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From theory to reality – Evaluation of suitable organisms and test systems for the biomonitoring of pharmaceuticals. Part I: Literature review

by

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Abstract

In a literature review aiming at identifying sensitive organisms, biological endpoints *in vivo*, and suitable *in vitro* test systems for a biomonitoring of pharmaceuticals, the database OEKOTOX was upgraded with effect data published for 90 pharmaceuticals of high priority until 2013. Lowest effect concentrations were identified and the respective studies checked for reliability. Reliable MECmax/LOECmin-values >0.1 for a minimum of 3 different biota classes were identified for diclofenac and, for 2 biota classes, for propranolol, sulfamethoxazole, bezafibrate, 17α-ethinlyestradiol, 17β-estradiol and oxytetracycline. The reliably most sensitive organisms were *Danio rerio*, *Oncorhynchus mykiss*, *Oryzias latipes*, *Elliptio complanata*, and *Potamopyrgus antipodarum*. The most sensitive endpoint was behavioural change. Furthermore, the state of knowledge in the field of *in vitro* testing of pharmaceutical effects as well as advantages and disadvantages of these biochemical and cell-based assays were reviewed. As a future perspective for a biomonitoring of pharmaceuticals, the development of mode of action-based *in vitro* test systems for β-blockers and NSAIDs are recommended. Signals obtained by these assays should be aligned with *in vivo* responses of the above mentioned sensitive organisms or environmentally relevant biota of German streams.

Kurzbeschreibung

In einer Literaturstudie mit dem Ziel, sensitive Organismen und organismische Endpunkte sowie geeignete *in vitro*-Testsysteme für ein Biomonitoring von Arzneimitteln zu identifizieren, wurde die bestehende Datenbank OEKOTOX um Wirkdaten für 90 Arzneimittel von hoher Priorität bis 2013 erweitert. Niedrigste Effektkonzentrationen wurden identifiziert und die jeweiligen Studien auf Reliabilität überprüft. Reliable MECmax/LOECmin-Werte >0.1 wurden für Diclofenac bei mehr als 3 Organismengruppen und für Propranolol, Sulfamethoxazol, Bezafibrat, 17α-Ethinlyestradiol, 17β-Estradiol und Oxytetrazyclin bei 2 Organismengruppen identifiziert. Als reliabel sensitivste Organismen erwiesen sich *Danio rerio*, *Oncorhynchus mykiss*, *Oryzias latipes*, *Elliptio complanata* und *Potamopyrgus antipodarum*. Der sensitivste Wirkendpunkt waren Verhaltensänderungen. Des Weiteren wurden der Wissensstand zu *in vitro*-Verfahren zum Nachweis von Arzneimittel-Effekten sowie Vor- und Nachteile dieser biochemischen und zellbasierten Assays recherchiert. Auf dieser Basis wird für ein künftiges Biomonitoring von Arzneimitteln empfohlen, Wirkstoffklassen-spezifische *in vitro*-Testsysteme für β-Blocker und nichtsteroidale Analgetika zu entwickeln und deren Signale mit *in vivo*-Reaktionen o.g. sensitiver bzw. für deutsche Fließgewässer relevanter Organismen abzugleichen.

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List of Abbreviations

A. victoria	Aequorea victoria
ADP	Adenosinediphosphate
ALPHA	Amplified Luminescent Proximity Homogeneous Assay
AMP	Adenosine monophosphate
AP	Alkaline phospatase
ATP	Adenosinetriphosphate
β-AR	β-adrenoceptor
BRET	Bioluminescence resonance energy transfer
C.elegans	*Caenorhabditis elegans*
CaM	Calmodulin
cAMP	Cyclic adenosinemonophosphate
CAS	Chemical Abstracts Service
CDCFDA	5′, 6′ Dicarboxy-2′,7′-dichlorodihydrofluorescein
CDCFDA	2′,7′-dichlorodihydrofluorescein
CFP	Cyan fluorescent protein
cGMP	Cyclic guanosinemonophosphate
CNG	Cyclic nucleotide-gated
COX	Cyclooxygenase
CRE	Cyclic adenosinemonophosphate response element
CREB	cAMP response element binding protein
DOI	Digital Object Identifier
E.coli	*Escherichia coli*
EC50	half-maximal effective concentration
EGFP	Enhanced green fluorescent protein
Epac	Exchange protein directly activated by cyclic adenosinemonophosphate
EYFP	Enhanced yellow fluorescent protein
FlincGs	fluorescent indicators of cyclic guanosinemonophosphate
FP	Fluorescence polarization
FRET	Fluorescence Resonance Energy Transfer or Förster Resonance Energy Transfer
G12/13	RhoA signalling G proteins
GCa-MPs	M13 domain of a myosin light chain kinase and calmodulin based genetically encoded calcium indicator
GFP	Green fluorescent protein
Gi	inhibitory G proteins

GlnK1	nitrogen regulatory protein P-II
GPCR	G protein-coupled receptor
Gq	Phospholipase C signalling G proteins
Gs	cAMP signalling G proteins
HCN2	Cyclic nucleotide-gated channel 2
HEK293	Human Embryonic Kidney 293 cells
HTRF	Homogeneous time resolved fluorescence
ICUE	Indicator of cAMP using Epac
LANCE	Lanthanide chelate exite
LOEC	Lowest observed effect concentration
LOEC min	Minimum of lowest observed effect concentrations reported
LogKOW	Octanol/water partitioning coefficient (logarithmic form)
MBP	Maltose binding protein
MEC	Measured environmental concentration
MECmax	Maximum of measured environmental concentrations reported
MOA	Mode of action
NAD	Nicotinamide adenine dinucleotide
NFAT-RE	Nuclear factor of activated T-cells response element
NF-κB	Nuclear Factor kappaB
NO	Nitrogen oxide
NOEC	No observed effect concentration
NSAID	Non-steroidal anti-inflammatory drug
PBT	persistent, bioaccumulating, and toxic
PEC	Predicted environmental concentration
PKA	Phospho kinase A
PKC	Phospho kinase C
PNEC	Predicted no effect concentration
RhoA	Ras homolog gene family member A
roGFPs	Redoxsensitive fluorescence protein indicators
rxYFPs	Redox yellow fluorescence protein sensors
SPA	Scintillation proximity assay
SRE	Serum response element
SRF-RE	serum response factor response element
t1/2	Half-life
TER	Toxicity exposure ratio

UBA	Umweltbundesamt der Bundesrepublik Deutschland
YFP	Yellow fluorescent protein

1 Summary

In order to identify sensitive organisms, biological endpoints in vivo and suitable *in vitro* test systems for the biomonitoring of pharmaceuticals, a two-part literature search was conducted.

In the first part, the database OEKOTOX established by Bergmann et al. (2011) [1] was upgraded with effect data published between 2011 and 2013 for 90 pharmaceuticals of high priority. From all available data the lowest effect concentrations were identified and the most sensitive organisms and effect endpoints were determined. In addition, effect data were assessed with respect to their relation to measured environmental concentrations (MECs) in German surface waters by calculations of risk quotients MECmax/LOECmin. Publications providing the lowest effect data were evaluated with respect to their reliability according to the criteria of Wright-Walters et al. (2011) [2]. Out of 72 publications investigated for their reliability, 9 were "reliable", 49 "conditionally reliable" and 14 "not reliable" (of which 4 were, in part, conditionally reliable).

Prior to the reliability check, the analgesics paracetamol, diclofenac and ibuprofen, the β-blocker propranolol, the antibiotics sulfamethoxazole and erythromycin and the lipid regulator gemfibrozil were identified as environmentally most relevant pharmaceuticals. The relevance was defined by MECmax/LOECmin-values >0.1 for a minimum of 3 different biota classes. After assessment of publication reliability, however, only for diclofenac MECmax/LOECmin-values >0.1 were found in more than 3 biota classes. As further important pharmaceuticals propranolol, sulfamethoxazole, bezafibrate, 17α-ethinlyestradiol, 17β-estradiol and oxytetracycline were identified with MECmax/LOECmin-values >0.1 for 2 biota classes.

Prior to the reliability assessment, the ciliate *Tetrahymena pyriformis*, zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), medaka (*Oryzias latipes*), the mussel *Elliptio complanata*, and the mudsnail *Potamopyrgus antipodarum* were identified as most sensitive organisms for pharmaceuticals. The most sensitive effect endpoints were chemotaxis, behavior, vitellogenin synthesis, growth rate, reproduction, histopathological alterations, molecular stress biomarkers, oxidative stress markers, receptor binding, and gene expression. All extremely low LOECs (in the range of pg/L) for chemotaxis alterations in *Tetrahymena pyriformis*, however, derive from only a single publication by Lang & Köhidai (2012) [3], the reliability of which, however, could not be proven.

In the second part, a literature search was conducted with the aim to evaluate the state of knowledge in the field of *in vitro* testing of pharmaceutical effects, to discover advantages and disadvantages of biochemical and cell-based assays and to suggest possibilities for the realisation of cell-based tools for a mode of action-based biomonitoring of pharmaceuticals. Promising approaches for β-adrenoreceptor blockers and cyclooxygenase-inhibitors (analgesics) are presented.

As a future perspective for a biomonitoring of pharmaceuticals, the development of mode of action-based in vitro test systems for β-blockers and NSAIDs are recommended. Their suitability to reflect in vivo responses of organisms which have been identified to be sensitive in part 1 of this study, or which are relevant for German aquatic ecosystems should be assessed, and they should be validated by in vivo studies to be conducted in parallel to their establishment.

2 Zusammenfassung

Mit dem Ziel, sensitive Organismen und organismische Endpunkte sowie geeignete in vitro-Testsysteme für ein Biomonitoring von Arzneimitteln zu identifizieren, wurde eine zweiteilige Literaturstudie durchgeführt.

Im ersten Teil wurde die von Bergmann et al. (2011) [1] erstellte Datenbank OEKOTOX um Wirkdaten für 90 Arzneimittel von hoher Priorität bis 2013 erweitert. Von allen verfügbaren Daten wurden die niedrigsten Effektwerte identifiziert und die sensitivsten Organismen bzw. Effektendpunkte bestimmt. Darüber hinaus wurden die Effektkonzentrationen zu gemessenen Umweltkonzentrationen in Deutschen Oberflächengewässern (MECs) in Beziehung gestellt und Risikoquotienten MECmax/LOECmin berechnet. Diejenigen Publikationen, in denen die empfindlichsten Endpunkte bzw. Organismen enthalten waren, wurden auf der Basis der von Wright-Walters et al. (2011) [2] genannten Kriterien auf Reliabilität geprüft. Von 72 auf Reliabilität überprüften Publikationen wurden 9 als „reliabel“, 49 als „bedingt reliabel“ und 14 als „nicht reliabel“ (von denen 4 in Teilen bedingt reliabel waren) klassifiziert.

Vor der Reliabilitätsprüfung wurden in der vorliegenden Literaturstudie als Pharmazeutika mit höchster Umweltrelevanz die Schmerzmittel Paracetamol, Diclofenac und Ibuprofen, der β-Blocker Propranolol, die Antibiotika Sulfamethoxazol und Erythromycin und der Lipidsenker Gemfibrozil identifiziert. Die Umweltrelevanz wurde auf der Basis eines Risikoquotienten (MECmax/LOECmin) >0.1 für mindestens 3 Organismengruppen) definiert. Nach erfolgter Reliabilitätsprüfung zeigten sich MECmax/LOECmin-Werte >0.1 bei mehr als 3 Organismengruppen jedoch nur noch für Diclofenac. Als weitere wichtige Pharmazeutika wurden Propranolol, Sulfamethoxazol, Bezafibrat, 17α-Ethinlyestradiol, 17β-Estradiol und Oxytetrazyclin mit MECmax/LOECmin-Werten >0.1 bei 2 Organismengruppen identifiziert.

Als sensitivste Organismen für Arzneimittel hatten sich vor der Reliabilitätsprüfung der Ciliat *Tetrahymena pyriformis*, der Zebrabärbling (*Danio rerio*), die Regenbogenforelle (*Oncorhynchus mykiss*), Medaka (*Oryzias latipes*), die Muschel *Elliptio complanata* und die Zwergdeckelschnecke *Potamopyrgus antipodarum* erwiesen. Die sensitivsten Effektendpunkte waren Chemotaxis, Verhalten, Vitellogeninsynthese, Wachstum, Reproduktion, histopathologische Veränderungen, molekulare und oxidative Stressmarker, Genexpression und Rezeptorbindung. Alle extrem niedrigen LOECs (im Bereich von pg/L) für die Beeinflussung der Chemotaxis bei *Tetrahymena pyriformis* stammen allerdings von einer einzigen Publikation von Lang & Köhidai (2012) [3], die sich in der durchgeführten Reliabilitätsprüfung als „nicht reliabel“ erwies, so dass der Zebrabärbling als bedeutendster Testorganismus und das Verhalten als sensitivster Wirkendpunkt nachrückt.

Im zweiten Teil der Studie wurde eine Literaturrecherche durchgeführt, die zum Ziel hatte, den Wissensstand im Bereich von in vitro-Verfahren zum Nachweis von Arzneimittel-Effekten zu beleuchten, Vor- und Nachteile von biochemischen und zellbasierten Assays herauszuarbeiten und Möglichkeiten für die Realisierung von in vitro Testsystemen für ein Wirkmechanismus-basiertes Biomonitoring von Arzneimitteln vorzuschlagen. Am Ende dieses Teils werden vielversprechende Wege zur Etablierung solcher Testsysteme für den Nachweis der Wirkungen von β-Blockern und Cyclooxygenase-Hemmern (Schmerzmitteln) dargestellt.

Für ein künftiges Biomonitoring von Arzneimitteln wird empfohlen, Wirkstoffklassen-spezifische in vitro-Testsysteme für β-Blocker und Schmerzmittel zu entwickeln. Durch parallel durchzuführende in vivo Untersuchungen soll überprüft werden, inwieweit diese Testsysteme geeignet sind, in vivo-Reaktionen von Organismen abzubilden, die in Teil 1 der vorliegenden Studie als sensitiv für Arzneimittel identifiziert wurden, oder die als relevant für deutsche Fließgewässer bekannt sind.

3 Introduction

Chemical analysis has regularly revealed the presence of human and veterinary pharmaceuticals in wastewater and surface water in nanogram to microgram per liter concentrations (e.g. Fent et al., 2006 [4], Brauch, 2011 [5]). Decisions on the environmental relevance of these substances usually rely on data recorded for current and future consumption rates, environmental concentrations, environmental fate and pathways (persistence), and ecotoxicological effects recorded in laboratory studies. The problem with this approach is the fact that ecotoxicological routine testing mainly focuses on acute or chronic unspecific toxicity, whereas pharmaceuticals, according to their envisaged specific action in man (human medicine) or animals (veterinary drugs) more likely exert specific effects based on their mode of action (MOA) (Brausch et al. 2012 [6]). Information on the effects of active pharmaceutical ingredient classes, either acting on targets or exerting unwanted adverse side effects are, therefore, mandatory prerequisites for an effect-directed monitoring of pharmaceuticals in aquatic ecosystems.

One focus of the present literature review study thus was on the question which organisms sensitively respond to pharmaceuticals and which sublethal parameters can be used as warning sentinels (biomarkers) to monitor action of pharmaceuticals in a sensitive and specific way.

In a comprehensive report of Bergmann et al. (2011) [1] a prioritization of human and veterinary pharmaceuticals was undertaken on the basis of data on the presence of compounds in the aquatic environment, their ecotoxicological effects, and their consumption rates up to the year 2011. This report lists analytical data for 274 ingredients and ecotoxicological effect data for 251 ingredients, all of them incorporated in both the MEC (Measured Environmental Concentrations) and ÖKOTOX (effect data) databases. Bergmann et al. (2011) [1] have outlined 24 substances with high priority for environmental monitoring programs. However, the databases also provide evidence that 31 pharmaceuticals with partially high and steadily increasing consumption rates lacked ecotoxicological data until 2009 resulting in a very high uncertainty regarding the assessment of their environmental relevance. Supplementary to the substances regarded as relevant for further research by Bergmann et al. (2011) [1], the German Federal Environment Agency (UBA) has identified further substances as to be of high priority for an evaluation with respect to possible environmental effects. In the present study, we therefore focused on a set of 90 substances, prioritized by Bergmann et al. (2011) [1] and UBA, for which literature was analyzed.

Bergmann et al. (2011) [1] concluded from their research that already the number of substances with high priority (24) would exceed the capacity of routine chemical monitoring. They therefore suggested having leading substances defined for particular ingredient classes that should be included in monitoring programs. The apparent but crucial drawback of such an approach, however, lies in the risk of overlooking effective concentrations of non-leading substances. In contrast to it, the use of tests that could visualize specific molecular interactions of chemicals exhibiting the same mode of action may enable a monitoring of an entire class of compounds. Particularly in view of the vast number of pharmaceuticals with increasing consumption rates but lacking ecotoxicological effect data, a mode of-action-specific *in vitro* assay is a matter of paramount interest; also because new and future compounds that act in the same way on the same molecular target can easily be traced, once such an assay has been established.

In vitro systems using highly sensitive fluorescence detection technologies are already used by the pharmaceutical industry to identify compound classes as promising candidates in the development of new pharmaceuticals (Eggeling et al., 2003) [7]. A biomonitoring on the basis of such specific mechanisms of action could be possible for e.g. non-steroidal anti-inflammatory

drugs (NSAIDs) or β-blockers. For these, the inhibition of the cyclooxygenase and the blocking of β-receptors could be used as mode of actions to be targeted. In contrast to Escher et al. (2005) [8] who use "*in vitro* Assessment of Modes of Toxic Action" in that sense that specific mode of actions defined by the test system itself are investigated (e.g. photosynthesis inhibition of pharmaceuticals by a photosynthesis inhibition assay), we thus favor an approach to use the specific target molecules for pharmaceutical classes (responsible for the pharmacological effect of the respective substance class) as monitoring tools. In the second part of this study, we therefore evaluated different *in vitro* test systems with respect to their suitability as future test systems in routine monitoring of pharmaceutical classes.

In summary, this study reviews (1) the current state of knowledge in effect-directed research and analyzes present data with focus on suitable compounds, organisms, and endpoints which may be combined in a monitoring approach, and (2) in addition, the state of knowledge for *in vitro* testing of pharmaceuticals as a prerequisite for the development of mode of-action-based monitoring tools.

