg/L	(Richards and Cole 2006)
adrenergic receptor	acebutolol	*Ceriodaphnia dubia*: 48h immobility tests	EC_{50} 50.9 mg/L	(Fraysse and Garric 2005)

Target	Substance or model substance	Ecotox test	LOEC or EC_x/LC_x	Reference
adrenergic receptor	atenolol	*Desmodesmus subspicatus*: Growth inhibition – 72 h	EC_{50} 620 mg/L	(Cleuvers 2005)
adrenergic receptor	atenolol	*Raphidocelis subcapitata*: Growth inhibition – 72 h	NOEC 128.8 mg/L	(Küster, Alder et al. 2010)
adrenergic receptor	atenolol	*Ceriodaphnia dubia*: 48h immobility tests	EC_{50} 33.4	(Fraysse and Garric 2005)
adrenergic receptor	atenolol	*Brachionus koreanus*: 24h exposure, icrease in the P-glycoprotein mRNA expression	NOEC 0.05 mg/L	(Rhee, Jeong et al. 2012)
adrenergic receptor	atenolol	*Danio rerio*: Larvae mortality – 96 h	LC_{50} 2.5 mg/L	(Sun, Xin et al. 2014)
adrenergic receptor	atenolol	*Oncorhynchus mykiss*: Haematocrit – 21 d	NOEC 0.001 mg/L	(Steinbach, Burkina et al. 2014)
adrenergic receptor	betaxolol	*Daphnia magna*: 48h immobility tests	EC_{50} > 300 mg/L	(Hernando, Petrovic et al. 2004)
adrenergic receptor	metoprolol	*Desmodesmus subspicatus*: Growth inhibition – 72 h	EC_{50} 7.3 mg/L	(Cleuvers 2003)
adrenergic receptor	metoprolol	*Lemna minor*: NOEC (Growth inhibition, frond area – 7 d) 100o	NOEC 100 mg/L	(Maszkowska, Stolte et al. 2014)
adrenergic receptor	metoprolol	*Daphnia magna*: 48h immobility tests	EC_{50} 2.6 mg/L	(Czech, Jośko et al. 2014)
adrenergic receptor	metoprolol	*Daphnia magna*: Body length of neonates; Body length of females; age at first reproduction; number of offspring	NOEC 0.0012 mg/L	(Dietrich, Ploessl et al. 2010)
adrenergic receptor	metoprolol	*Danio rerio*: Coagulation of embryo, abnormalities, unsuccessful hatching, deformations and growth retardation – 72 h	EC50 31 mg/L	(van den Brandhof and Montforts 2010)
adrenergic receptor	metoprolol	*Daphnia magna*: acceleration of the heart beat rate	LOEC 26.7 mg/L	(Villegas-Navarro, Rosas-L et al. 2003)
adrenergic receptor	metoprolol	*Oncorhynchus mykiss*: 28d exposure	LOEC 1 µg/L changes in liver: reduction of glycogen stores	(Triebskorn, Casper et al. 2007)