4 Materials and Methods

4.1 Literature search and data collection

The literature search is divided into two parts.

In the first part, effect data for 90 pharmaceuticals defined as relevant according to either Bergmann et al. (2011) [1] or UBA were collected for several biota classes (bacteria, protists, plants/algae, mollusks, crustaceans, insects, fish, tetrapods, others incl. communities). The list of pharmaceuticals and the priority classes are shown in table 1. The prioritization of UBA is based on MECs obtained from the German counties, consumption rates (tendencies between 2002 and 2012), effect values of UBA-internal studies or literature, suspicion to be a PBT compound, degradability and metabolism. The prioritization of Bergmann et al. (2011) [1] is only based on literature data which resulted in partial differences between the two lists of priority substances available.

The search was restricted to literature published between 2011 and 2013, since all earlier published data have been analyzed by Bergmann et al. (2011) [1]. For the search, Web of Knowledge (including the databases "Web of Science®", "BIOSIS Citation Index℠", "BIOSIS Previews®", "MEDLINE®" and "Journal Citation Reports®") were used. The following search items were defined:

For Reviews: *pharma** AND *eco**, limited by *"review"*

For single substances: the specific search entry for each pharmaceutical (see table 1) combined with the search terms: *aqua** OR *eco** OR *tox**

The following research areas were seen as irrelevant and therefore excluded from the literature search:

Anesthesiology, Anthropology, Biomedical, Social Sciences, Biophysics, Business Economics, Communication, Computer Science, Criminology Penology, Critical Care Medicine, Cultural Studies, Demography, Dentist, Oral Surgery Medicine, Dermatology, Education, Educational Research, Electrochemistry, Emergency Medicine, Energy Fuels, Engineering, Ethnic Studies, Food Science Technology, General Internal Medicine, Genetics Heredity, Geography, Geology, Geriatrics, Gerontology, Government, Law, Health Care Sciences, Services, History, Imaging Science, Photographic Technology, Infectious Diseases, Information Science, Library Science, Instruments Instrumentation, Integrative Complementary Medicine, International Relations, Legal Medicine, Materials Science, Mathematical Computational Biology, Mathematics, Medical Informatics, Medical Laboratory Technology, Meteorology Atmospheric Sciences, Nursing, Nutrition Dietetics, Obstetrics, Gynecology, Oncology, Ophthalmology, Orthopedics, Otorhinolaryngology, Parasitology, Pediatrics, Pharmacology, Pharmacy, Physics, Polymer Science, Psychiatry, Psychology, Radiology, Nuclear Medicine, Medical Imaging, Rehabilitation, Research Experimental Medicine, Respiratory System, Social Issues, Sociology, Sport Sciences, Substance Abuse, Surgery, Transplantation, Tropical Medicine, Urology, Nephrology, Virology

In the second part, literature searches were conducted in PubMed using the following search items:

High throughput screening (title) & review (publication type)
HTS technolog & review (publication type)*
HTS technolog (title)*
*fluorescence based techni**
cell based assay & review (publication type)*

cell based reporter (title)*
cell based screening & review (publication type) & cell based (title)
fluorescent biosensor & review (publication type)*
fluorescent protein & review (publication type) & reporter**
signalling dynamics in living cell & review (publication type)*

Data published between 1995 and 2013 were analyzed.

Table 1: List of pharmaceuticals with defined priority classes. The prioritization of UBA is based on MECs obtained from the German counties, consumption rates (tendencies between 2002 and 2012), effect values of UBA-internal studies or literature, suspicion to be a PBT compound, degradability and metabolism. The prioritization of Bergmann et al. (2011) [1] is only based on literature data which resulted in partial differences between the two lists of priority substances available.

Pharmaceutical	Search entry	Priority class (UBA)	Priority class (Bergmann)	Priority class (final)	Class	CAS
Sartanic acid	sartan*	P		Pu	antihypertensive	
14-Hydroxyclarithromycin	hydroxyclarithromycin*	P		Pu	Antibiotic	110671-78-8
17alpha-Ethinylestradiol	ethinyl* estradiol*	P	P	P	Contraceptive	57-63-6
17beta-Estradiol	estradiol* AND pharma*		P	Pb	Hormone	50-28-2
4-N-Methylaminoantipyrin	methylaminoantipyrin*	P		Pu	Analgesic	519-98-2
6(carboxymethoxy)-4-(2-chlorophenyl)-5-(ethoxycarbonyl)-2-methylpyridine-3-carboxylic acid	amlodipin*	P		Pu	antihypertensive	
Acetylsalicylic acid	acetylsalic*				NSAID	50-78-2
Aciclovir	aciclovir*		?	?	antiviral drug	59277-89-3
Allopurinol	allopurinol*				xanthine oxidase inhibitor	315-30-0
AMDOPH	AMDOPH		(P)	(P)	analgesic	
Amlodipine	amlodipin*	P		Pu	antihypertensive	88150-42-9
Amoxicilline	amoxicillin*	P	P	P	antibiotic	26787-78-0
Atenolol/ Atenolol acid	atenolol*, tenormin*	P		Pu	Betablocker	29122-68-7

Azitromycin	azitromycin*, zithromax*, azithrocin*, azin*	P		Pu	Antibiotic	83905-01-5
Bezafibrate	bezafibrat*		(P)	(P)	lipid regulator	41859-67-0
Bisoprolol	bisoprolol*	P	?	Pu	betablocker	66722-44-9
Capecitabin	capectiabin*, xeloda*	P		Pu	cytostatic	15361-50-9
Carbamazepine	carbamazepin*	P	P	P	anticonvulsant	298-46-4
Cefaclor	cefaclor* OR cefachlor*		?	?	antibiotic	53994-73-3
Cefuroxime axetil	cefuroxim* axetil*		?	?	antibiotic	64544-07-6
Chloramphenicole	chloramphenicol*		P	Pb	antibiotic	56-75-7
Chlortetracycline	chlortetracyclin*		P	Pb	antibiotic	57-62-5
Ciprofloxacin	ciprofloxacin*	P	P	P	antibiotic	85721-33-1
Clarithromycine	clarithromycin*	P	P	P	antibiotic	81103-11-9
Clindamycin	clindamycin*	P	?	Pu	antibiotic	18323-44-9
Clopidogrel	clopidogrel*		?	?	antiplatelet agent	113665-84-2
Clotrimazole	clotrimazol*		(P)	(P)	antimycotic	23593-75-1
Deltamethrin	deltamethrin*, decamethrin*	P		Pu	antiparasitics	52918-63-5
Desflurane	desfluran*		?	?	anesthetic	57041-67-5
Diatrizoic acid	diatriz* OR iotalam* OR amidotriz*		(P)	(P)	contrast agent	737-31-5
Diazepam	diazepam*		(P)	(P)	antidepressant/ antipsychotic	439-14-5
Diclofenac	diclofenac*	P	P	P	NSAID	15307-86-5
Dienogest	dienogest*, visanne*	P		Pu	gestagen	65928-58-7
Dipyridamole	dipyridamol*		?	?	antiplatelet agent	58-32-2
Doxycycline	doxycyclin*		P	Pb	antibiotic	564-25-0

Duloxetine	duloxetin*, cymbalta*	P		Pu	antidepressant/ antipsychotic	116539-59-4
Entacapone	entacapon*		?	?	catecholamin-O-Methyltransferase-Inhibitor	130929-57-6
Eprosartan	eprosartan*		?	?	antihypertensive	133040-01-4
Erythromycin	erythromycin*		P	Pb	antibiotic	114-07-8
Gabapentin	gabapentin* OR neurotin*		?	?	anticonvulsant	60142-96-3
Gemfibrozil	gemfibrozil*, lopid*	P		Pu	lipid regulator	25812-30-0
Glutaral	glutaral*		?	?	desinfectant (Glutaraldehyd)	111-30-8
Hydrochlorothiazide	hydrochlorothiazid*		?	?	antihypertensive	58-93-5
Ibuprofen	ibuprofen*	P	(P)	P	NSAID	15687-27-1
Imatinib	imatinib*, gleevec*, glivec*	P		Pu	cytostatic	152459-95-5
Indometacin	indometacin* OR indomethacin*		(P)	(P)	NSAID	53-86-1
Iohexol	iohexol* OR omnipaq*	P	(P)	P	contrast agent	66108-95-0
Iomeprol	iomeprol*	P	(P)	P	contrast agent	78649-41-9
Iopamidol	iopamidol*		(P)	(P)	contrast agent	60166-93-0
Iopromide	iopromid* OR ultravist*		(P)	(P)	contrast agent	73334-07-3
Ivermectin	ivermectin*, stromectol*	P		P	antiparasitic	70288-86-7
Lamotrigin	lamotrigin*, lamictal*	P		Pu	anticonvulsant	84057-84-1
Levetiracetam	levetiracetam*		?	?	anticonvulsant	102767-28-2
Lincomycin	lincomycin*		(P)	(P)	antibiotic	154-21-2
Mesalazine	mesalazin*		?	?	NSAID	89-57-6
Mestranol	mestranol*		P	Pb	contraceptive	72-33-3
Metamizole	metamizol*	P		Pu	analgesic	68-89-3
Metformin	metformin*	P		Pu	antidiabetic	657-24-9
Metoprolol	metoprolol*	P		Pu	betablocker	51384-51-1

Naproxen	naproxen*	P	P	P	NSAID	22204-53-1
Norethisterone	norethisteron*		P	Pb	contraceptive	68-22-4
Opipramol	opipramol*		?	?	antidepressant/ antipsychotic	315-72-0
Oxytetracycline	oxytetracyclin*		P	Pb	antibiotic	79-57-2
Pantoprazole	pantoprazol*		?	?	proton pump inhibitor	102625-70-7
Paracetamol	paracetamol* OR acetaminophen*		P	Pb	analgesic	103-90-2
Piperacillin	piperacillin*		?	?	antibiotic	61477-96-1
Pregabalin	pregabalin*		?	?	anticonvulsant	148553-50-8
Primidone	primidon*		P	Pb	anticonvulsant	125-33-7
Propranolol	propanolol* OR propranolol*		(P)	(P)	betablocker	525-66-6
Quetiapine	quetiapin*	P	?	Pu	antidepressant/ antipsychotic	11974-69-7
Ramipril	ramipril*		?	?	antihypertensive	87333-19-5
Roxithromycine	roxithromycin*		P	Pb	antibiotic	80214-83-1
Sevelamer	sevelamer*		?	?	phosphate binding drug	52757-95-6
Simvastatin	simvastatin*	P	?	Pu	lipid regulator	79902-63-9
Strontium ranelate	strontium ranelat*		?	?	osteoporose agent	135459-90-4
Sulbactam	sulbactam*		?	?	antibiotic	68373-14-8
Sulfadimethoxine	sulfadimethoxin*		P	Pb	antibiotic	122-11-2
Sulfadimidine	sulfadimidin* OR sulfamethazin*		P	Pb	antibiotic	57-68-1
Sulfamethoxazole	sulfamethoxazol*	P	P	P	antibiotic	723-46-6
Tazobactam	tazobactam*		?	?	antibiotic	89786-04-9
Telmisartan	telmisartan*		?	?	antihypertensive	144701-48-4

Tetracycline	tetracyclin*		P	Pb	antibiotic	60-54-8
Tiamulin	tiamulin*	P	P	P	antibiotic	55297-95-5
Tilidine	tilidin*		?	?	analgesic	51931-66-9
Torasemide	torasemid* OR torsemid*		?	?	diuretic drug	56211-40-6
Tramadol	tramadol*, ryzolt*, ultram*	P		Pu	analgesic	27203-92-5
Valproic acid	valpro*		?	?	anticonvulsant	99-66-1
Valsartan	valsartan* OR angiotan* OR diovan*	P	?	Pu	antihypertensive	137862-53-4
Venlafaxine	venlafaxin*		?	?	antidepressant/ antipsychotic	93413-69-5

P: high priority according to Bergmann et al. (2011) [1] and UBA;
Pu: high priority according to UBA
Pb: high priority according to Bergmann et al. (2011) [1]
(P): medium priority according to Bergmann et al. (2011) [1]
?: substances requiring further information
none: substances without priority according to Bergmann et al. (2011) [1]

4.2 Created library and data files

4.2.1 Endnote library

All references analyzed were included into an Endnote library ("EndNote Library – Pharmaceuticals – Literature study part 1 / 2 /3") in the format ".CIW".

The three partners involved in the literature search used the following labels for their citations:

BER 1-x: GWT Dresden (R-: Review, A-: Additional Information, I-: Irrelevant)

SCH 1-x: University Tübingen (R-: Review, A-: Additional Information, I-: Irrelevant)

FRE 1-x: STZ Frey

The library contains the following folders:

Part 1:

Tuebingen - relevant studies: Studies on vertebrates, plants, protozoans and bacteria, which were directly included into the database.

Tuebingen - additional information: Studies, mainly reviews, on vertebrates, plants, protozoans and bacteria, which were used as help for the general interpretation of the data, but not directly included into the database.

Tuebingen - irrelevant studies: Studies, which were analyzed but were not included into the database.

Tuebingen- evaluation not possible: Studies, for which an evaluation was not possible because the full text was not available and the abstract did not contain sufficient information.

Dresden - relevant studies: Studies on invertebrates, which were directly included into the database.

Dresden - additional information: Studies, mainly reviews, on invertebrates, which were used as help for the general interpretation of the data, but not directly included into the database.

Dresden - irrelevant studies: Studies, which were analyzed but were not included into the database.

Part 2:

Mannheim - in vitro techniques: Studies on in vitro techniques, which were used for the second part of the literature study.

Part 3:

Additional literature used for this report

4.2.2 OEKOTOX$_{upgrade}$

In part 1 of the study, all publications available for the 90 substances were analyzed and evaluated with respect to the following criteria:

Name of effective substance, investigated form of the substance (salt/conjugate), synonyms, CAS number, LogK_{ow}, molar mass, substance class, field of application, effective substance or medical product, single substance or mixture, test organisms (species), biota group, field/lab/mesocosm test, effect endpoint, population relevance yes/no, standard method yes/no, test concentration in water (µg/L, mol/L), test concentration in sediment (if necessary), applied dose (if necessary), duration of test, acute/chronic/sub-acute, chemical analyses present, accumulation data present, bioaccumulation factor, NOEC (µg/L, µmol/L), LOEC (µg/L, µmol/L), EC_{10} (µg/L, µmol/L), EC_{50} (µg/L, µmol/L), citation, DOI, full text available, Endnote label, chemical present in priority list yes/no, comments, reliability analysis (only for selected publications).

In case no effect was observed in the study, the highest concentration tested (without effect) has been included as „NOEC" without corresponding LOEC. Furthermore, numerous studies reported effects already at the lowest tested concentration. These values are recorded as LOECs without a corresponding NOEC. It should be kept in mind that there is a possibility that lower values than the reported may also cause an effect.

Besides data for the priority substances defined in table 1, data for several other pharmaceuticals is also reported in the database. These effects were reported in the analyzed publications besides the ones for the target substances, and therefore included. However, the data sets for these substances are not complete, since they are mere "byproducts" of the original search.

All data were included into the database OEKOTOX$_{upgrade}$

A separate row was created for every chemical, species and endpoint used in each study. Each single row was defined as a "database entry".

Because many publications report on multiple chemicals, organisms or endpoints, the total number of database entries is much higher than the number of publications.

4.2.3 "Evaluation database" for assessment of data

With the aim to evaluate the collected data with respect to (1) their suitability for the study, (2) data number per pharmaceutical and organism group, (3) most sensitive organisms, (4) lowest effect concentrations, (5) mixture toxicity, and (6) population relevance, the "evaluation database" has been created.

In data sheet (4) the lowest and second lowest effect data and measured environmental concentrations (MECs) obtained either from the MEC database of Bergmann et al. (2011) [1] or UBA were included with the aim to assess the environmental relevance of the lowest effect data. However, not for all 90 substances both values MECs and LOECs were available. As a result, only for 32 substances a risk quotient (MECmax/LOECmin) could be calculated as a proxy for their environmental relevance. These substances were: paracetamol, tramadol, amoxicilline, chloramphenicole, chlortetracycline, ciprofloxacin, doxycycline, erythromycin, lincomycin, oxytetracycline, sulfadimethoxine, sulfadimidine, sulfamethoxazole, carbamazepine, diazepam, clotrimazole, ivermectin, atenolol/ atenolol acid, metoprolol, propranolol, 17alpha-ethinylestradiol, 17beta-estradiol, norethisterone, diatrizoic acid, bezafibrate, gemfibrozil, acetyl cysteine, acetylsalicylic acid, diclofenac, ibuprofen, indometacin, naproxen. For tetracycline, quetiapine, venlafaxine and deltamethrin no MECs were available.

With the aim to compare mixture toxicity data with LOECs for isolated substances, the "mixture toxicity" datasheet was created. The data sheet "population relevance" has been used as a basis to quantify available data with relevance for the population level.

5 Results

5.1 Effects of pharmaceuticals *in vivo*

5.1.1 Number of publications and database entries

Altogether, 452 publications were analyzed for 90 pharmaceuticals. 325 papers contained data for vertebrates, plants/algae, protozoa, or bacteria (analyzed by the University of Tübingen), 179 papers contained data for invertebrates (analyzed by the GWT, TU Dresden) , and 50 of them contained data for invertebrates and either vertebrates, plants, protozoa or bacteria (analyzed by both, University of Tübingen and GWT TU Dresden). 232 of these publications were of relevance for the database, 95 provided additional information, 134 were not relevant, and for 6 publications the evaluation was not possible, since the full PDF was either not available or did not contain enough information to analyze the study.

All in all, 1678 entries were included in the database OEKOTOXupgrade (1434 for vertebrates, plants, protozoa and bacteria and 244 for invertebrates). Because many studies investigated multiple chemicals and endpoints, the number of database entries is much higher than the number of studies.

Figure 1 and Figure 2 show that most of the studies were conducted with antibiotics, followed by NSAIDs, anticonvulsants, antiparasitics, β-blockers and contraceptives, and that the number of publications per pharmaceutical class is reflected by the number of database entries.

5.1.2 Number of publications per organism group

Most publications on effects of pharmaceuticals in biota have been found for fish, followed by mollusks, plants/algae, crustaceans, and bacteria (Figure 3). In general, this distribution pattern for the publication number is reflected by the number of database entries per organism group (Figure 4), however, for plants/algae more data were available per publication than for mollusks, and for bacteria more database entries have been conducted than for crustaceans.

5.1.3 Evaluation of data

5.1.3.1 Most sensitive biota classes for pharmaceuticals

In a first step, the lowest and second lowest effect data were analyzed with respect to their relation to distinct organism classes (Figure 5).

It became evident that most of the lowest plus second lowest effect values were recorded for protozoans (represented by only a single test species: *Tetrahymena pyriformis*). Mollusks were shown to be very sensitive for anticonvulsants, antipsychotics, lipid regulators, analgesics (other than NSAIDs), hormones and contraceptives. The LOECs were in the range of ng/L – lower µg/L values.

Lowest or second lowest effect values for fish (several species) were determined for hormones, mycolytica, anticonvulsants, antiparasitics and lipid regulators. Also for them, the LOECs are in the range of ng/L - lower µg/L values.

Bacteria and plants were shown to be highly sensitive to antibiotics, crustaceans were very sensitive to antiparasitics.

In a second step, data were analyzed in a more detailed way by determining the number of pharmaceuticals for which defined LOECs (< 0.1, 1 or 10µg/L) were reported for the investigated species in order to identify the most sensitive species for pharmaceuticals.

Figure 1: Number of publications per substance class

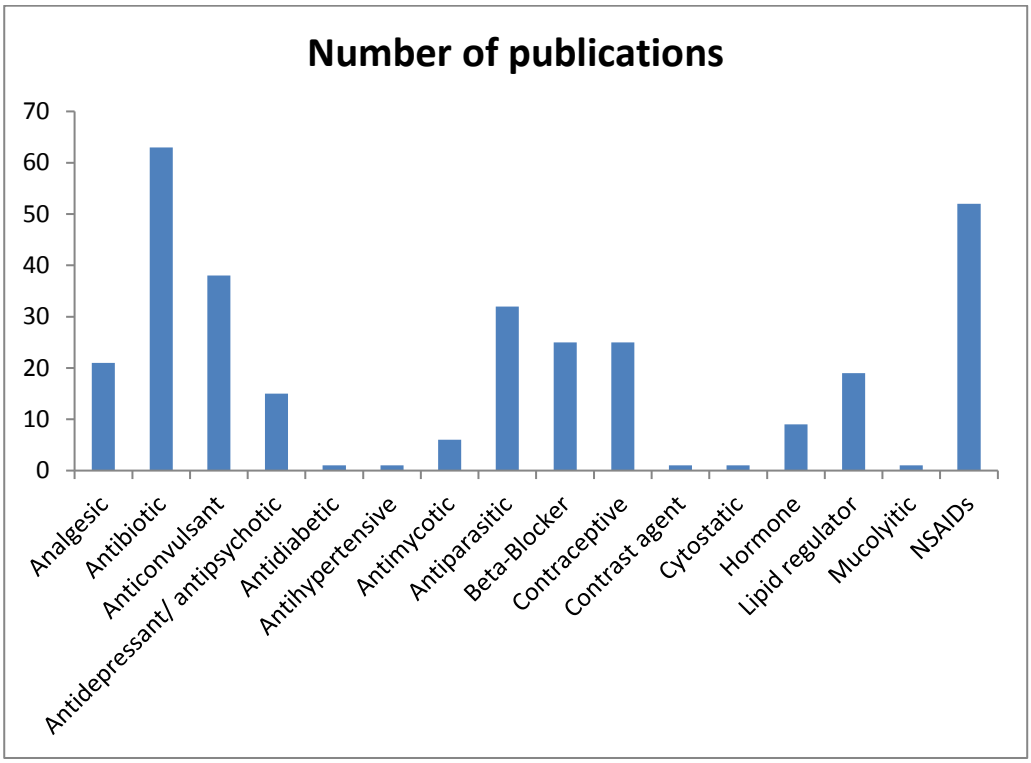


Figure 2: Number of database entries per substance class

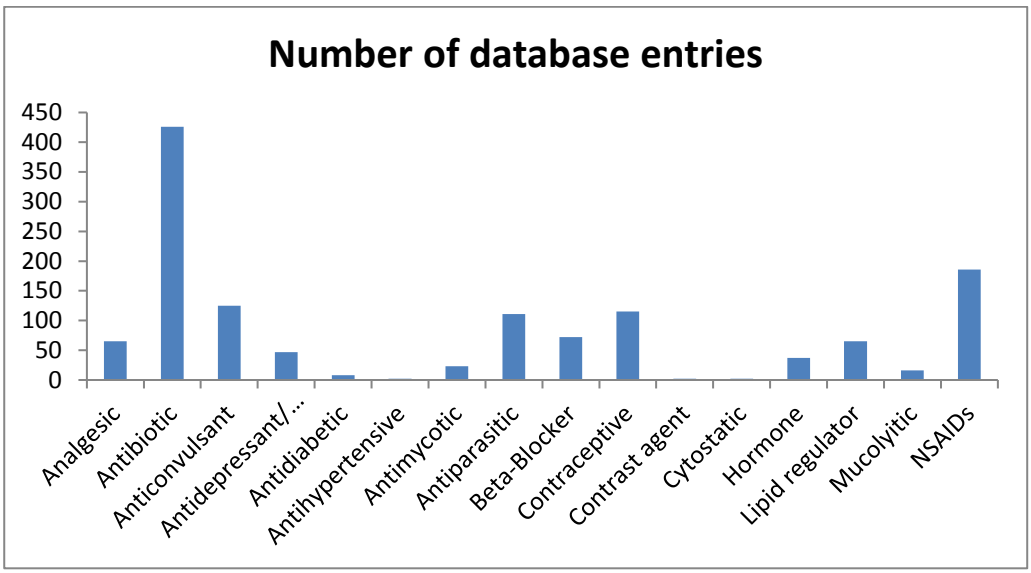


Figure 6 shows that the most sensitive species with LOECs < 0.1 µg/L for 2-8 pharmaceuticals were the ciliate *Tetrahymena pyriformis*, zebrafish *(Danio rerio)*, rainbow trout *(Oncorhynchus mykiss)*, medaka *(Oryzias latipes)*, the mussel *Elliptio complanata* and the mudsnail *Potamopyrgus antipodarum*.

All extremely low LOECs (in the range of pg/L) for *Tetrahymena pyriformis* derive from only a single publication by Lang & Köhidai (2012) [3], the reliability of which, however, could not be proven (5.1.5). Additionally, the conclusion on the most sensitive species is biased by the fact that these species are also among the most frequently used test organisms.

Figure 3: Number of publications per organism group

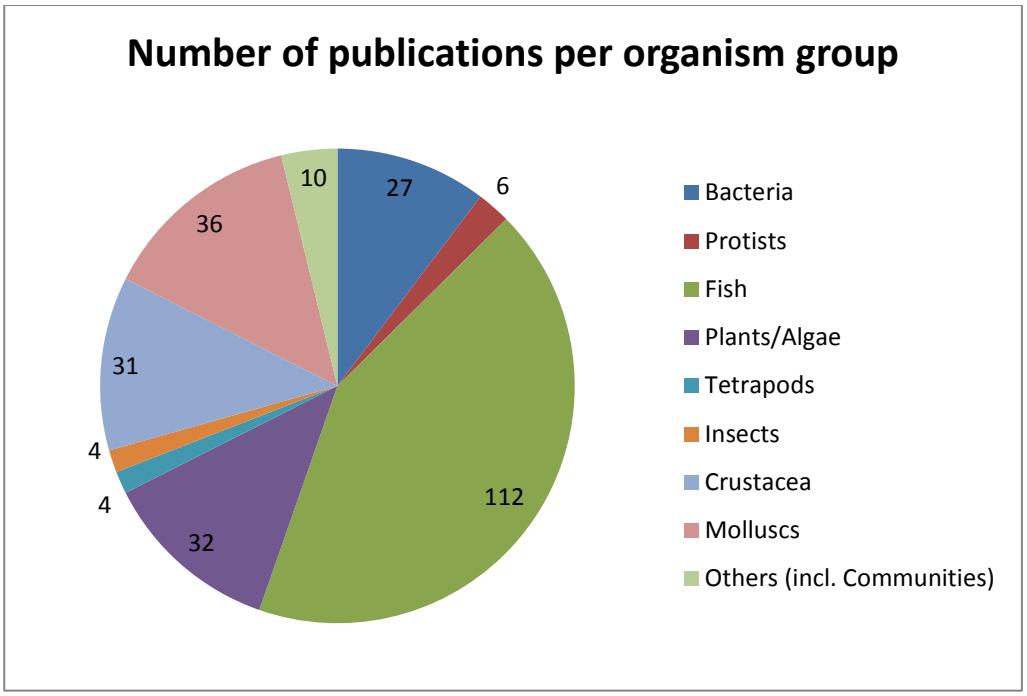
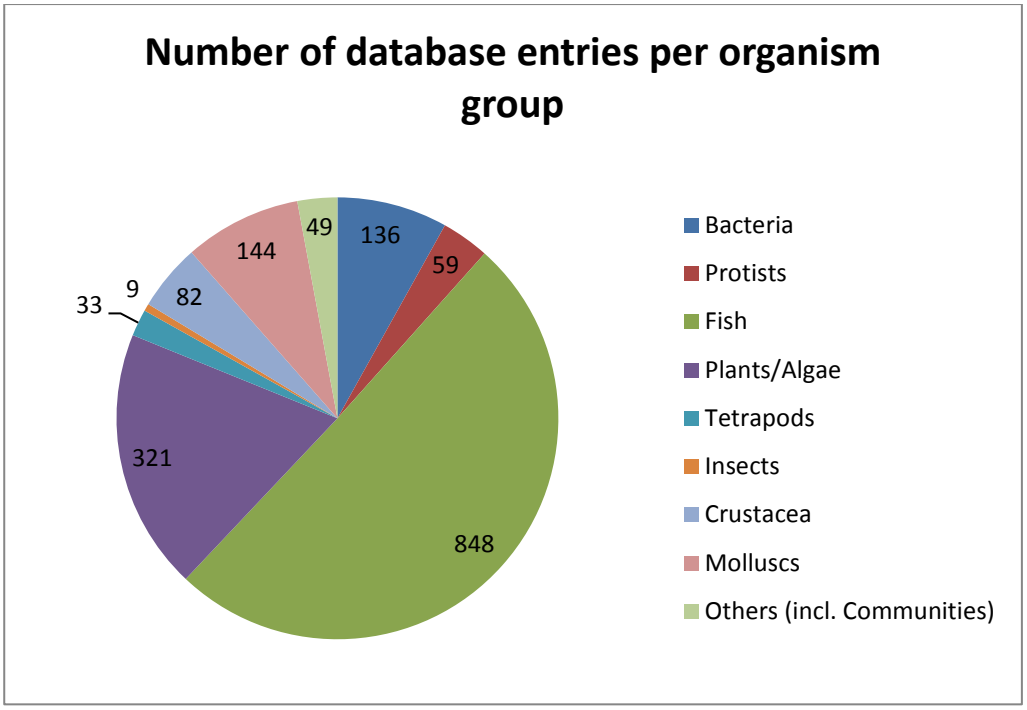


Figure 4: Number of database entries per organism group

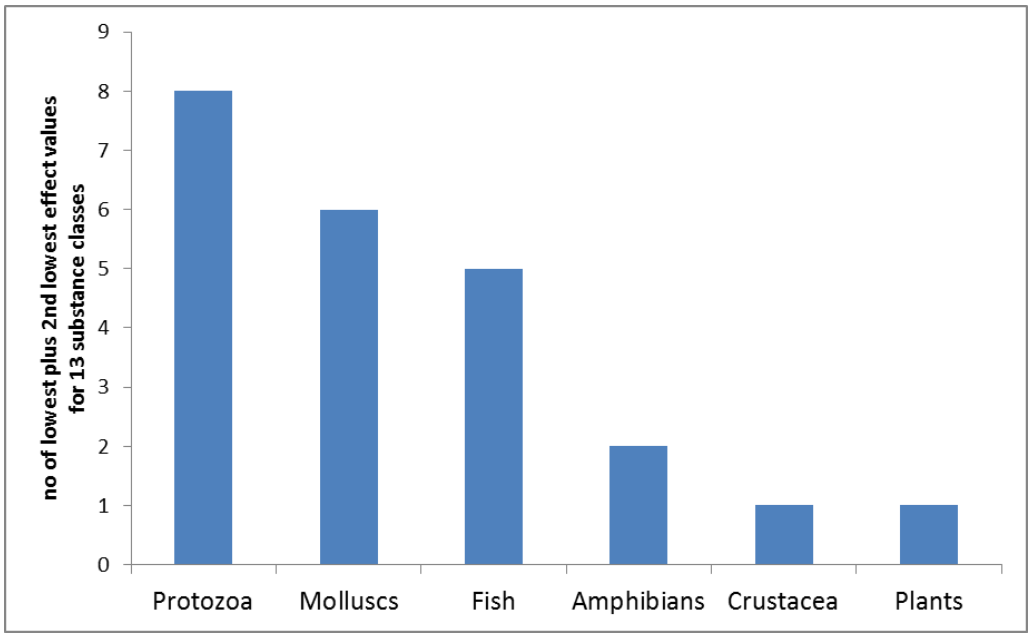


5.1.3.2 Most sensitive biota classes for pharmaceuticals

In order to identify most sensitive effect endpoints for pharmaceuticals, data were analyzed with respect to the number of pharmaceuticals with defined LOECs (< 0.1, 1 or 10µg/L) for the

investigated effect endpoints. Figure 7 makes evident that the most sensitive endpoints with LOECs < 0.1 µg/L for 3-8 pharmaceuticals were chemotaxis, behavior, vitellogenin induction, growth rate, reproduction, histopathological alterations, molecular stress biomarkers, oxidative stress markers, receptor binding, and gene expression.

Figure 5: Number of lowest plus second lowest effect values for 13 pharmaceutical classes



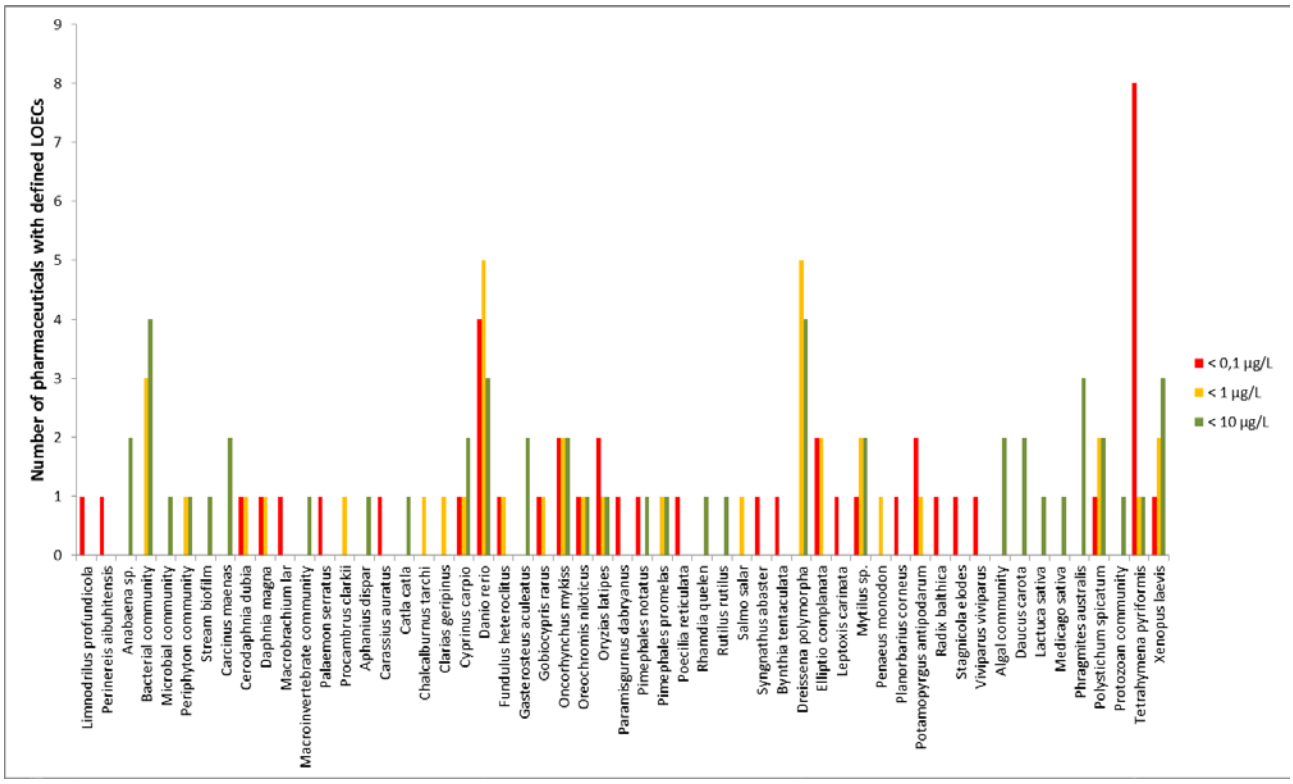
5.1.3.3 Identification of pharmaceuticals of highest relevance

To identify the risk exerted by chemicals for the aquatic environment, different types of risk quotients are calculated in ecological risk assessment (PEC/PNEC ratios for industrial chemicals and pharmaceuticals, TER for pesticides). The PEC/PNEC ratio is defined by (1) the PEC, which represents the concentration of a chemical supposed to occur in the environment (predicted environmental concentration), and (2) the PNEC (predicted no effect concentration) which is calculated on the basis of the no observed effect concentration in any group of biota (NOEC), divided by a safety factor which itself depends on the size and quality of the data for different trophic levels. A possible risk for the environment is indicated by a risk quotient larger than 1.