Target	Substance or model substance	Ecotox test	LOEC or EC_x/LC_x	Reference
adrenergic receptor	metoprolol	*Daphnia magna:* 9 day exposure - growth, fecundity, heart rate and metabolic rate	LOEC for heat rate 3.1 mg/L,	(Dzialowski, Turner et al. 2006)
adrenergic receptor	metoprolol	*Dreissena polymorpha:* 7d exposure, metallothionein mRNA upregulation, mRNA up-regulation in gills was found for P-glycoprotein, catalase and superoxide dismutase were up-regulated in the digestive gland indicating oxidative stress	LOEC (metallothionein mRNA) 0.534 µg/L	(Contardo-Jara, Pflugmacher et al. 2010)
adrenergic receptor	nadolol	*Ceriodaphnia dubia*: 48h immobilization test	EC_{50} 163.4 mg/L	(Fraysse and Garric 2005)
adrenergic receptor	oxprenolol	*Ceriodaphnia dubia*: 48h immobility tests	EC_{50} 10.1 mg/L	(Fraysse and Garric 2005)
adrenergic receptor	practolol	*Thamnocephalus platyurus*: acute test , 1h	EC_{50} 115mg/L	(Nalecz-Jawecki and Persoone 2006)
adrenergic receptor	propranolol	Rainbow trout cell lines: EROD assay	EC_{50} 453 µM cytotoxicity, EC_{50} 27 µM EROD	(Laville, Aït-Aïssa et al. 2004)
adrenergic receptor	propranolol	*C. dubia*: Acute lethality – 48 h	LC_{50} 0.8 mg/L	(Huggett, Brooks et al. 2002)
adrenergic receptor	propranolol	*C. dubia*: Reproduction-7 d	NOEC 0.009 mg/L	(Ferrari, Mons et al. 2004)
adrenergic receptor	propranolol	*Mytilus galloprovincialis:* Catalase activity in digestive gland, 7d	NOEC 3.10 mg/L	(Franzellitti, Buratti et al. 2011)
adrenergic receptor	propranolol	*Oryzias latipes:* Larvae mortality – 96 h	EC_{50} 11.4 mg/L	(Kim, Ishibashi et al. 2009)
adrenergic receptor	propranolol	*Oryzias latipes*: Female steroid concentration- 2 weeks	NOEC 0.001 mg/L	(Huggett, Brooks et al. 2002)
adrenergic receptor	propranolol	*Ceriodaphnia dubia*: 48h immobilization test	EC_{50} 1.4 mg/L	(Fraysse and Garric 2005)

Target	Substance or model substance	Ecotox test	LOEC or EC_x/LC_x	Reference
adrenergic receptor	propranolol	*Daphnia magna*: 9 day exposure - growth, fecundity, heart rate and metabolic rate	LOEC for heat rate 0.055 mg/L	(Dzialowski, Turner et al. 2006)
adrenergic receptor	propranolol	*Cyclotella meneghiniana*: Growth inhibition – 96 h	EC_{50} 0.24 mg/L	(Ferrari, Mons et al. 2004)
adrenergic receptor	propranolol	*Cyclotella meneghiniana*: Growth inhibition – 96 h	NOEC 0.094 mg/L	(Ferrari, Mons et al. 2004)
adrenergic receptor	propranolol	*Skeletonema pseudocostatum*: Growth inhibition – 72 h	EC_{50} 0.24 mg/L	(Petersen, Heiaas et al. 2014)
adrenergic receptor	propranolol	*A. fischeri*: Bioluminescence inhibition – 30 min	EC_{50} 61 mg/L	(Ferrari, Mons et al. 2004)
adrenergic receptor	propranolol	*Mytilus galloprovincialis*: 10 day exposure; feeding rate	LOEC 147 µg/L	(Solé, Shaw et al. 2010)
adrenergic receptor	propranolol	*Mytillus edulis trossulus* (Baltic Sea blue mussels): exposure 7 days, Byssus strength, Scope for growth, mortality	LOEC 1000 µg/L	(Ericson, Thorsén et al. 2010)
angiotensin receptor	valsartan	*D. subspicatus*: 72 growth inhibiotn test	NOEC 85 mg/L	(Bayer, Asner et al. 2014)
angiotensin receptor	valsartan	*Oncorhynchus mykiss*: 96h mortality	LC_{50} >100 mg L	(Bayer, Asner et al. 2014)
angiotensin receptor	valsartan	*Daphnia magna*: 48h immobilisation assay	EC_{50} > 580 mg/L	(Bayer, Asner et al. 2014)
angiotensin receptor	olmesartan	*Danio rerio*: 48h	EC_{50} > 120 mg/L	(Bayer, Asner et al. 2014)
angiotensin receptor	olmesartan	*Daphnia magna*: 48h immobilisation assay	EC_{50} > 120 mg/L	(Bayer, Asner et al. 2014)
angiotensin receptor	olmesartan	*Desmodesmus subspicatus*: 72h growth inhibition	NOEC 60 mg/L	(Bayer, Asner et al. 2014)
angiotensin receptor	losartan	brown mussel *Perna perna*: exposure for 96h; induction of CYP450 like activity and glutathione S-transferase in mussel gills	LOEC 300 ng/L	(Cortez, Souza et al. 2018)

3.8.2 Potential strategies to assess the specific risk of cardiologically active substances

The literature review identified that studies with cardiologically active substances were limited to a number of 13 substances, with 3 different molecular targets, i.e. the HMG-CoA- reductase (statins), the adrenergic receptor, and the angiotensin receptor.