In the present study, the calculation of the risk quotient slightly differs from this routine procedure: For those pharmaceuticals, for which both Measured Environmental Concentrations (MECs) (either from Bergmann et al., 2011 [1] or UBA; summarized in data sheet "MECs & LOECs") and LOEC values were available, MEC/LOEC quotients were calculated as a proxy for the environmental relevance of LOECs by using the highest available MEC (MEC_{max}) and the lowest LOEC ($LOEC_{min}$). In contrast to the established prospective risk assessment procedure, we used LOECs (lowest observed effect concentrations) instead of NOECs. This can be justified by the necessity to identify threshold values for effects as a prerequisite for pharmaceutical monitoring. A further reason is that our literature survey aims at identifying pharmaceuticals of environmental concern retrospectively, and thus had to take into consideration concentrations measured in the environment (MECs). Finally, only for 37% of the LOECs corresponding NOECs were available. If NOECs were used, the database would have become too small for the envisaged analyses.

For the calculations of $MEC_{max}/LOEC_{min}$ values also data from Bergmann et al. (2011) [1] were analysed.

Figure 6: Number of pharmaceuticals with defined LOECs for investigated species



All extremely low LOECs (in the range of pg/L) for chemotaxis derive from the above mentioned study of Lang & Köhidai (2012) [3] who used *Tetrahymena pyriformis* as test organism. As mentioned above, the reliability of this study, however, could not be proven (shown in chapter 5.1.5).

Figure 8 makes evident that most pharmaceuticals with LOECs leading to risk quotients above 0.1 were antibiotics and NSAIDs followed by β-blockers and lipid regulators, analgesics different from NSAIDs and contraceptives/hormones.

When analyzing data with focus on $MEC_{max}/LOEC_{min}$ values for the respective biota classes (Figure 9) it became evident that most pharmaceuticals, for which risk quotients >0.1 were calculated were investigated in plants/algae and invertebrates closely followed by vertebrates. For bacteria, only few risk quotient > 0.1 were found.

Figure 7: Number of pharmaceuticals with defined LOECs for investigated effect endpoints

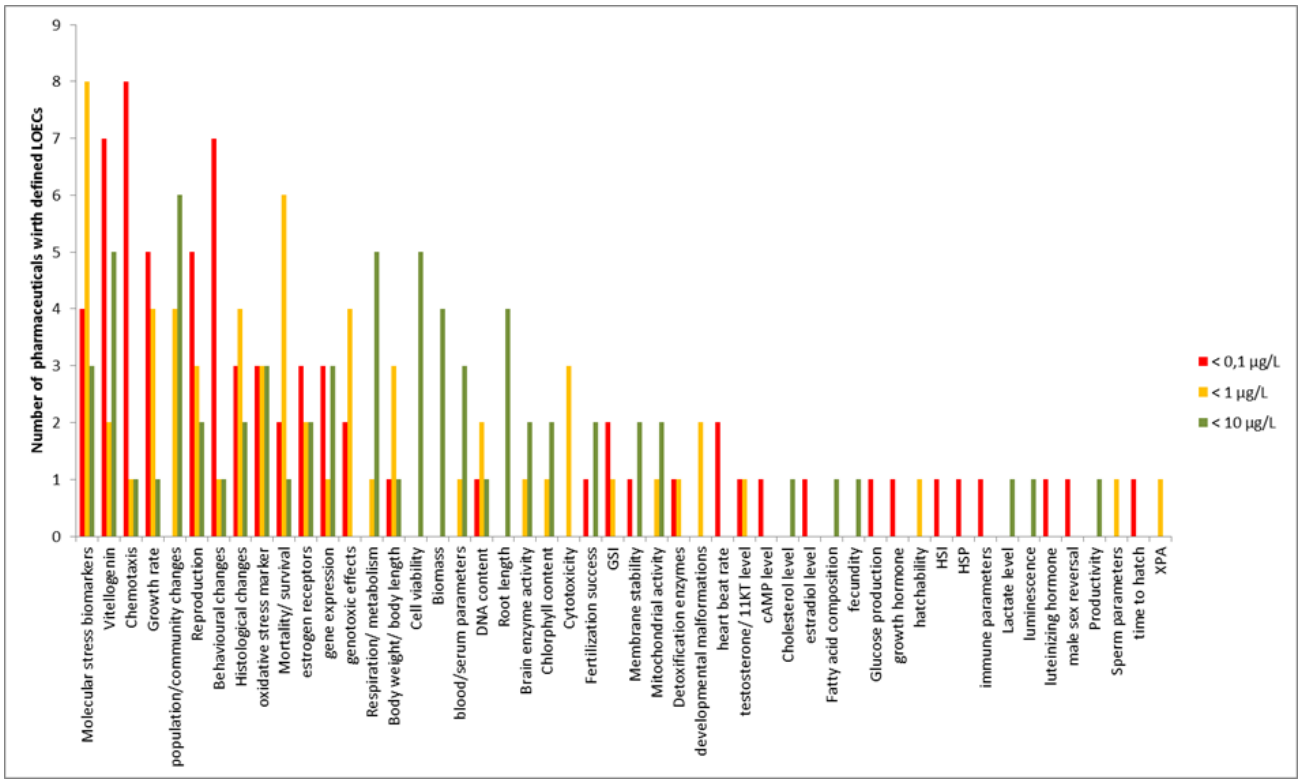
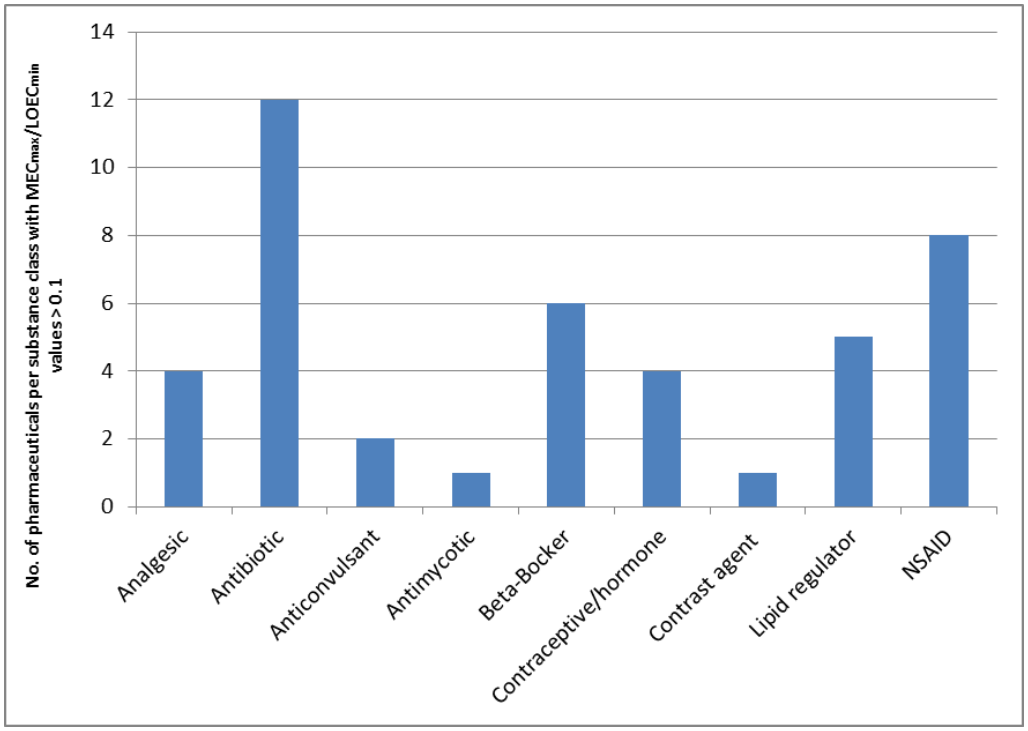


Figure 8: Number of pharmaceuticals per pharmaceutical class with MECmax/LOECmin values >0.1



For the following substances, the risk quotient values were >1 (>0.1):

Vertebrates: >1: atenolol, carbamazepine, diclofenac, 17α-ethinylestradiol, 17β-estradiol, gemfibrozil, ibuprofen, metoprolol, paracetamol, propranolol (>0.1: bezafibrate).

Invertebrates: >1: bezafibrate, carbamazepine, diclofenac, erythromycin, 17α-ethinylestradiol, ibuprofen, ivermectin, oxytetracycline, paracetamol, propranolol, sulfamethoxazole (>0.1: ciprofloxacin, gemfibrozil, 17β-estradiol)

Plant/Protozoa: >1: acetylsalicylic acid, diatrizoic acid, diclofenac, erythromycin, lincomycin, metoprolol, naproxen, oxytetracycline, paracetamol, propranolol, sulfadimidine, sulfamethoxazole (>0.1: clotrimazole, ibuprofen)

Bacteria: >1: paracetamol, erythromycin, sulfadimidine, sulfamethoxazole (>0.1: diclofenac, gemfibrozil).

Figure 9: Number of pharmaceuticals per pharmaceutical class with $MEC_{max}/LOEC_{min}$ values >0.1

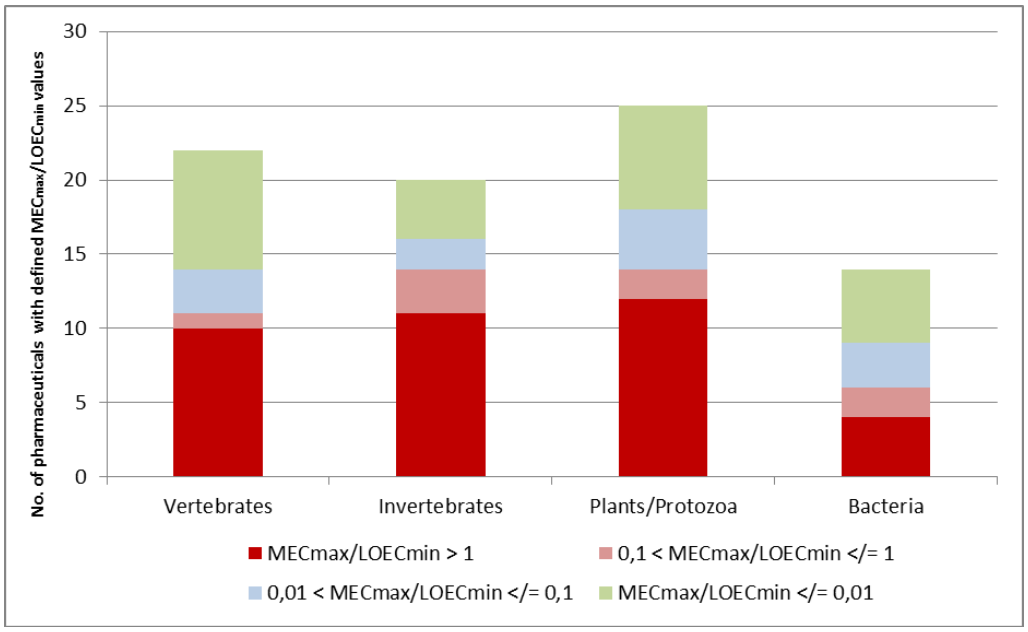


Table 2 makes evident that, according to the calculated risk quotients, the pharmaceuticals of highest relevance (with $MEC_{max}/LOEC_{min}$>0.1) in at least three different biota classes were:

Paracetamol (analgesic, acetaminophen),

Diclofenac, ibuprofen (analgesic, NSAIDs - non-steroidal anti-inflammatory drugs)

Propranolol (β-blocker)

Sulfamethoxazole, Erythromycin (antibiotics)

Gemfibrozil (lipid regulator)

Table 2: Priority pharmaceuticals for which $MEC_{max}/LOEC_{min}$ values were >0.1 in 1, 2, 3 or 4 biota groups before reliability evaluation, including data from $OEKOTOX_{upgrade}$ and Bergmann et al. (2011) [1]

$MEC_{max}/LOEC_{min}$>0.1 in 4 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 3 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 2 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 1 organismic group
Paracetamol	Ibuprofen	Bezafibrate	Acetylsalicylic acid
Diclofenac	Propranolol	Carbamazepine	Atenolol
	Sulfamethoxazole	17β-Estradiol	Ciprofloxacin
	Gemfibrozil	17α-Ethinylestradiol	Clotrimazole
	Erythromycin	Metoprolol	Diatrizoic acid
		Oxytetracycline	Lincomycin
		Sulfadimidine	Naproxen
			Ivermectin

5.1.3.4 Data for mixtures

In general, only for a few cases, a direct comparison between LOECs for an isolated substance and its toxicity in a pharmaceutical mixture could be realized, since data differed with respect to species, effect endpoints or test designs. However, for a few substances, such a comparison was possible (summarized in Table 3)

The table makes evident that, dependent on - at least - the chemical tested and the effect endpoint under investigation, the toxicity of a single pharmaceutical could be lower, higher or equal to its toxicity when applied in a mixture with other compounds.

However, since no mode of action-based effect endpoints were investigated in any of these studies, the specific contribution of the respective substance to the toxicity of the chemical mixture cannot be quantified.

Table 3: Comparison of pharmaceutical effect concentrations applied either isolated or mixture of pharmaceuticals

	Species	Effect	LOEC single	Effect conc. Mixture (LOEC)	Reference
Paracetamol	*Danio rerio*	reproduction output	10	0,5	Galus et al., 2013a, b [9, 10]
	Danio rerio	mortality	0,5	10	Galus et al., 2013a, b [9,10]
Propranolol	*Mytilus galloprovincialis*	cAMP-level, PKA activity	0,0003	NOEC 0,0003	Franzellitti et al. 2011, 2013 [11,12]
Diclofenac	*Dreissena polymorpha*	molecular stress marker	0,3	0,1	Parolini et al, 2013 [13]

5.1.3.5 Data with population relevance

All data collected in the present study were investigated with respect to their population relevance. We defined data as being relevant for the population level as containing information on effects related to (1) community changes, (2) reproduction, fecundity, fertility, embryo

development, sex ratio, intersex, imposex (summarized as “reproduction”), (3) behavior including mating behavior, (4) growth, and (5) survival / mortality.

Altogether, 106 studies and 561 database entries were found to be related to population-relevant endpoints. Figure 10 shows that most database entries contained information of influences of pharmaceuticals on survival and mortality, growth and reproduction. Studies related to behavioral and community changes were in the minority. Hormone-like acting substances did induce population-relevant effects already in relatively low concentrations.

5.1.4 Reliability of publications

For those studies which contained data for the most relevant pharmaceuticals (lowest LOECs) and for the most sensitive endpoints and organisms, the reliability was investigated according to the criteria of Wright-Walters et al. (2011) [2].

These are the following:

“1) A thorough description of the experimental design, including exposure regime and replication,

2) Analytical confirmation of test concentrations

3) Description of ecologically relevant endpoints and all supplemental

morphological information collected

4) Use of test procedures that are based, at least generally, on internationally accepted procedures and practices. Newly developed test procedures must be able to be repeated, and meet all other required criteria

5) Clear linkage of reported findings with the exact experimental design, and

6) Sufficient reporting of results, including system performance, toxicity results, and statistical methods employed to ascertain how the data support the conclusions that are drawn“

If criteria 1, 2, 3 and 6 were met the study was designated as “reliable”. If only 3 of these criteria were met, it was designated as “conditionally reliable”, If less than 3 criteria were met or mistakes became obvious, the study was designated to be “not reliable”.

Consideration was also given to whether the studies were conducted according to GLP and whether both NOEC and LOEC values were provided; however, these criteria did not influence the decision about reliability.

Taking all together, 72 publications have been checked for reliability. Nine of them were reliable, 49 conditionally reliable and 14 not reliable (of which 4 were, in part, conditionally reliable). In

Table 4, the results of the reliability assessment are summarized. For studies which were not reliable, further information is given in Table 5. Except for the study of Fairchild et al. (2011) [14], none of the investigations were conducted under GLP.

Figure 10: Number of database entries with population-relevant endpoints

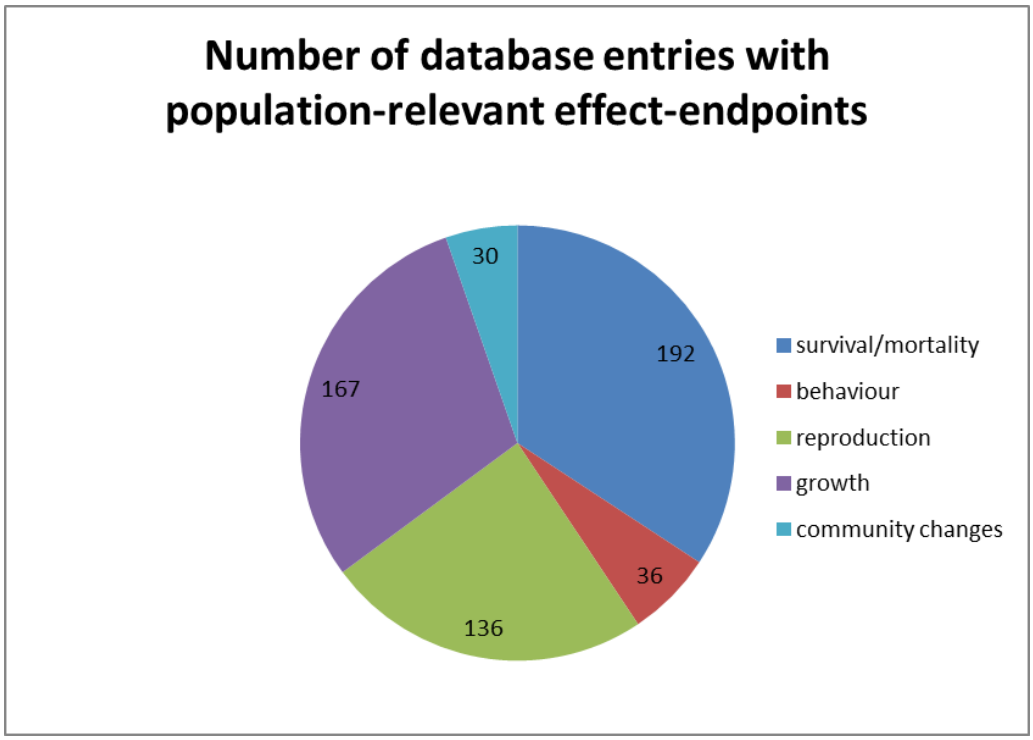


Table 4: Publications checked for reliability, reliability criteria met, and decision on reliability

Reference	Reliability criteria met	Reliability
Antunes et al., 2013 [15]	1,3,6	conditionally reliable
Backhaus et al., 2011 [16]	3, (6)	not reliable
Bajet, 2012[17]	1,3,6	conditionally reliable
Benstead, 2011 [18]	1,2,3	conditionally reliable
Boltes et al., 2012 [19]	1,3,6	conditionally reliable
Boonstra et al., 2011 [20]	1,2,3	conditionally reliable
Chandra et al., 2012 [21]	1,3,6	conditionally reliable
Chen et al., 2012 [22]	1,3, 6	conditionally reliable
Claessens et al., 2013 [23]	1,3,6	conditionally reliable
Contardo-Jara, 2011 [24]	1,2,3	conditionally reliable
Doyle et al., 2013 [25]	1,3,6	conditionally reliable
Fairchild et al., 2011 [14]	1,2,3,6	reliable
Feito et al., 2012 [26]	1,3,(6)	conditionally reliable (mitochondrial activity, DNA content), not reliable (lipid peroxidation, chlorophyll content)
Feito et al., 2013 [27]	1,3, (6)	conditionally reliable (DNA-content), not reliable (mitochondrial activity)
Finn et al., 2012 [28]	2,3,6	conditionally reliable
Fong & Hoy, 2012 [29]	1,3,6	conditionally reliable
Franzellitti et al., 2011 [11]	1,3,6	conditionally reliable
Franzellitti et al., 2013 [12]	1,3,6	conditionally reliable
Gagné et al., 2012 [30]	2,3,6	conditionally reliable

Galus et al., 2013 [10]	(1),2,3,(6)	not reliable
Gonzalez-Rey, 2011 [31]	1,3,6	conditionally reliable
Gust et al., 2012 [32]	1,3,6	conditionally reliable
Gust et al., 2013 [33]	1,3,6	conditionally reliable
Hallgren et al., 2011 [34]	(1),3,6	conditionally reliable
Hallgren et al., 2012 [35]	1,2,3,6	reliable
Hillis et al., 2011 [36]	1,3,6	conditionally reliable
Hoffmann & Kloas, 2012 [37]	1,3,6	conditionally reliable
Huynh Thi et al., 2012 [38]	1,2,3,6	reliable
Ings et al., 2012 [39]	1,3,6	conditionally reliable
Johns et al. 2011 [40]	1,3,6	conditionally reliable
Kaptaner et al., 2011 [41]	1,3,6	conditionally reliable
Lang & Köhidai, 2012 [3]	1,3,(6)	not reliable
Lange et al., 2012 [42]	2,3,6	conditionally reliable
Lawrence et al., 2012 [43]	1,3,6	conditionally reliable
Lei et al., 2013 [44]	1,3,6	conditionally reliable
Liu et al., 2011 [45]	1,3,6	conditionally reliable
Liu et al., 2011 [46]	1,2,3,6	reliable
Madureira et al., 2011 [47]	1,3,6	conditionally reliable
Meina et al., 2013 [48]	1,3,6	conditionally reliable
Notch &Mayer, 2013 [49]	3,(6)	not reliable
Oliveira et al., 2012 [50]	1,3,6	conditionally reliable
Ozdemir et al., 2011 [51]	(1),3,6	conditionally reliable
Parolini et al., 2011 [52]	3,6	not reliable
Parolini et al., 2011 [53]	1,3,6	conditionally reliable
Parolini et al., 2013 [13]	3,6	not reliable
Ragugnetti et al., 2011 [54]	1,3,6	conditionally reliable
Reyhanian et al., 2011 [55]	1,3,6	conditionally reliable
Ribeiro et al., 2012 [56]	1,3,6	conditionally reliable
Rocco et al., 2012 [57]	(3), 6	not reliable
Rocco et al., 2012 [58]	(3), (6)	not reliable
Saravanan et al., 2011 [59]	1,3,6	conditionally reliable
Sarria et al., 2011 [60]	1,3,(6)	conditionally reliable,
Shen et al., 2012 [61]	1,2,3,6	conditionally reliable
Shi et al., 2012 [62]	1,3,(6)	conditionally reliable (chronic test), not reliable (acute test)
Silva et al., 2012 [63]	1,2,3,6	reliable
Skolness et al., 2012 [64]	1,2,3,(6)	reliable
Sponchiado et al. 2011 [65]	3,6	not reliable
Stange et al., 2012 [66]	1,3,6	conditionally reliable
Stange et al., 2012 [67]	1,3,(6)	cond. reliable (reproduction), not reliable (gene expression)
Thomas et al., 2012 [68]	1,3,6	conditionally reliable
Toumi et al., 2013 [69]	1,2,3,6	reliable

van Leeuwen et al., 2012 [70]	(1),3,6	conditionally reliable
Veach et al., 2012 [71]	1,3,6	conditionally reliable
Wang & Gunsch, 2012 [72]	1,2,3,6	reliable
Wang et al., 2011 [73]	1,3,6	conditionally reliable
Wu et al., 2012 [74]	1,3,6	conditionally reliable
Yan et al., 2013 [75]	1,2,3,6	reliable
Yergeau et al., 2012 [76]	(1), 6	not reliable
Yonar et al., 2011 [77]	1,3,6	conditionally reliable
Zhang & Gong, 2013 [78]	1,3,6	conditionally reliable
Zhang etal., 2012 [79]	1,3,6	conditionally reliable
Zhang et al, 2012 [80]	1,3,6	conditionally reliable

Table 5: Further information on pharmaceuticals tested, test organisms, effect endpoints and reasons for lacking reliability for not reliable studies

Reference	Pharmaceutical tested	Test organism	Effect endpoint	Reason for lacking reliability
Backhaus et al 2011 [16]	Fluoxetine Propranolol Clotrimazole	Periphyton community	Inhibition of total pigment content (biomass)	Lacking replicates for some test substances, but not specified (N= 1 to N=5). Results from other studies were involved for calculations of some concentration-response-curves without representing them.
Feito et al 2012 [26]	Diclofenac	*Danio rerio* *Polystichum spicatum*	Lipid peroxidation Chlorophyll autofluorescence	Partly not reliable for lipid peroxidation in zebrafish and Chlorophyll content in Polystichum due to lacking concentration-effect relationships. .
Feito et al 2013 [27]	Venlafaxine	*Polystichum spicatum*	Mitochondrial activity	Partly not reliable for mitochondrial activity due to lacking concentration-effect relationships.

Galus et al 2013 [10]	Paracetamol Venlafaxine Carbamazepine Gemfibrozil	*Danio rerio*	Reproductive output Embryonic mortality Developmental malformations Histopathological changes Plasma estradiol level Blood 11-Ketotestosterone level	Experimental mistake obvious with high concentrations of pharmaceuticals in control treatments, for Gemfibrozil higher than the treatment "low". This information can be obtained from the "Supplementary data", and it is mentioned in one sentence of the discussion. The effective concentrations highly differ from the nominal concentrations for which results are presented. For acetaminophen, e.g., the effective concentrations were only about 10% of the nominal concentrations.
Láng & Köhidai 2012 [3]	Acetylsalicylic acid Diclofenac Fenoprofen Ibuprofen Naproxen Paracetamol Erythromycin Lincomycin Sulfamethoxazole Trimethoprim Metoprolol Propranolol Timolol Diatrizoic acid	*Tetrahymena pyriformis*	Growth rate Chemotactic behaviour	Calculation of EC50 values for growth inhibition unclear; no concentration-effect relationships for chemotaxis; authors recommend themselves to use the test system not as a quantitative, but a qualitative assay to prove for environmental effects of chemicals.
Notch & Mayer 2013 [49]	17alpha-ethinylestradiol	*Danio rerio*	Embryonic vitellogenin mRNA embryonic Cyp1a mRNA embryonic XPC mRNA (genome repair pathway) embryonic XPA mRNA (genome repair pathway)	Lacking replicates and insufficient description of exposition conditions. Effects for XPC/XPA-mRNA und CYP1a-mRNA which only occurred after 24h or 48 h. The authors wanted to show that some effects disappear after longer exposure times. Effects for vtg-mRNA remained stable for the entire exposure time.
Parolini et al 2011 a [52]	Ibuprofen	*Dreissena polymorpha*	Molecular stress biomarkers	Lacking replicates. Chemical analysis only in stock solution.
Parolini et al 2013 [13]	Ibuprofen Diclofenac Paracetamol	*Dreissena polymorpha*	Molecular stress biomarkers	Lacking replicates. Chemical analysis only in stock solution.

Rocco et al 2012 a [57]	Gemfibrozil	*Danio rerio*	Comet assay Diffusion assay RAPD-PCR	Insufficient description of experimental design (replica, organisms, test concentrations); results from controls not presented.
Rocco et al 2012 b [58]	Lincomycin Erythromycin	*Danio rerio*	Micronucleus testComet assay	Insufficient description of experimental design (replica, organisms, test concentrations); no significant effects.
Shi et al 2012 [62]	Clotrimazole	*Xenopus laevis*	Embryo mortalityEmbryo body length	lacking concentration-effect relationships in acute test
Sponchiado et al 2011 [65]	17beta-estradiol	*Oreochromis niloticus*	Micronucleus test Nucleus abnormalities Comet assay	Lacking replicates. Exposure from 5 to 35 days, but no renewal of test substances.
Stange et al 2012b [67]	17alpha-ethinylestradiol	*Potamopyrgus antipodarum*	Gene expression (estrogen receptor)	Effects on gene expression of estrogen receptor only occurred after 7 days, but not after 28 days, lacking time-effect relationship.
Yergeau et al 2012[76]	Erythromycin Sulfamethoxazole Sulfadimidine Gemfibrozil Erythromycin Sulfamethoxazole Sulfadimidine Gemfibrozil	Bacterial community	Microbial community composition (DNA level)	Insufficient description of experimental design (substances, solvents for stock solution, organisms). Exposure time 8 weeks without renewal of test substances.

5.1.5 Consequences of reliability assessment for the described results

Our literature study revealed *Tetrahymena pyriformis* as the most sensitive test organism for pharmaceuticals prior to the reliability check of the publications. This assessment, however, is based on the fact, that for this protozoan extremely low LOECs are reported for a total of 8 substances by Láng & Köhidai (2012) [3] who used "chemotaxis" as an effect endpoint. Since the reliability assessment could not prove the reliability of this publication, three fish species (zebrafish, rainbow trout and medaka) can be identified as most sensitive organisms for pharmaceuticals followed by bivalves and snails. The publications which provide the lowest LOECs for these species did successfully pass the reliability assessment. Table 6, however, also makes evident, that for the three fish species the database was much larger (in total 489 database entries) than for the mentioned molluscan species (only 8 database entries). Also for crustaceans which represent ecologically important species of aquatic ecosystems only few data are available compared to fish.

With respect to the determination of the most important pharmaceuticals for environmental effects the reliability assessment had a more important influence on the final result. In chapter 5.1.3.3 all data available from the OEKOTOX and the $OEKOTOX_{upgrade}$ databases were assessed with the result summarized in table 2. Since the publications of the OEKOTOX database analyzed by Bergmann et al. (2011) [1], however, were not at our disposal for reliability checks, we could only compare the results for the most important pharmaceuticals based on data of the

OEKOTOX$_{upgrade}$ database prior (Table 7) and after reliability assessment (Table 8). The results of the analyses based on data from OEKOTOX$_{upgrade}$ only before reliability evaluation did not differ much from those when the OEKOTOX data were included. . This is due to the fact that the original OEKOTOX database mainly reports on EC_{50} values and includes only a low number of LOECs. Only a slight difference becomes obvious for Metoprolol with $MEC_{max}/LOEC_{min}$>0.1 in only 1 organismic group instead of 2. After assessment of publication reliability, however, only for diclofenac $MEC_{max}/LOEC_{min}$-values >0.1 were found in >3 biota classes. As further important pharmaceuticals propranolol, sulfamethoxazole, bezafibrate, 17α-ethinlyestradiol, 17β-estradiol and oxytetracycline were identified with $MEC_{max}/LOEC_{min}$-values >0.1 for 2 biota classes.

Table 6: Assessment of data for most sensitive species with respect to reliability of publications

	Number of publications	Number of database entries	Number of database entries with LOEC ≤0.1	Number of reliable database entries with LOEC ≤0.1	Lowest LOEC [µg/L]	Lowest reliable LOEC [µg/L]
Tetrahymena pyriformis	1	28	13	--	1,51E-05 (Láng & Köhidai 2012) [3]	--
Zebrafish (Danio rerio)	36	333	36	21	0,0104 (Lange et al 2012) [42]	0,0104 (Lange et al 2012) [42]
Rainbow trout (Oncorhynchus mykiss)	18	92	5	5	2,66E-03 (Ings et al 2012) [39]	2,66E-03 (Ings et al 2012) [39]
Medaka (Oryzias latipes)	8	64	12	12	0,001 (Lei et al 2013) [44]	0,001 (Lei et al 2013) [44]
mussel (Elliptio complanata)	2	4	3	3	0,04 (Gust et al, 2012) [32]	0,04 (Gust et al, 2012) [32]
mudsnail (Potamopyrgus antipodarum)	2	4	4	3	0,05 (Stange et al, 2012a) [66]	0,05 (Stange et al, 2012) [66]
Daphnia magna	12	42	2	2	0,011 (Toumi et al, 2013) [69]	0,011 (Toumi et al, 2013) [69]
Gammarus spec	6	11	0	0	1 (Boonstra et al, 2011) [20]	1 (Boonstra et al, 2011) [20]

Table 7: Priority pharmaceuticals before reliability evaluation, only based on data from OEKOTOXupgrade

$MEC_{max}/LOEC_{min}$>0.1 in 4 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 3 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 2 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 1 organismic group
Paracetamol	Ibuprofen	Bezafibrate	Acetylsalicylic acid
Diclofenac	Propranolol	Carbamazepine	Atenolol
	Sulfamethoxazole	17β-Estradiol	Ciprofloxacin
	Gemfibrozil	17α-Ethinylestradiol	Clotrimazole
	Erythromycin	Oxytetracycline	Diatrizoic acid
		Sulfadimidine	Lincomycin
			Naproxen
			Metoprolol

Table 8: Priority pharmaceuticals after reliability evaluation, only based on data from $OEKOTOX_{upgrade}$

$MEC_{max}/LOEC_{min}$>0.1 in 4 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 3 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 2 organismic groups	$MEC_{max}/LOEC_{min}$>0.1 in 1 organismic group
Diclofenac		Propranolol	Ibuprofen
		Sulfamethoxazole	Paracetamol
		Bezafibrate	Carbamazepine
		17α-Ethinylestradiol	Erythromycin
		17β-Estradiol	Gemfibrozil
		Oxytetracycline	Sulfadimidine
			Atenolol
			Ciprofloxacin

5.1.6 Summary of part 1

The literature survey conducted in the present project reviewed publications on pharmaceutical effects in the environment from January 2011 - July 2013, and thus completed the database OEKOTOX established by Bergmann et al. (2011) [1] with the database $OEKOTOX_{upgrade}$. An Endnote library was created which contains all publications analyzed. In addition, a data evaluation sheet was created as a base for the identification of the most sensitive organisms, the most sensitive effect endpoints, and the ecologically most relevant pharmaceuticals.

For data published between 2011 and 2013, the following results were found:

- Most studies were conducted with antibiotics, followed by NSAIDs, anticonvulsants, antiparasitics, β-blockers and contraceptives.
- Most of the research data are related to pharmaceutical effects in fish, followed by effects in mollusks, plants/algae, crustaceans, and bacteria.

- After the reliability evaluation three fish species (zebrafish, rainbow trout and medaka) were identified as most sensitive organisms for pharmaceuticals followed by bivalves and snails.
- Sensitive effect endpoints were behavior, vitellogenin induction, growth rate, reproduction, histopathological alterations, molecular stress biomarkers, oxidative stress markers, receptor binding, and gene expression

In order to evaluate the lowest and the second lowest effect values from both the OEKOTOX and the $OEKOTOX_{upgrade}$ database with respect to their ecological relevance, risk quotients were calculated as $MEC_{max}/LOEC_{min}$. Risk quotients >0.1 were defined as to be of ecological relevance. The analyses provided the following results:

- Most pharmaceuticals for which risk quotients >0.1 were calculated were investigated in plants/algae and invertebrates closely followed by vertebrates. For bacteria, only few risk quotient >0.1 were found.
- Antibiotics, analgesics (NSAIDs), and β-Blockers were the pharmaceutical classes for which data with the highest ecological relevance were found.
- After reliability assessment diclofenac was identified as the pharmaceutical of highest relevance (with $MEC_{max}/LOEC_{min}$ >0.1 in > 3 biota classes). For propranolol, sulfamethoxazole, bezafibrate, 17α-ethinlyestradiol, 17β-estradiol and oxytetracycline $MEC_{max}/LOEC_{min}$-values >0.1 were calculated for 2 biota classes.

Only in a few cases, the toxicity of an isolated substance could be compared to its toxicity in a pharmaceutical mixture. It could be shown that, at least dependent on the chemical tested and the effect endpoint under investigation, the toxicity of a single pharmaceutical can either be lower, higher, or equal to its toxicity when applied in a mixture together with other compounds. Since no mode of action-based effect endpoints were investigated it was impossible to quantify the specific contribution of the respective substance to the toxicity of the chemical mixture under investigation.

106 studies and 561 database entries were found to be related to population-relevant endpoints with most data on survival/mortality followed by growth and reproduction. Studies related to community changes and behavioral endpoints were in the minority. Direct population relevance, for example induced changes in the composition of bacterial, protozoan or algal communities could only be shown in few studies.

From part 1 of this literature review the following shortcomings could be identified:

- More studies which fulfill the reliability criteria are necessary, especially more chemical analyses should be integrated
- Only few data for invertebrates are available
- Data for ecologically relevant crustaceans are lacking
- Data on sediment toxicity are lacking
- More population-relevant community data are necessary
- In order to be able to identify the contribution of isolated pharmaceuticals to chemical mixtures, mode of action-based effect endpoints have to be investigated.

5.2 *In vitro* test systems

5.2.1 Introduction

I*n vitro* assays developed for the monitoring of pharmaceuticals can be broadly categorized into biochemical assays and cell-based assays. In biochemical and cell based assays, homogeneous assay readouts are preferred over non homogeneous readouts since they do not require washing steps.