No data on the standard tests required for the ERA according to the EMA guideline were found in the literature.

Interestingly, plants possess a similar target to HMG-CoA reductase, i.e. HMGR. A study of Brain, Reitsma (2006) indicated that *Lemna gibba* was indeed sensitive to the treatment with statins. Thus, the inclusion of a *Lemna sp*. study seems to be appropriate for the assessment of substances acting by a similar MoA.

Furthermore, chronic endpoints were determined to be much more sensitive than acute toxicity, and LOECs in the µg/L (development of *Nicocra spinipes*, Dahl et al., 2006; abnormal development in zebrafish embryos, Riberio 2015) to the ng/L range were identified (reproduction in *Gammarus locusta*, Neuparth et al., 2014).

As the main goal of statins is the reduction of the cholesterol levels, it might be that these substances also possess endocrine effects, as cholesterol functions as the backbone of the steroids estrogen and testosterone. As especially zebrafish react very sensitive to imbalanced steroid levels, studies on the development of the sex ratio might be appropriate.

Angiotensin II receptor antagonists, the sartans, did not result in acute toxicity to aquatic organisms. Responses of *Oncorhynchus mykiss*, *Daphnia magna* and *Desmodesmus subspicatus* were in the mg/L range. Also the development of the sea urchin seems not to be affected by low concentrations of the sartan valsartan and effects were only determined in the mg/L range.

Beta-blockers, which act on the adrenergic receptors, might result in responses in fish or in bivalves, as similar receptors to their mammalian counterparts are present. Performed test, which resulted in effects, include the assessment of the glycogen stores in trout liver (Triebskorn et al., 2007), or the assessment of cAMP signalling, growth or feeding rate in mussels (Franzellitti et al., 2011; Ericson et al., 2010, Sole et al., 2010). One physiological biomarker furthermore seems to be sensitive to beta-blockers, i.e. the heart rate of *Daphnia magna*. The heart rate and blood flow can be furthermore easily assessed in the FET, which might be another test system for the assessment of the effect of beta-blockers. A response of zebrafish embryos after treatment with beta-blockers was for example demonstrated by Bittner, Teixido et al. (2018), who demonstrated an effect of metoprolol, labetalol, and propanolol on heart rate and swimming behaviour of zebrafish embryos.

Thus, for the substance class of statins we suggest the following:

a) Performance of plant studies, e.g. the studies according to the OECD TG 221 with *Lemna sp*., as these organisms seem to be especially sensitive

b) Studies determining the embryo/larval development of sensitive species (e.g. zebrafish, more sensitive species could be determined by a SeqAPASS analysis)

c) FSDT according to the OECD TG 234 (probably inadequate as no literature available that supports this assumption).

For the class of sartans, no appropriate test strategy could be suggested.

For the class of beta-blockers we suggest the following:

a) Assessment of glycogen levels in fish: Glycogen levels can be analyzed semi quantitatively by cytopathology of the liver or by different glycogen assays for biological tissues or fluids with a colorimetric read out or by the methods (with iodine or with anthrone) described by Van der Vies, (1954). The assessment of the glycogen levels could be included in the required FELS study, in order to obtain material for gycogen measurements. Thus, this endpoint would not require additional animal studies.

b) Application of bivalves as test organism, assessment of growth rate

c) FET or *Daphnia magna* for the assessment of heart rate

4 Work package 3

In work package 3 substances from each substance class were selected, in order to compare the assessment according to the guideline on the environmental risk assessment of medicinal products for human use (EMEA/CHMP/SWP/4447/00 corr 2,), with the test strategies suggested in this report.

For the selection of substances two criteria were defined to be crucial:

a) Data on the environmental risk assessment for algae growth inhibition, daphnia reproduction and fish early life stage test should be available in EPARs.

b) For the specific mode of action of each substance, literature on model substances acting on the same specific target should be available.

With the available data on the substances and the model substances it is possible to review, whether the refined test strategy leads to more sensitive test results, compared to the already existing data (Table 10).