Biochemical assays are developed to characterize compounds that interact with an isolated target in an artificial environment. Biochemical assays are target-based and historically have been the mainstay of substance screening in the pharmaceutical industry. Such *in vitro* assays include the assessment of enzymatic activity (e.g., for kinases [81], proteases [82], transferases [83]), receptor-ligand binding (e.g., for G-protein coupled receptors (GPCRs) [84] and nuclear receptors [85]) or protein-protein interactions [86]. Biochemical assays are often directed to and specific for a recombinant purified target of interest and are conducted in homogeneous reaction media. However, not all targets can be purified or prepared suitably for biochemical measurement. For example methods that measure ion channel activities across a biological membrane require two ionic compartments with different ion concentrations separated by a lipid bilayer in which the ion channels reside. Ion channel assays are therefore conducted in cell based assay systems. Additionally, the activity of a small molecule in a reconstituted *in vitro* assay does not always translate into the same activity in a cellular context, due to requirements for cellular cofactors, issues of membrane permeability and other reasons.

Cell-based assays have emerged as a more physiological alternative to assays involving purified proteins. Cell-based assays interact with regulatory networks and feedback control mechanisms. In contrast to biochemical target-based assays, cell-based assays can target an outcome of a pathway in the physiological environment of a cell enabling amplification of a specific signal. Examples of cell-based assays include functional assays (e.g., second messenger mobilization after GPCR activation [87, 88]) and reporter gene assays [89, 90]. Cell-based assays allow for the selection of compounds that can cross cellular membranes, a prerequisite for biological activity towards multicellular organisms.

Homogeneous assays can be categorized into radioactive and non-radioactive assays. Although radioactive assays are decreasing on the market due to the need of radioactive waste disposal, this technology is unlikely to disappear completely. Homogeneous radioactive assay technologies include scintillation proximity assay (SPA) (Amersham Pharmacia Biotech) and FlashPlates™ (NEN Life Science Products, Boston, MA). With these approaches, the target of interest is immobilized onto a solid support (e.g. SPA beads or FlashPlate™ surface) that contains a scintillant [91]. When a radiolabelled molecule binds to the target molecule, the radioisotope is brought in close proximity to the solid support, and the energy transfer between the emitted beta particle and the scintillant results in the emission of light. Radioisotopes which remain unbound to the target and thus free in solution are too distant from the scintillant and, consequently, the released beta particles dissipate their energy solely into the aqueous environment. SPA has been used in a wide variety of applications and has become a standard technique in high throughput screening labs. The technology has been applied to kinases [92, 93] and other enzymes [94] and is widely used for the detection of ligand-receptor interactions [95]. FlashPlate™ technology is similar to SPA but the solid surface is a microtiter plate rather than a bead. FlashPlate™ applications include the detection of cAMP levels [96] and ligand-receptor interactions [97]. Radiometric assays have the advantage to be relatively sensitive but, however, show also numerous disadvantages including limited reagent stability, relatively long read-times and waste disposal.

Most common homogeneous assay readouts are non-radioactive and optical, measuring absorbance and luminescence. Luminescence is the emission of light from any substance, and occurs from electronically excited states. Luminescence is formally split up into two categories (fluorescence and phosphorescence) depending on the nature of the excited state. In excited singlet states, the electron in the excited orbital is paired (by opposite spin) to the second electron in the ground-state orbital. Return to the ground state is spin-allowed and occurs rapidly by emission of a photon. Typical fluorescence lifetimes are around some nanoseconds. Many fluorophores display sub-nanosecond lifetimes. Because of the short timescale of fluorescence, measurement of the time-resolved emission requires sophisticated optics and electronics. In spite of the added complexity, time-resolved fluorescence is widely used because of the increased information available from the data, as compared with stationary or steady-state measurements. Additionally, advances in technology have made time-resolved measurements easier, even when using microscopes. Phosphorescence is emission of light from triplet excited states, in which the electron in the excited orbital has the same spin orientation as the ground-state electron. Transitions to the ground state are forbidden so that phosphorescence lifetimes are typically milliseconds to seconds. Transition metal–ligand complexes, which contain a metal and one or more organic ligands, display mixed singlet–triplet states. These transition metal–ligand complexes display intermediate lifetimes of hundreds of nanoseconds to several microseconds. These optical properties can be exploited by a number of detection methods.

5.2.2 Fluorescence Spectroscopy

Fluorescence spectroscopy can be applied to a wide range of questions in the chemical and biological sciences. The measurements can provide information on a wide range of molecular processes, including the interactions of solvent molecules with fluorophores, rotational diffusion of biomolecules, distances between sites on biomolecules, conformational changes, and binding interactions. Recently, the usefulness of fluorescence has been expanded by advances in technology for development of stable cell lines and cellular imaging. These advances in technology have been decreasing costs for previously complex *in vitro* assay development.

5.2.2.1 Steady-State and Time-Resolved Fluorescence

Fluorescence measurements can be broadly classified into two types of measurements: steady-state and time-resolved. Steady-state measurements, the most common type, are those performed with constant illumination and observation. Here, the sample is illuminated with a continuous beam of light, and the fluorescence intensity or emission spectrum is recorded. The second type of measurement is time-resolved, which is used for measuring intensity decays or anisotropy decays. For these measurements, the sample is exposed to a pulse of light, where the pulse width is typically shorter than the decay time of the sample. Time-resolved measurements contain information on the timescale of conformational changes. Time resolved fluorescent measurements furthermore enable separation of signals emitted by long-lived transition metal–ligand complexes from other unspecific signals generated by short-lived intermediates.

5.2.2.2 Fluorescence Polarization

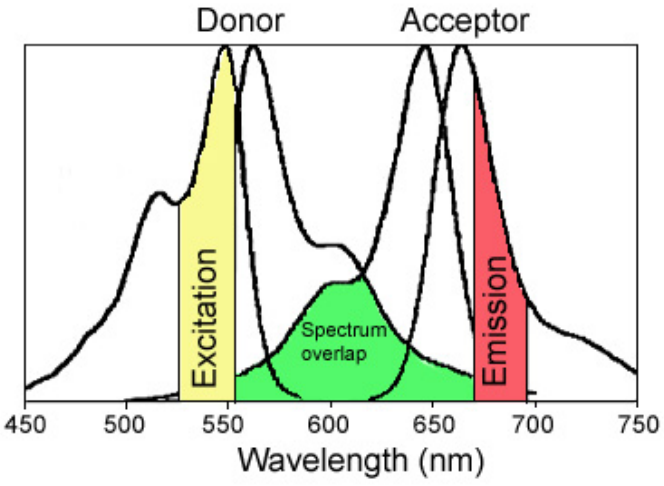
Fluorophores absorb light along a particular direction with respect to the molecular axes. The extent to which a fluorophore rotates during the excited-state lifetime determines its polarization or anisotropy. The phenomenon of fluorescence polarization can be used to measure the apparent volume (or molecular weight) of proteins. This measurement is possible because larger proteins (or protein complexes) rotate more slowly than small ones. Hence, if a protein

binds to another protein, the rotational rate decreases, and the anisotropies increase. The application of plane-polarized light in fluorescence polarization can be used to measure a probe's rotational perturbations, thereby enabling simple assay designs dependent on a single labelled ligand [98, 99]. For example fluorescein- and rhodamine-labelled small molecules are suitable for quantifying the associations with proteins via changes in fluorescence polarization.

5.2.2.3 Fluorescence Resonance Energy Transfer

FRET is an electrodynamic phenomenon occurring between a donor molecule in the excited state and an acceptor molecule in the ground state. The donor molecules typically emit radiation at shorter wavelengths that overlap with the absorption spectrum of the acceptor (Figure 11). The extent of energy transfer is determined by the distance between the donor and acceptor, and the extent of spectral overlap. The distance at which FRET is 50% efficient is called the Förster distance, which is typically in the range of 20 to 60 Å. Förster distances ranging from 20 to 90 Å are convenient for studies of biological macromolecules. These distances are comparable to the size of biomolecules and/or the distance between sites on multi-subunit protein complexes. Any condition that affects the D–A distance will affect the transfer rate, allowing the change in distance to be quantified. FRET can be used to measure the distance between a site on a protein and a membrane surface, the association between protein subunits, and the lateral association of membrane-bound proteins.

Figure 11: Fluorescence Resonance Energy Transfer (FRET).



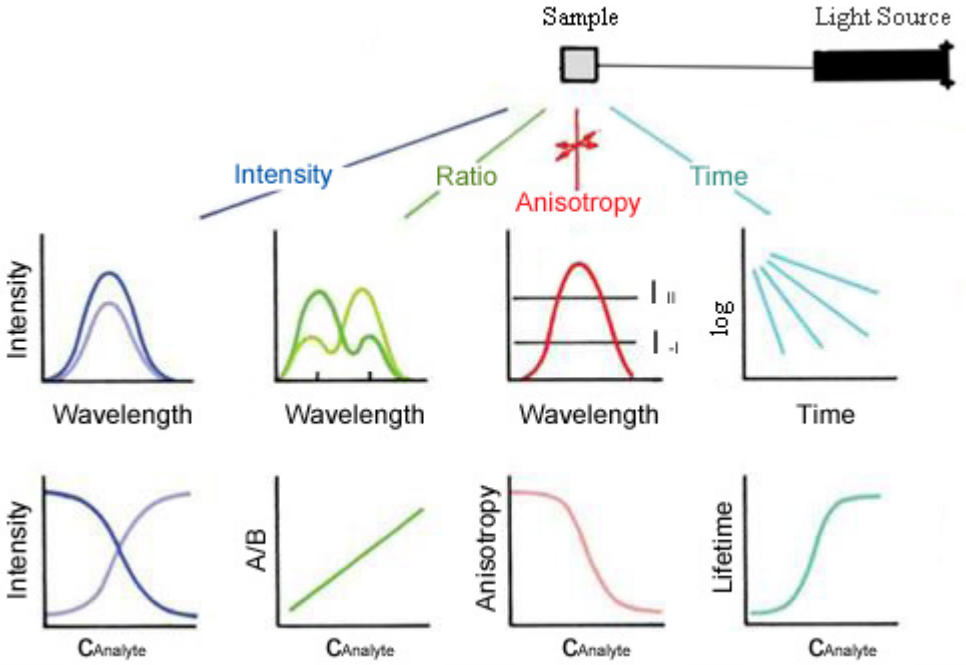
FRET occurs between a donor in an exited state and an acceptor in the ground state. The extent of energy transfer is determined by the distance between the donor and acceptor, and the extent of spectral overlap.

5.2.3 Fluorescence Sensing

There are several observables for fluorescence sensing (Figure 12). The most direct sensing method uses changes in the fluorescence intensity of the probe in response to the analyte. However, it is often inconvenient to use changes in fluorescence intensity for measurements due to the following reasons: Measurements which are independent of fluorophore concentration can be accomplished using wavelength-ratiometric probes, which display shifts in the absorption or emission spectra upon binding of the analyte. Another ratiometric method is fluorescence polarization or anisotropy. In this case the analyte causes a change in the anisotropy of the label. Anisotropy measurements are frequently used in competitive immunoassays, in which the actual analyte displaces a labeled substitute that is bound to specific antibody. This results in a decrease in the anisotropy of the analyte. Anisotropy values are calculated using the ratio of

polarized intensity measurements. The use of an intensity ratio makes the anisotropy measurements independent of fluorophore concentration as long as the measurements are not distorted by auto-fluorescence or poor signal-to-noise ratio. Fluorescence lifetimes can also be used for sensing.

Figure 12: Spectral observables for fluorescence sensing.



Sensing is performed using intensities, intensity ratios, anisotropies and time-domain lifetimes [100].

5.2.3.1 Low molecular weight Sensors

Low molecular weight sensors are available for sensing pH, chloride, oxygen, carbon dioxide, Ca^{2+}, Mg^{2+}, and other parameters. A survey of the literature revealed that a large number of chelate molecules can be used for imaging intracellular concentrations of cations. Calcium probes are perhaps the most widely used intracellular indicators. The salt forms of these dyes do not diffuse across cell membranes, so that the cells need to be labelled by microinjection or electroporation. 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid based chelates are also available with esterified carboxy groups, the so-called acetoxymethyl esters. In this form, the dyes are less polar, and, hence, passively diffuse across cell membranes. Once being inside the cell the acetoxymethyl esters are cleaved by intracellular esterases, and the negatively charged probe is trapped in the cells.

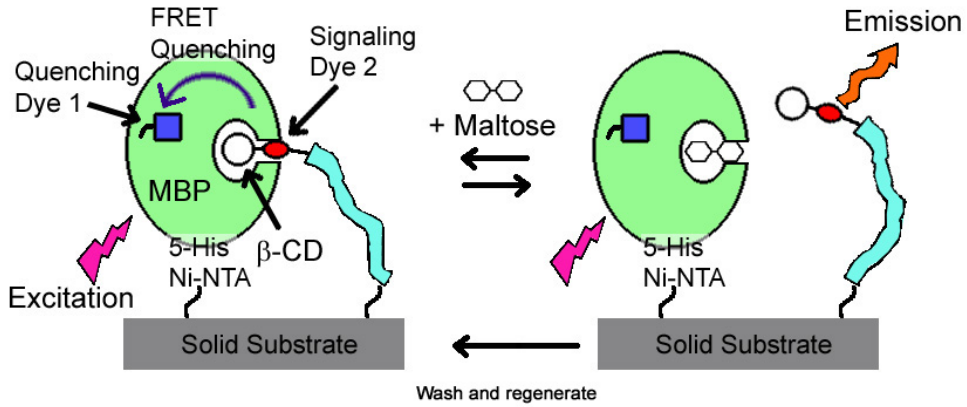
5′, 6′ Dicarboxy-2′,7′-dichlorodihydrofluorescein (CDCFDA) is a fluorogenic probe which is oxidized by reactive oxygen species in living cells. The cell-permeable reagent is very weakly fluorescent while in a reduced state and upon oxidation exhibits strong fluorogenic signal. After uptake into the cell cytoplasmic esterases cleave the esters trapping the resultant charged CDCF inside the cell. CDCF is a co-substrate for peroxidases and could be used for sensing of peroxidase activities [101].

5.2.3.2 Protein Sensors

The combination of recombinant technologies with chemical cross linking enables manifold creation of protein based fluorescent sensors. One example is a MBP (maltose binding protein from E. coli) based sensor based on FRET (102). This example also illustrates the increasingly

sophisticated chemistry of sensors. This maltose sensor is bound to a neutravidin surface by a biotinylated linker. MBP is labelled with a non-fluorescent acceptor quenching dye. The donor is a signalling dye, which is also bound to the surface by a specialized linker. This linker contains a cyclodextrin that binds to MBP. These components are bound to the surface by a biotinylated DNA linker arm that allows changes in rigidity. When the linked cyclodextrin is bound to the binding pocket of MBP the donor is quenched because this binding brings the donor dye in close proximity to the quenching dye. Addition of maltose displaces cyclodextrin from MBP, resulting in increased donor intensity (Figure 13). These results describe a general strategy for surface-bound sensors that are adjustable and yield large changes in intensity. This approach is likely to be used in sensors for a wide variety of analytes.

Figure 13: Surface bound maltose sensor.



Schematic of a surface-bound maltose sensor based on the maltose-binding protein from E. coli and FRET [102].

5.2.3.3 Green fluorescent protein variants

A revolution in the field of live cell imaging occurred following the development of genetically encodable fluorescent tags to specifically label a protein-of-interest within the cellular milieu. This allowed researchers to develop fluorescent biosensors that are able to track signalling molecules within their endogenous environment. This “enlightenment” in the field of cell biology was sparked by the discovery, and subsequent cloning, of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. For simplifying the emission spectra and improving the spectral properties of *A. victoria* GFP for biosensor applications, the wild-type protein has been mutated in several ways. There are several important factors that must be considered when employing GFP family members as genetically encodable fluorescent tags. These include both physical and biological parameters like protein folding efficiency, stability, intrinsic brightness, photo-stability, spectral properties and environmental sensitivity.

The discovery and development of GFP family members exhibiting either red- or blue-shifted excitation/emission spectra allows fluorescence proteins to be used for a number of FRET-based applications. If prior excitation of the donor fluorophore is generated as by-product of an enzymatic reaction, such as the oxidation of luciferin, this is termed “bioluminescence resonance energy transfer” (BRET). These processes represent highly sensitive methods for measuring protein-protein interactions and conformational changes within individual proteins.

Although BRET signals are generally dimmer than those generated by FRET, this approach offers several potential advantages over FRET for live cell applications [103]. Because BRET does not require excitation of the donor fluorophore for energy transfer to occur, it does not elicit cellular auto-fluorescence. As a consequence, BRET exhibit signal-to-noise ratios that are much

higher than those obtained using FRET. Likewise, the chemiluminescent nature of the excitatory light used for BRET simultaneously eliminates two of the primary sources of error associated with FRET-based measurements: spectral bleed-through (donor emission into the acceptor channel and excitation of acceptor molecules by the donor excitation wavelength) and photobleaching of the donor molecules by excitatory light. Finally, since the donor and acceptor molecules used for BRET generate luminescent and fluorescent signals, respectively, the expression levels of each component can be measured independently of the other. Thus BRET-based probes hold potential for live cell applications, including some unique applications in cell based compound screening [104] and *in vivo* pharmacodynamics studies for drug candidates.

Relatively large fluorescent proteins could be replaced by short peptide sequences designed to bind small molecule probes [105]. This can result in restoring activity which was lost due to bulky fluorescent protein fusion tags. However the chemical modifying of the peptide sequences for generation of fluorescent peptides is laborious and modifying reagents need to cross cell membranes. Therefore it is more convenient to use genetically encodable fluorescent proteins to build fluorescent biosensors for probing cellular components within their native environment. Such biosensors have been used to monitor a multitude of cellular processes, offering valuable insights into the dynamic nature of the signalling, metabolic and other regulatory networks that govern cell function. The design and application of a series of fluorescent biosensors range from relatively simple sensors designed to measure changes in the expression to more complex reporter systems designed to probe biochemical processes, such as second messenger turnover and enzyme activities within their native cellular environment [106].