For the neuroactive substances, many substances were already excluded by applying the two criteria. For the specific targets sodium channel, serotonin and dopamine receptor, literature on different model substances acting on the targets were found. The model substances for these targets are for example Fluoxetine and Venlafaxine (serotonine receptor), and Carbamazepine (sodium channel).

The substances Vortioxetine and Iloperidone, both acting on the 5HT2A (serotonin) & D2 (dopamine) receptors, and Lorcaserin targeting the 5HT2C serotonin receptor were selected. Furthermore Eslicarbazepine with the target sodium channel was selected. Another substance may be selected with a target on which not much information is available, in order to test if a refined risk assessment strategy is applicable also to neuroactive substances with a different MoA. Here, Dimethyl fumarate with the enzyme target heme-oxygenase (HO-1) or Varenicline with the acetylcholine receptor as target, would be appropriate test substances. As indicated in chapter 3.6.2, behavioural tests with at least two taxonomic classes (i.e. fish and invertebrates), are suggested (Table 10).

The oncologically active substances, which met the two criteria were mainly cytostatics with the target group kinase. Here three substances target the same kinase (CDK4, CDK6), Palbociclib, Ribociclib and Abemaciclib, allowing a direct comparison whether the refined risk assessment results in the same sensitivity when the substances act on the same target. Furthermore, Bosutinib monohydrate can be tested, as the target is a tyrosine kinase (BCR-ABL) or Vandetanib acting on a different set of kinases (RET, VEGFR1, VEGFR2, VEGFR3, EGFR). The model substances identified in the literature were Imatinib mesylate or Erlotinib. In contrast to the cytostatic mode of action, also the cytotoxic modes of action would be of interest. In this case, Cabazitaxel acting on the beta tubulin or Panobinostat with the specific target deacetylase (DAC) would be appropriate targets. The model substance Paclitaxel also acts on beta tubulin, while no model substance actin on the deacetylase was identified.

The test strategy for oncologically active substances follows the strategy described in chapter 3.7.2. Briefly, we suggest a tiered approach with a Comet assay as first tier, and a chronic test battery with actively dividing organisms. Even though the Comet assay is already required for the approval of human pharmaceuticals, we suggest the test with appropriate systems, i.e. with a zebrafish liver cell line or with individuals of *Ceriodaphnia dubia*. For chronic studies, we suggest algae test according to the OECD TG 201 and growth inhibition tests with the test species *Lemna sp.* according to OECD TG 221.

For the cardiologically active substances even less substances met the two criteria, for the selection of test substances. Here, only for two targets information on model substances were found. Pitavastatin acting on the HMG-CoA reductase and Valsartan targeting the angiotensin receptor were selected. Model substances for HMG-CoA reductase were Artorvastatin or Simvastatin, and for the angiotensin receptor, Valsartan itself or Olmesartan were identified. The use of Valsartan as test substance is considered beneficial, as the results could be directly compared to the results obtained in the literature. Thus, as validation of the literature data is already included. For the rather unknown targets, the enzyme factor Xa with the substances Apixaban or Edoxaban or Dronedarone HCl acting on ion channels (sodium, potassium & calcium) could be tested. The test strategy for cardiologically active substances is described in chapter 3.8.2. The strategy includes studies with the test species *Lemna sp*., as this test organism seems to be especially sensitive to statins. For sartans, assessment of the glycogen level in fish liver cells, assessment of the growth rate in bivalves, or determination of the heart rate in fish embryos or *Daphnia magna*.

Table 10: Potential test substances for the practical testing of the developed tailored risk assessment strategies.

Substance	Target
Neuroactive substance	
Vortioxetine	serotonin (5-HT)(3A) receptor, h5-HT(7) receptor, h5-HT(1B) receptor, h5-HT(1A) receptor, 5-HT transporter
Iloperidone	5HT2A (serotonin) & D2 (dopamine) receptors
Lorcaserin	Dopamine-2 (D2), 5-HT2A, 5HT1A, 5-HT7 receptors, alpha-2C adrenergic receptor
Eslicarbazepine	sodium channel
Dimethyl fumarate	heme oxygenase 1 (HO-1)
Varenicline	acetylcholin receptor
Oncologically active substances	
Palbociclib	deacetylase (DAC)
Panobinostat	deacetylase (DAC)
Ribociclib	kinase CDK4, CDK6
Abemaciclib	kinase CDK4, CDK6
Bosutinib monohydrate	tyrosine kinase BCR-ABL
Vandetanib	Kinase RET, VEGFR1, VEGFR2, VEGFR3, EGFR
Cabazitaxel	beta tubulin
Cardiologically active substances	
Pitavastatin	3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase
Valsartan	angiotensin II receptor

Substance	Target
Apixaban	factor Xa
Edoxaban	factor Xa
Dronedarone HCl	factor Xa

5 Discussion

This literature review intended to identify the specific problems caused by human pharmaceuticals of a new generation (NMEs) and potential tailored risk assessment strategies. The highest number of pharmaceuticals belonged to the categories 'Neurology', 'Oncology' and 'Infections'. However, as antibiotics are already specifically mentioned in the new draft EMA guideline, and are thus already under specific consideration, we decided to include the category 'Cardiology' instead for the definition of a TRA.

During the review of the literature, it was identified that there was only limited information on ecotoxicological effects of NMEs. Thus, we focussed on information on ecotoxicological effects of model substances of relevant MoAs. The quality of the identified literature was checked by the application of modified CRED scores.

Another aspect which impedes the interpretation of the data was that the effects of the model substances in the literature studies did not allow an assignment to a specific MoA. The most sensitive species was thus not identical even for substances with similar MoA. A potential explanation is that the pharmaceuticals do not only interact by their primary MoA. Also other structural elements of the substances, which are ineffective in the human target, could influence the effect in non-target organisms in different ways. Other than for endocrine substances, these secondary effects might be more effective than the indicated MoA. The same is true also for the SeqAPASS analyses. A high homology of the target did not directly allow a conclusion on the most sensitive species, as targets for other structural elements are not defined. Nevertheless, we suggest performing this analysis as it is easily performed and provides basic information on the susceptibility of any species investigated.

5.1 Excluded MoAs

For several substance classes, an environmental concern with respect to the action limit of 0.01 µg/L already exists, e.g. for substances with sexual endocrine MoA. According to this, a specific risk assessment strategy for these substances is already into force. Thus, sexual endocrine substances were excluded from this literature review. Furthermore, a recent UBA project is aimed to develop a TRA for progestins and glucocorticoids, which are likely to act on the reproductive axis of fish as the most sensitive endpoint. Thus, these substance classes, for which some new pharmaceuticals are marketed, were excluded from further investigations.

Furthermore, the EMA guideline as well as the new draft already considers the effects of antimicrobials and specifically recommends studies to be performed for these substance category. The guideline recognizes that effect assessment at lower trophic levels (bacteria, algae, aquatic invertebrates) is sufficiently sensitive for antimicrobials. Thus, fish tests are not required. The guideline recommends studies according to the OECD TG 201 (Algae, Growth Inhibition Test) with a blue-green algae (Cyanophyta, e.g. Anabaena flos-aquae or Synechococcus leopoliensis) or with the green algae Raphidocelis subcapitata. As there is not validated scientific evidence for an increased sensitivity of cyanobacteria compared to green algae, it would be beneficial to perform the studies according to OECD TG 201 with at least two species, covering both taxonomic groups. Furthermore, also studies according to the OECD TG 211 should be performed (Daphnia reproduction study). Effects on microbial communities should be assessed with the OECD TG 209 (Activated Sludge, Respiration Inhibition Test). Thus, also for this substance class, a tailored approach would not deliver substantial more information compared to the approach suggested in the EMA guideline.

5.2 Fate studies do not require a tiered testing strategy based on the MoA of the pharmaceutical

The EMA guideline requires mandatory fate studies for the ERA of pharmaceuticals (Phase II Tier A). These mandatory studies include adsorption/ desorption studies using the batch equilibrium method with three soils and two sludges, according to the OECD TG 106. Furthermore, the ready biodegradability test according to the OECD TG 301 is required. The results of these studies are necessary to determine if further studies are required and to perform PEC calculations for soil and sediment. If a substance might enter the groundwater, the DT50 value has to be additionally derived from an OECD TG 308 study (Aerobic transformation in aquatic sediment systems). If the substance enters the soil, the degradation in soil according to the OECD TG 307 has to be determined instead. For this study, different soils have to be used.