Fluorescence protein based transcriptional reporters have proven to be valuable tools for studying transcriptional activities. One of earliest uses of GFP as a biological probe involved the *in vivo* visualization of promoter activation in the nematode *C. elegans*. During these studies, GFP DNA was placed under the control of a promoter and GFP gene expression was measured during different stages of nematode development [107]. During these studies, GFP DNA was placed under the control of a promoter and GFP gene expression was measured during different stages of nematode development. Over the years, similar studies have been conducted in a variety of cellular contexts, offering valuable information about the activation and regulation of cellular promoters. The stability of GFP inside the cell (t1/2= ~1 day) [108] allows the activation of weak promoters to be measured. The persistence of GFP molecules long after transcription masks transient changes in gene expression. One way to overcome this limitation is to fuse a degradation sequence, such as a domain from mouse ornithine decarboxylase, to the fluorescence protein indicator [109]. Using this strategy, the fluorescent half-life of an EGFP-mouse ornithine decarboxylase chimera was reduced nearly 12-fold to approximately two hours. The increased temporal resolution afforded by this probe allowed the observation of transient changes in NF-κB-mediated gene expression that could not be observed using longer-lived EGFP reporters.

Through protein engineering efforts, fluorescent protein can be generated in such a way that its spectral properties are altered in response to specific cellular factors. Fluorescent protein based reporters that change their spectral properties in response to cellular parameters have also been used extensively to study the regulation of many cellular processes. While in some cases these types of sensors exploit photophysical properties intrinsic to the fluorescent protein tags themselves, in others, the spectral properties of the chromophore are altered by distorting the architecture of the fluorescent protein using integrated protein sequences that are sensitive to the signalling molecule under study.

Engineered molecular switches, which are constructed based on a modular design, consist of a “receiver” module that specifically recognizes the small-molecule-of-interest linked to a

"switching" module that converts the binding event into a conformational change. By combining the molecular switch with an appropriate reporter unit (either by grafting it into the fluorescence protein itself or by flanking the switch region with complementary FRET pairs) small molecule-dependent changes in the sensor unit can be translated into fluorescence readout from the reporter unit. Using this basic design, researchers have constructed a diverse set of fluorescence protein based biosensors capable of probing a large number of small molecule analytes involved in cellular signalling.

Single Fluorophore Sensors

Halide and pH Sensors

Unlike wild-type *A. victoria* GFP, whose spectral properties remain relatively unchanged over a wide range of physiological conditions, the mutations introduced into many engineered fluorescence protein variants often render them sensitive to fluctuations in the cellular environment. For example, because of changes in their internal hydrogen bonding networks, the chromophores of EGFP and several EYFP family members fluoresce very weakly in the protonated state. As a consequence, cellular components that promote protonation, such as protons or halide ions, can lead to markedly reduced fluorescence intensities. This intrinsic property has been exploited to measure several important cellular parameters, including pH and chloride ion concentrations.

The YFP variant YFP (H148Q) exhibits a dramatic reduction in its fluorescence intensity at elevated halide concentrations. This property has been exploited to monitor fluctuations in chloride ion concentration in cells overexpressing the chloride transporter cystic fibrosis transmembrane regulator. These studies demonstrated that YFP (H148Q) faithfully reports chloride flux through the cystic fibrosis transmembrane regulator in response to elevations in intracellular cyclic AMP, providing an attractive alternative to chemical halide dyes [30]. While EGFP (pKa = 6.15) is well-suited for the detection of pH fluctuations within acidic organelles, EYFP (pKa = 7.1) is preferable in more basic environments like the mitochondrial matrix where equilibrium pH values are closer to 8.0 [111].

Redox Potential Sensors

Sensor units can be constructed by introducing residues on the surface of a fluorescence protein variant that renders it sensitive to a particular cellular parameter. This is the basis for redoxsensitive fluorescence protein indicators, termed roGFPs, which use disulfide bond formation between pairs of strategically placed cysteine residues on the surface of GFP to alter the β-barrel architecture of the protein [112]. In the case of roGFP2, structural analysis suggests that the formation of a disulfide bond between the engineered cysteine residues promotes reorganization of two β-strands in close proximity to the chromophore, causing subtle rearrangements in the residues surrounding the chromophore. These conformational changes ultimately shift the equilibrium between the neutral and phenolic states of the chromophore, resulting in reciprocal changes in the excitation maxima at 400 and 480 nm in response to changes in the redox potential [113]. As genetically encodable and genetically targetable redox sensors, roGFP family members as well as the related rxYFPs, have proven to be valuable tools for measuring the redox potential of many subcellular compartments, including the cytosol [114]

Calcium Sensors

The circularly permutated EGFP-based GCa-MPs [115] are among the most popular genetically encodable Ca^{2+} indicators. These fluorescence proteins were generated by fusing the carboxy terminus of calmodulin with the calmodulin binding peptide M13. In these reporters the switch is grafted into a single fluorescence protein variant. In the presence of Ca^{2+}, the CaM-M13

interaction induces conformational changes in the reporter that result in a change in fluorescence intensity of the chromophore. Such Ca^{2+} sensors have been used to measure Ca^{2+} fluxes in a variety of cell types, offering valuable insights into the timing and regulation of Ca^{2+} transients during many cellular processes.

cGMP Sensors

A series of fluorescent indicators of cGMP (FlincGs) based on the regulatory domain of protein kinase G was developed [116]. These sensors consist of a circularly permutated EGFP variant fused to two cGMP binding sites derived from various regions of the protein kinase G regulatory domain. To probe changes in cGMP concentration over a wide range of conditions, FlincG family members have been engineered to bind cGMP with KD's ranging from 35 nM to 1.1 µM. Nucleotide binding induces conformational changes in the protein kinase regulatory domain that lead to a corresponding increase in the fluorescence intensity of the reporter. Importantly, each of the FlincG probes also exhibits a high degree of selectivity for cGMP relative to the chemically similar second messenger cAMP. This specificity for cGMP, coupled with their rapid and reversible binding kinetics and relatively good pH stability, makes FlincGs well-suited for measuring cGMP inside cells.

Reactive Oxygen Species Sensor

A hydroperoxide sensor was generated by inserting circularly permutated EYFP into the regulatory domain of a hydroperoxide sensing bacterial protein [117]. This hydroperoxide sensor exhibits reciprocal changes in the emission intensity at 520 nm when excited by 405 and 488 nm light. Using this sensor, fast and reversible changes in the fluorescence intensity of circularly permutated EYFP in a cell line after exposure to 50 µM hydroperoxide was observed.

A circularly permuted yellow fluorescent protein can be used for sensing superoxide radical anion, the primal reactive oxygen species generated by the electron transport chain. The fluorescence emission (at 515 nm) of purified circularly permutated YFP when excited at 488 nm is five times brighter under strong oxidizing conditions compared to strong reducing conditions, indicative of a large dynamic range. Extensive *in vitro* experiments revealed the superoxide selectivity of circularly permutated YFP over other physiologically relevant oxidants and metabolites. Compared to the fully reduced state, circularly permutated YFP fluorescence displays a 250% increase by oxygenation and a full 420% increase by the superoxide radical anion. The superoxide radical anion associated increase in circularly permutated YFP fluorescence is completely reversed by subsequent addition of copper/zinc-superoxide dismutase. By contrast, circularly permutated YFP emission is unchanged by hydroxyperoxide and peroxynitrite, and is decreased by hydroxyl radical and nitric oxide. Other metabolites tested, including physiological levels of Ca^{2+}, ATP, ADP, $NAD(P)^+$, and NAD(P)H, all exert negligible or only marginal effects [118]. Unlike GFP-based redox biosensors the fluorescence emission of circularly permutated YFP is unaltered when the redox potential varies between -319 mV and -7.5 mV. The reversibility of circularly permutated YFP permits real-time measurements of dynamic changes in superoxide levels in living cells.

ATP/ADP ratio sensing

Perceval, an ATP/ADP indicator is based upon the bacterial ATP-binding protein, GlnK1, and a circularly permutated version of Venus [119]. In the presence Mg-ATP, the T-loop is converted from an extended structure to a highly compact form that is believed to relieve strain on the integrated circularly permutated Venus module. In Perceval, T-loop closure leads to reciprocal changes in circularly permutated Venus emission intensity when excited with 405 and 490 nm light. Therefore, Perceval offers the advantage of a radiometric readout. Mg-ADP also elicits a change in the emission ratio of the probe; however, because of incomplete loop closure, this

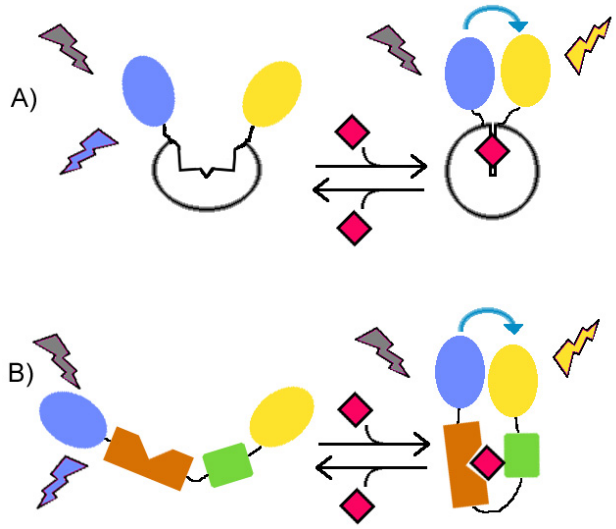
change is only about half that observed upon Mg-ATP binding. As a consequence, Perceval can be calibrated to measure the relative ratio of ATP to ADP inside cells based upon competition between the two molecules for T-loop binding. As a proof of principle, the authors demonstrated that Perceval could detect global metabolic changes caused by the inhibition of glycolysis or the modulation of external glucose levels [119].

Membrane Potential sensing

Fluorescence protein based voltage sensors modulate fluorescence intensity of the fluorescence protein reporter module by placing it between regions of an ion channel or voltage-sensitive protein that undergoes a conformational change in response to changes in the membrane potential [120]. A Cerulean-based voltage sensor, that utilizes conformational changes in the voltage-sensitive domain of the voltage sensor containing phosphatase from *Ciona intestinalis* to affect the fluorescence intensity of Cerulean [121]. These properties make this voltage sensor well-suited to measure the fast neuronal electrical signals often observed during signal propagation.

Beside single fluorophore sensors like the above mentioned, more complex sensor units are required to convert changes in other cellular parameters into a fluorescence output. A "molecular switch" can be derived from a conformational change intrinsic to an endogenous protein or it can be generated via an engineered switch (Figure 14).

Figure 14: Molecular switch.



A molecular switch can be generated by either (A) a conformational change intrinsic to a protein domain or (B) by an engineered conformational change driven by interactions between a receiver module (grey block) and a switching module (green block). In each case, the conformational change is converted to a change in FRET efficiency (blue arrow) by altering the distance and/or orientation of the attached fluorescence protein reporter units.

FRET / BRET based Sensors

FRET-based sensors exist as uni- and bimolecular reporter systems, each of which utilizes a molecular switch to convert activity-dependent changes in the reporter into a measurable FRET response. In each case the molecular switch is designed to alter the distance or relative orientation of a FRET pair in response to specific cellular conditions. For instance, whereas most unimolecular sensors rely upon a conformational change to reposition their fluorophores in space, bimolecular probes typically bring their FRET pairs into close proximity via protein-protein interactions. An advantage of a bimolecular design is that the reporter typically exhibits

a larger dynamic range than its unimolecular counterpart. In the case of bimolecular reporter systems the stoichiometries between the donor and acceptor are variable. Therefore more sophisticated measures of FRET efficiency, such as donor fluorescence recovery after acceptor photo bleaching and fluorescence lifetime imaging, must be used.

FRET based cAMP Sensor

Several FRET-based biosensors have been developed to better understand how the concentrations of cAMP fluctuate over time and throughout the cell. These include the unimolecular reporter system derived from various portions of the guanine nucleotide exchange factor, exchange protein directly activated by cAMP (Epac) [122]. Several intramolecular cAMP reporters have been developed based upon cAMP effector Epac1 and Epac2. These reporters using Epac (ICUE) [123, 124], CFP-Epac (δDEP-CD)-YFP [125], Epac1-camps and Epac2-camps [122] all exhibit decreasing FRET following cAMP binding. Presumably, the binding of cAMP induces an intrinsic conformational change in Epac isoforms that liberates the catalytic domain from intrasubunit allosteric inhibition, thereby altering the distance and relative orientation of their flanking CFP/YFP FRET pairs. Furthermore, mutations have been introduced to abolish the guanine exchange factor activity of Epac, thereby reducing the effects that overexpression of the biosensor may have on cellular signalling pathways. cAMP sensor studies have begun to uncover important details about the role of subcellular compartmentalization in shaping cAMP dynamics during GPCR activation.

BRET based cAMP Sensor

By replacing the CFP donor in Epac-based sensors with luciferase, cAMP reporters were created that utilize BRET as the fluorescent readout [126]. Though their emission intensity has prevented BRET-based sensors from being used to measure biochemical changes at the subcellular level, BRET-based cAMP reporters have proven to be powerful tools for examining drug effects in cell populations in a medium- to high-throughput manner and for screening fluorescent compounds whose excitation/emission profiles preclude the use of FRET-based probes. For instance, using a BRET-based cAMP sensor derived from the second generation ICUE reporter, ICUE2, the impact of nine clinically effective antipsychotics on D2 class dopamine receptor activity was examined [127].

FRET based GPCR activation Sensor

The GPCR superfamily, which represents the largest family of proteins involved in signal transduction, is composed of structurally-similar receptor proteins characterized by seven R-helical membrane-spanning domains. As the primary upstream activators of many intracellular signalling pathways, GPCRs play a key role in converting extracellular stimuli, such as hormones and neurotransmitters, into an intracellular response. The deregulation of GPCRs is also critical to the etiology of many diseases. In fact, roughly one half of the drugs on the market today target GPCRs. For gaining insights into spatial and temporal aspects of GPCR activation and signalling as well as into the mechanistic basis of the signalling process itself, several FRET-based sensors have been created to study receptor activation in the context of single, living cells [128]. Upon ligand binding, GPCR family members act as guanine nucleotide exchange factors that facilitate the exchange of GDP for GTP in the GR subunit of associated trimeric G-protein complexes. Nucleotide exchange promotes the dissociation of the GR subunit from the Gβγ subunits of the complex which, in turn, leads to the activation of downstream effectors, such as phospholipase C and transmembrane adenylate cyclases involved in phosphoinositide and cAMP metabolism. To measure activation-induced conformational changes in GPCR family members, several groups have fused CFP and YFP color variants to the third intracellular loop and the C-termini of the receptor molecule to yield sensitive GPCR activation sensors [129]. These sensors,

which all exhibit reproducible decreases in emission ratio following agonist stimulation, have been used to study GPCR activation kinetics in the presence of full agonists, partial and even inverse agonists. In contrast to agonists generating a decrease in FRET inverse agonists appear to cause an increase in FRET that may be attributed to distinct conformational states of the receptor [130]. Effects of beta blockers bisoprolol, metoprolol and carvedilol in inhibition β1-adrenoceptor isoforms in living cells was measured in real time by FRET using a cyan fluorescent protein, Cerulean fused to the carboxy terminus of the human β1-adrenoceptor and a yellow fluorescent protein inserted into the third intracellular loop [131].

FRET based protein kinase activity Sensor

Protein kinase activity reporters utilize an engineered molecular switch based upon a modular design. A consensus phosphorylation site specific for the kinase of interest serves as the "receiving segment" while a phosphoamino acid binding domain functions as the "switching segment". These regions are concatenated together by a flexible linker and sandwiched between a fluorescence protein FRET pair. Whereas the length of the linker and the choice of FRET pairs influence the dynamic range of the reporter, the "receiving and switching segments" contribute to its specificity and reversibility. This basic modular design has been applied to a number of protein kinases, including protein kinase A. Genetically encoded A-kinase activity reporters are reversible and targetable reporters allowing real-time imaging of protein kinase A activity, and are valuable for analyzing compartmentalized kinase activities [132].

Coupled FRET based NO Sensor

A coupled fluorescent indicator system was used for reporting NO release. The FRET based cGMP indicator "CGY" exhibits a rapid and reversible decrease in emission ratio in response to elevations in cGMP produced by NO-dependent activation of soluble guanylyl cyclase. Since it is estimated that a single NO molecule generates nearly 6,000 cGMP molecules/min, this indicator system is extremely sensitive to NO release [133]. In fact, this indicator system is able to detect NO concentrations in the picomolar range, several orders of magnitude lower than direct NO fluorescent indicators.

Protein Fragment Complementation Sensor

Fluorescence based approaches has paralleled the study of GPCR oligomerization. GPCRs are the largest family of cell surface receptors and are the targets many clinical drugs (e.g. β-blockers and antipsychotics). Bimolecular fluorescence complementation relies on the generation of a fluorescent signal from two non-fluorescent fragments of a fluorescence protein when brought in close proximity by fusion partners. Receptor activation through binding of a ligand agonist proceeds by conformational rearrangement within the transmembrane helical domain as the receptor switches from an inactive to an active state, which in turn activates heterotrimeric G proteins. Activated G proteins regulate the levels of intracellular second-messenger molecules (Ca^{2+}, cAMP, phoshoinositides, cGMP), which modulate signalling cascades involving kinases such as PKA, PKC and others. The 2012 Nobel Prize in Chemistry was awarded to Brian Kobilka and Robert Lefkowitz for their work that was crucial for understanding GPCR function.

Proteins interacting with GPCRs have been shown to modulate receptor expression, membrane targeting, and desensitization. Among these, arrestins are well characterized scaffolding proteins that are notably involved in receptor internalization after ligand activation. As part of a high-content protein fragment complementation assay study based on fluorescence protein complementation, interaction between β-arrestins and the β2-adrenoceptor was detected in cells treated with β2-adrenoceptor agonists, thus providing a measure of receptor activation and internalization [134]. These studies demonstrate the applicability of fluorescent protein

fragment complementation assays for the detection of drug-induced changes in GPCR interactions. Likewise, non-fluorescent protein fragment complementation assays based on β-galactosidase fragment complementation can also be used for the detection of GPCR-β-arrestin interactions.