Thus, the current EMA guideline provides a detailed description which approach has to be followed in the case that results indicate no biodegradation and no transformation. Furthermore, negative results, i.e. no biodegradation and no transformation of the substance, would lead to a more detailed testing, and a refined PEC calculation. The results of the above mentioned studies directly contribute to the refined PEC calculation, and substances which are not biodegradable or which are persistent in any of the compartments have to be labelled appropriately and the applicant should propose adequate precautionary and safety measures. This indicates that the risk to miss potentially persistent substances is small compared to the chance to miss potentially ecotoxic substances.

5.3 The stability of biologicals in the environment and the environmental relevance

In the offer, we propose to place special emphasis on biopharmaceuticals. Biopharmaceuticals (or biologicals) are substances, which are manufactured in or extracted from biological sources.

After the EMA guideline was placed into force, 470 pharmaceuticals are approved. 114 pharmaceuticals belong to the type of biopharmaceuticals. These encompass antibodies, enzymes, or other types of peptides/proteins, which potentially act as receptor agonist or antagonist, enzyme inhibitor or as inhibitor of tumor growth factors. As described in chapter 3.1, the sensitivity depends on the homology between the biological target in humans and in the non-target organisms. In most cases, antibodies are designed to act very specific on their target, and a cross-reactivity to other species is very unlikely. The above mentioned effects due to other structural elements are not applicable to antibodies, as the structural composition of antibodies in heavy and light chains, and variable and non-variable regions always follow the same principle. Furthermore, antibodies are subjected to many different instability mechanisms, which can be divided into chemical and physical instabilities (Le Basle, Chennell et al. 2019). The most frequent chemical degradation is for example oxidation, which happens in the presence of oxidants, for example light. Antibodies are also susceptible to fragmentation (disruption of disulfide bonds), especially in the hinge region between the antibody binding fragment and the constant fragment. In the environment, antibodies are more exposed to physical instabilities, triggered by temperature or pH, resulting in denaturation of the protein and loss of the higher-order structure.

Other peptides/proteins with enzymes or receptors as target proteins might be of higher interest, and should be considered similar to small molecules. For these biologicals, the MoA has to be considered. To be nevertheless able to discriminate small synthetic molecules from biologicals, we have marked the substances differently in Table 3 (b = biological; s = synthetical).

Thus, a general TRA for biologicals cannot be proposed, and the TRA should consider the MoA and the target rather than the nature of the pharmaceutical.

However, there is a general argument that the majority of biologicals is comparably instable in the environment, and thus do not pose a risk to non-target organisms. This assumption is for example supported by Straub (2010), who tested eight different protein and peptide therapeuticals, belonging for example to the group of recombinant hormones (e.g. Neorecormon), recombinant antibodies (e.g. Tocilizumab, Bevacizumab), or synthetic peptides (e.g. Enfuviritide). With these substances, a manometric respirometry test according to the OECD TG 301F or a closed bottle test according to the OECD TG 301D was performed for the determination of the ready biodegradability. The results of this study indicated that all peptides and proteins tested in this study were ready biodegradable, with > 70% degradation at test end. Further personal communication confirmed the ready biodegradability of proteins and peptides (see also https://www.roche.com/sustainability/environment/environmental-risk-assessment-downloads.htm).

One biological was designed as siRNA (small interfering RNA). SiRNAs are small, single- or double-stranded RNA molecules with a length of 20 to 25 base pairs. They are designed to covalently bind to single-stranded RNA molecules, thus inhibiting its translation into proteins, which results in a degradation of mRNA of a specific target protein.

Specifically, the pharmaceutical Patisiran is applied for the treatment of transthyretin-mediated amyloidosis and results in a dose-dependent knockdown of transthyretin in liver. This approach is very specific and a mismatch in the complementary RNA sequence results in no binding of the siRNA molecule, and finally in no degradation of the target mRNA. However, it is assumed that non-target effects of siRNA appear. These are either triggered by the delivery formulation (cationic lipids) or by incomplete binding of the siRNA to the mRNA, which does not result in degradation, but in an inhibition of translation (Fedorov, Anderson et al. 2006). To avoid these off-target effects, which might not only result in adverse effects in non-target organisms but which is also critical for the patients, chemical modification patterns are introduced.

While the off-target effects of the delivery formulations are difficult to predict, the similarity of RNA sequences in non-target organisms can be reviewed, e.g. by a blast research identifying similar mRNA sequences. However, if these off-target effects finally result in an effect in a non-target organism could not be predicted beforehand.

5.4 Potential tailored risk assessment strategies

The literature review demonstrated that there is a lack of information on specific endpoints for different classes of human pharmaceuticals. While e.g. endocrine disruptors show specific effects in different organisms, which are partly unequivocally allocated to endocrine disruption (like a reversal of the sex ratio in fish), similar specific endpoints are lacking for other substance classes. Endocrine mediated effects usually occur at very low concentrations, and the acute to chronic ratio is quite high. Thus, specific endpoints, which can be unequivocally assigned to the endocrine disruptive properties of the substances, could be defined. Usually, these specific analyses could be performed at concentrations which are much below the concentrations effective for example in a FELS. This is not the case for other substance classes, for which acute and substance-specific effects occur in a similar concentration range. For example, cardiologically active substances act on the heart rate and the blood flow in fish embryos, effects which could result in mortality at higher concentrations. The test concentrations applied in more specific assays would be identical to the test concentrations applied in a FELS, and the results might be similar. Thus, it is difficult to distinguish these effects from systemic effects.

Furthermore, the benefit of more specific tests is questionable if the FELS would result in an effective ERA. A more comprehensive study comparing acute, chronic effects assessed by FELS studies, and substance-specific chronic effects would help to identify assays which are more sensitive than FELS studies and which would result in a proper identification of the substance class-specific risk.

We also observed that there is a lack of comprehensive data, which allow comparison of results obtained in standard studies required by the EMA guideline with effects in studies specifically designed to assess effects of a pharmaceutical. Studies applying alternative assays are mainly conducted with a limited number of references substances, for which no EPAR exists, and vice versa. Thus, it was not possible to identify if an alternative testing strategy was more or less sensitive than the standard study. We also observed that substances which belong to the same substance class do not always results in a similar order of sensitivity when applied in a similar testing battery. This might be due to effects which are likely a result of chemical residues or interactions with other targets.

Thus, we suggest to initially create a solid data base, by parallel performance of the standard studies and the more specific assays identified in this literature review.

The suggested test strategies in this review, which were described for the three substance classes cardiology, oncology and neuroactive substances, should to date complement the established test strategy described by the EMA (Doc. Ref. EMEA/CHMP/SWP/4447/00 corr 2), rather than replacing it. Partly, the tests required by EMA can be used as basis and further endpoints can be examined, e.g. the glycogen level in fish in combination with the fish early life stage test (OECD210). The outcome of the additional endpoints can be very beneficial in order to improve the risk assessment for distinct active pharmaceutical ingredients.

With sufficient data on new ecotoxicological test e.g. fish embryo test, behavioural tests or tests with bivalves, a comparison of sensitivity with existing data from the environmental risk assessment can be performed. Subsequently, less sensitive tests or endpoints can be excluded for the respective substance group in order to improve the risk assessment.

However, not for every substance class this tailored risk assessment can be performed. Only if sufficient data on potential sensitive endpoints and targets is available in literature, a tailored risk assessment can be developed. Thus, we suggest to continously review new literature in order to increase the data base for potential new testing approaches and the identification of new test strategies.

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A Appendix

A.1 CRED evaluation

Table 11: Selected CRED criteria for the evaluation of ecotoxicity data for reliability and relevance (table modified according to Moermond et al 2015).

Number	Evaluation criteria for reliability and relevance	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
Evaluation for Reliability						
General information						
1	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?					
2	Are appropriate controls performed (e.g. solvent control, negative and positive control)?					
	Test compound					
3	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?					
4	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?					
	Test organism					
5	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?					
6	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?					
	Exposure conditions					
7	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?					

Number	Evaluation criteria for reliability and relevance	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
8	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?					
9	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?					
10	Is the exposure duration defined?					
11	Are chemical analyses adequate to verify concentrations of the test substance over the duration of the study?					
	Statistical Design and Biological Response					
12	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?					
13	Are appropriate statistical methods used?					
Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)						

Evaluation for Biological Relevance

	General information					
14	Is the species tested relevant for the compartment under evaluation?					
15	Are the organisms tested relevant for the tested compound?					
16	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?					
17	Are appropriate life-stages studied?					
18	Are the experimental conditions relevant for the tested species?					
19	Is the exposure duration relevant and appropriate for the studied endpoints and species?					

Number	Evaluation criteria for reliability and relevance	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Exposure relevance					
20	Is the tested exposure scenario relevant for the substance?					
21	Is the tested exposure scenario relevant for the species?					
Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)						

Table 12: Summary of the CRED evaluation with the final scores for reliability and relevance. R1/C1 = Reliable, R2/C2 = Reliable with restrictions, R3/C3 = Not reliable, R4/C4 = Not assignable.

Reference	Reliability	Relevance
Neurology		
Barrozo et al. 2015	R1	C1
Bisesi et al. 2014	R1	C1
Bossus et al. 2014	R1	C1
Bownik et al. 2018	R2	C1
Brandão et al. 2013	R2	C1
Brooks et al. 2003	R4	C4
Campos et al. 2012	R1	C1
Castro-Catala et al. 2017	R1	C1
Fong et al. 2012	R1	C1
Foster et al. 2010	R2	C1
Galus et al. 2013	R2	C1
Guler and Ford, 2010	R2	C1
Hazelton et al. 2014	R1	C1
Hossain et al. 2019	R1	C1
McDonald et al. 2011	R2	C1
Melvin, 2017	R1	C1
Minguez et al. 2016	R1	C1
Nassef et al. 2010	R1	C1
Painter et al. 2009	R2	C1
Péry et al. 2008	R2	C1
Rivetti et al. 2019	R1	C1
Schultz et al. 2011	R1	C1
Simão et al. 2019	R1	C1
Stanley et al. 2007	R2	C1
Valenti et al. 2012	R2	C1
Villeneuve et al. 2010	R1	C1
Weinberger & Klaper, 2014	R2	C1
Yang et al. 2014	R1	C1

Reference	Reliability	Relevance
Cardiology		
Bayer et al. 2014	R2	C1
Brain et al. 2006	R2	C1
Cleuvers, 2003	R2	C1
Contardo-Jara et al. 2010	R1	C1
Cortez et al. 2018	R1	C1
Czech et al. 2014	R2	C1
Dahl et al. 2006	R1	C1
Dietrich et al. 2010	R1	C1
Dzialowski et al. 2006	R2	C1
Ellesat et al. 2010	R1	C1
Ellesat et al. 2012	R1	C1
Ericson et al. 2010	R1	C1
Ferrari et al. 2004	R4	C4
Franzellitti et al. 2011	R1	C1
Fraysse & Garric, 2005	R2	C1
Hernando et al. 2004	R4	C4
Huggett et al. 2002	R2	C1
Kim et al. 2009	R2	C1
Küster et al. 2010	R4	C4
Laville et al. 2004	R2	C1
Macreadie et al. 2006	R3*	C2*
Maszkowska et al. 2014	R1	C1
Nalecz-Jawecki & Persoone, 2006	R2	C1
Neuparth et al. 2014	R1	C1
Petersen et al. 2014	R1	C1
Rhee et al. 2012	R1	C1
Ribeiro et al. 2015	R1	C1
Richards & Cole, 2006	R2	C1
Solé et al. 2010	R1	C1
Steinbach et al. 2014	R1	C1

Reference	Reliability	Relevance
Sun et al. 2014	R2	C1
Triebskorn et al. 2007	R2	C1
van den Brandhof & Montforts, 2010	R1	C1
Villegas-Navarro et al. 2003	R2	C1
Yamamoto et al. 2014	R3*	C3*
Oncology		
Besse et al. 2012	R4	C4
Bialk-Bielinska et al. 2017	R2	C1
Booker et al. 2014	R4	C4
Kovacs et al. 2016	R1	C1
Mahony et al. 2013	R2	C1
Novak et al. 2017	R1	C1
Parrella et al. 2014	R1	C1
Ribas et al. 2017	R2	C1
Russo et al. 2018	R1	C1
Zounkova et al. 2007	R2	C1
Zounkova et al. 2010	R2	C1

* The literature was classified as not relevant and not reliable and was therefore excluded from further evaluation.