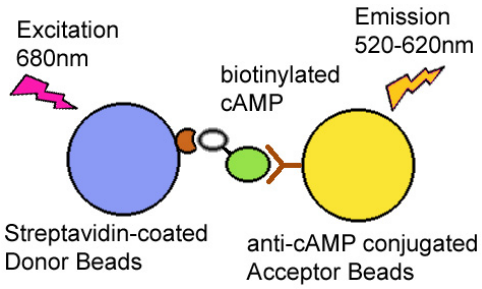
5.2.4 Immunoassays

Immunoassays constitute a large and diverse family of assays. The basic idea is to couple the association of antibody with antigen to some other event that yields an observable spectral change. Various mechanisms are possible, including energy transfer, anisotropy, delayed lanthanide emission, or the use of enzymes to amplify the signal. The use of antibodies as analytical tools can be traced to the development of radioimmunoassays by Berson and Yalow, which resulted in a Nobel Prize. Since then immunoassays have been widely used, but are now based mainly on fluorescence detection.

5.2.4.1 cAMP AlphaScreen® (PerkinElmer)

The acronym ALPHA stands for Amplified Luminescent Proximity Homogeneous Assay. The assay contains two bead types, donor beads and acceptor beads. Both bead types provide reactive aldehyde groups for conjugating biomolecules to the bead surface. Donor beads contain a photo-sensitizer, phthalocyanine, which converts ambient oxygen to an excited form of O_2, singlet oxygen, upon illumination at 680 nm. Singlet oxygen has a limited lifetime prior to falling back to ground state. Within its 4 µsec half-life, singlet oxygen can diffuse approximately 200 nm in solution. If an acceptor bead is within that proximity, energy is transferred from the singlet oxygen to thioxene derivatives within the acceptor bead, subsequently culminating in light production at 520–620 nm (Figure 15). In the absence of an acceptor bead, singlet oxygen falls to ground state and no signal is produced.

Figure 15: AlphaScreen cAMP assay.



Competitive assay type where endogenous cAMP produced in whole cells competes with biotinylated cAMP for binding to an anti-cAMP antibody conjugated to the Acceptor beads. [www.perkinelmer.de]

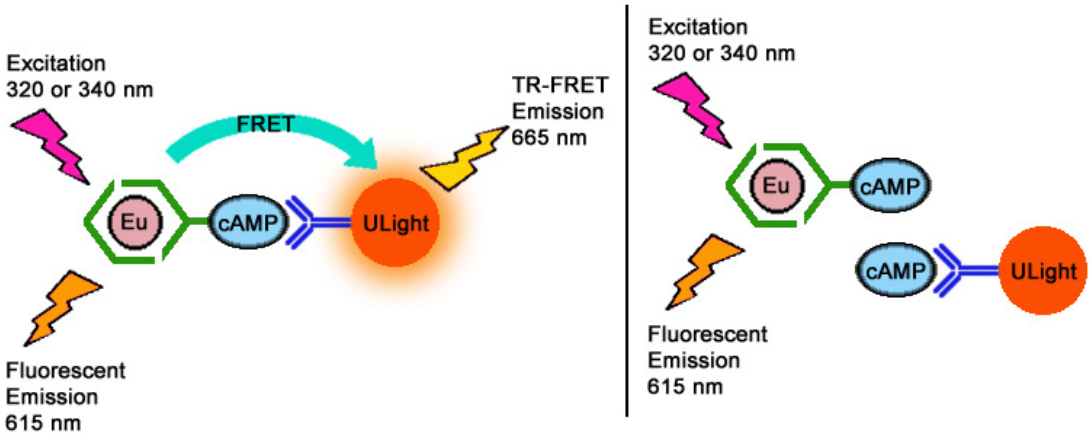
5.2.4.2 cAMP LANCE® (PerkinElmer)

LANCE stands for Lanthanide chelate exite. This assay is a homogeneous time-resolved fluorescence energy transfer immunoassay designed to measure cAMP produced upon modulation of adenylyl cyclase activity. The assay is based on the competition between a Europium-labelled cAMP tracer and sample cAMP for binding sites on cAMP-specific antibodies labelled with a FRET acceptor dye (ULight). When the ULight anti-cAMP antibody is bound to the Eu-cAMP tracer, excitation at 340 nm excites the Europium. The energy is transferred to the ULight-labelled antibody. The fluorescence measured at 665 nm will decrease in the presence of cAMP from test samples, and resulting signals will be inversely proportional to the cAMP concentration of a sample (Figure 16).

5.2.4.3 cAMP HTRF (CisBio International)

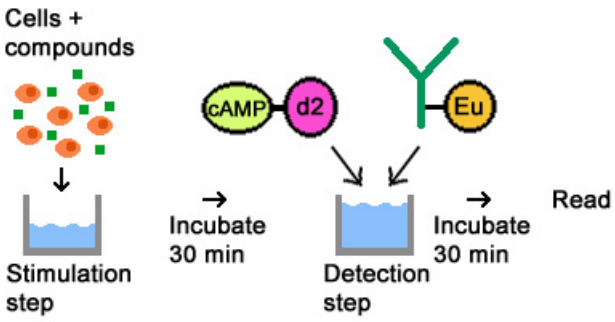
HTRF (homogeneous time resolved fluorescence) combines standard FRET technology with time-resolved measurement of fluorescence. The cAMP assay is based on a competitive immunoassay using cryptate-labelled anti-cAMP antibody and d2-labelled cAMP (Figure 17).

Figure 16: LANCE cAMP assay.



Competitive assay type where endogenous cAMP produced in whole cells competes with a cAMP antibody complex. [www.perkinelmer.de]

Figure 17: HTRF cAMP assay.



Competitive assay type where endogenous cAMP produced in whole cells competes with a cAMP d2 complex. Donor: Europium cryptates (Eu^{3+}cryptate), Acceptor: d2 (an organic motif of approximately 1 kDa) [www.htrf.com]

5.2.4.4 cAMP Screen (Life Technologies)

The cAMP Screen is a competitive immunoassay. Cell lysates are incubated with a cAMP-AP conjugate and an anti-cAMP antibody in the secondary antibody-coated assay plate. The resulting immune complexes are captured in the plate. The captured immune complexes are washed to remove unbound cAMP-AP, and the resulting signal is measured in a luminometer.

5.2.5 Reporter Assays

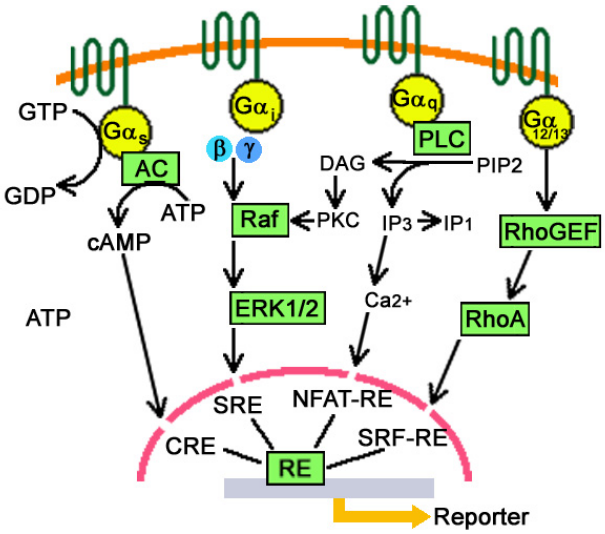
Cell based reporter assays provide a cost effective platform for sensing promoter activities. Reporter gene constructs contain a promoter element which regulates the expression of a selected reporter protein. Commonly used reporters are enzyme proteins with activities linked to colorimetric or luminescent readouts such as luciferase, alkaline phosphatase, β-galactosidase, β-lactamase or fluorescent proteins such as GFP variants. The advantages of reporter gene assays include the wide linearity and sensitivity of the technique and a large signal to background ratio, making them suitable for amplification of a single signal induced by a receptor agonist.

GPCR activation is well known to alter gene transcription via responsive elements for second messengers including the cAMP response element (CRE), the nuclear factor of activated T-cells response element (NFAT-RE), the serum response element (SRE) and the serum response factor response element (SRF-RE, a mutant form of SRE), all of which are located within gene promoter regions. G-protein-dependent reporter gene assays were developed using second messenger responsive elements upstream of a minimal promoter, which in turn regulate the expression of a selected reporter protein (Figure 18).

Reporter gene assays are also easy to set up and can be scaled down to extremely low assay volumes. Despite these advantages, some concerns have been raised, such as the requirement for long incubation periods, difficulty in antagonist detection due to reporter accumulation and the higher potential for false positives because the signal event is distal from receptor activation.

Concerns about the long incubation time and accumulation of reporter have been addressed through the use of destabilized reporters. The higher false positive rate due to the distal signalling event could be partially resolved with by the co-expression of a constitutively expressed internal control, so compounds non-specifically affecting gene transcription could be ruled out [135].

Figure 18: Receptor binding and G-protein-dependent reporter gene assays.



Schematic representation of receptor binding and major pathways activated by different G proteins. RE symbols a specific second messenger responsive element upstream of a minimal promoter, which in turn regulate the expression of a selected reporter protein. [Promega Corporation]

5.2.6 β-adrenoceptor assays

Activation of cardiac β-adrenoceptors by endogenous catecholamines plays a key role in the regulation of cardiac function. The heart contains at least 2 β-adrenoceptor subtypes, termed β1-AR and β2-AR, and may also contain β3-ARs. Stimulation of the β1-AR represents the strongest endogenous mechanism for increasing contractility and beating frequency of the mammalian heart [136]. In human heart failure, which has become one of the leading causes of death and hospitalization, the sympathetic nervous system is chronically activated to overcome the loss of cardiac output. While this initially leads to compensation through a short-term increase in cardiac function, chronic stimulation of the cardiac β-AR system contributes to progression of the disease. These detrimental effects of chronic β-adrenergic signalling are attributed to the β1-AR subtype. Consequently, the development of receptor antagonists has resulted in the single most effective therapeutic regimen to treat heart failure [137]. Beta blockers are part of most commonly prescribed drugs in Germany.

β-adrenoceptors are a class of GPCRs. The subtype β1-AR is linked to Gs protein which in turn is linked to adenylate cyclase (Figure 18). Downstream effectors of cAMP include cAMP response element and cAMP-dependent protein kinase, which mediate some of the intracellular events following hormone binding.

Measuring of β-adrenoceptor activity could be done using *in vitro* assays like tagged-ligand binding, GTPγS binding, CRE reporter, cAMP or conformation based FRET assays.

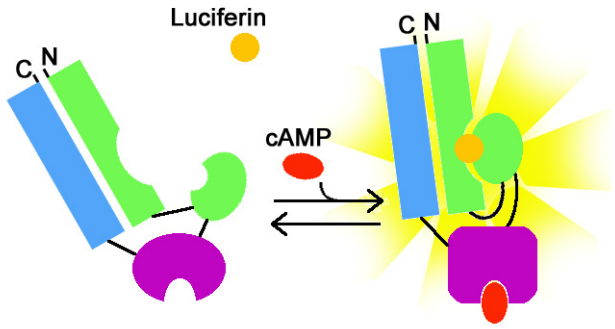
5.2.6.1 Tagged ligand binding assays

Tagged ligand binding assays are based on antibodies binding to cAMP. Commercial available are several competitive assays like cAMP AlphaScreen (5.2.4.1), cAMP LANCE (5.2.4.2), cAMP HTRF (5.2.4.3) or cAMP screen (5.2.4.4). All assays measure cAMP in cellular extracts, therefore cells expressing β-adrenoceptor has to be lysed first.

5.2.6.2 cAMP luminescent biosensor assay

A novel protein biosensor is described, which uses a cAMP-binding domain from PKA coupled to a circularly permuted form of *Photinus pyralis* luciferase. Upon binding of cAMP the luciferase undergoes conformational change from an open less reactive to a closed form resulting in high luciferase activity (Figure 19) [138]. The real-time cAMP luminescent biosensor assay allows the sensitive detection of GPCR mediated signalling through the second messenger cAMP. In contrast to lytic assays with multiple samples and procedural steps, cAMP dynamics can be followed from a population of living cells with greatly reduced time and labour. When compared to existing biosensor designs, this evolved biosensor construct provides increased dynamic range, making it better suited to monitor a broad range of receptor behaviours such as treatment with full, partial, or inverse agonists.

Figure 19: cAMP luciferase assay.



Schematic representation of the cAMP luciferase assay [138]

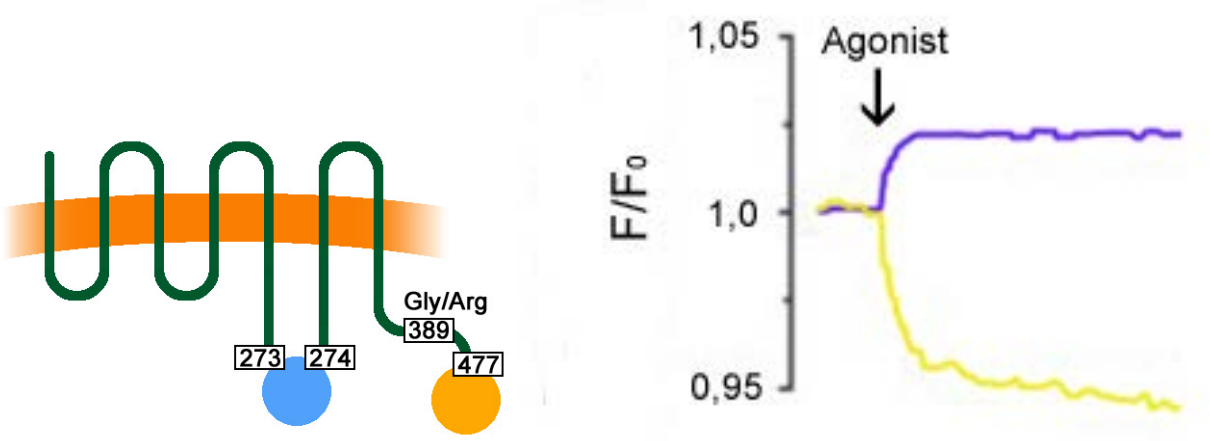
5.2.6.3 cAMP CRE reporter assays

After activation of the β-adrenoceptor adenylyl cyclase is activated by a coupled Gs protein. Adenylyl cyclase catalyzes the formation of cAMP which activates PKA. PKA in turn initiates the downstream kinase cascade. One of the end steps of this cascade is the phosphorylation of the cAMP response element binding protein. The phosphorylated CREB can bind promoter regions containing the 5'-TGACGTCA-3' sequence i.e. the cAMP response element, and in consequence induces downstream gene transcription. An externally-introduced reporter gene usually contains a specific promoter and a reporter gene DNA. The promoter can be made artificially by fusion of CRE elements with a minimal promoter sequence. For real time imaging a CRE- EGFP reporter system was developed, which could be used for monitoring real time transcription under a fluorescent microscope or by flow cytometry [139].

5.2.6.4 Receptor conformation based FRET assay

Beside FRET based cAMP Sensorand BRET based cAMP Sensors a FRET approach is described were the human β1-adrenoceptor was fused at the carboxyterminus to a mutant CFP (Cerulean) and a YFP inserted into the third intracellular loop (Figure 20). After binding of the agonist norepinephrine a conformational shift leads to a decrease of FRET signal. The β-blocker bisoprolol, metoprolol, and carvedilol led to an active change of the receptor conformation resulting in an increase in the FRET ratio, suggesting inverse agonist behaviour [131].

Figure 20: Development of a β-1 adrenoceptor FRET sensor.



After binding of the agonist norepinephrine a conformational shift leads to an increase of distance of the two fluorescent proteins resulting in a decreasing FRET effect [131].

5.2.6.5 cAMP gated ion channel

cAMP produced in subcellular compartments near the plasma membrane can be monitored in HEK cell lines expressing the rat wild-type (GenBank CAA39135.1) or several mutant olfactory CNG channel proteins after recombinant adenovirus infection [140]. Forskolin induced cAMP synthesis resulted in Ca^{2+} influx in the cells. Using the wild-type channel, a forskolin induced modest Ca^{2+} influx could be measured after pre-treatment of the cells with phosphodiesterase inhibitors only. Site specific mutations of the wild-type protein resulted in special mutant channel proteins with enhanced cAMP sensitivity and specificity [140].

5.2.6.6 HCN2 cAMP FRET assay

A cAMP FRET sensor based on a single cAMP binding domain of the hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2) enabled studies of spatial and temporal cAMP dynamics after β1- and β2- adrenoceptor stimulation in freshly isolated adult cardiomyocytes [141].

5.2.7 Cyclooxygenase assays

Cyclooxygenase-1 and cyclooxygenase-2 are bifunctional enzymes that carry out two sequential reactions in spatially distinct but mechanistically coupled active sites: the double dioxygenation of arachidonic acid to prostaglandin G2 and the reduction of prostaglandin G2 to prostaglandin H2. Arachidonic acid oxygenation occurs in the cyclooxygenase active site, and prostaglandin G2 reduction occurs in the peroxidase active site. Prostaglandin H2 diffuses from the COX proteins and is transformed by different tissue specific isomerases to prostaglandins (PGE2, PGD2, PGF2α, PGI2) and thromboxane A2 (TxA2).

Although crude and purified preparations of cyclooxygenase isoenzymes have been used in characterizing inhibitors, several studies indicate that for unknown reasons the potency and selectivity of inhibitors determined using intact cells expressing cyclooxygenase isoenzymes differ from values established using cell-free cyclooxygenase preparations. For example, ibuprofen is approximately 10-fold more potent against cyclooxygenase-2 in intact cells than against cyclooxygenase-2 activity in broken cells [142].

Several functional COX assays using purified or recombinant enzymes have been reported and include an oxygen consumption assay, a peroxidase co-substrate oxidation assay, a radiolabelled chemical inhibition assay, and an enzyme-linked immunosorbant assay.

5.2.7.1 Oxygen consumption assay

COX enzymatic activity was determined by measuring oxygen consumption at 37°C in an oxygraph chamber using an YSI Model 53 oxygen monitor [143]. Tests were performed using isolated protein.

5.2.7.2 Peroxidase co-substrate oxidation assay

Peroxidase activity of the cyclooxygenase could be measured in a peroxidase co-substrate oxidation assay using guaiacol, heme and hydroperoxide [144]. Assays were performed with recombinant purified protein.

5.2.7.3 COX Immunoassay

COX activity in protein solutions can be measured using a commercial Prostaglandin E2 competitive immunoassay kit. The kit uses a monoclonal antibody to Prostaglandin E2 in a competitive manner. Prostaglandin E2 from the sample competes with an alkaline phosphatase Prostaglandin E2 fusion molecule (#ADI-901-001 Enzo Life Sciences).

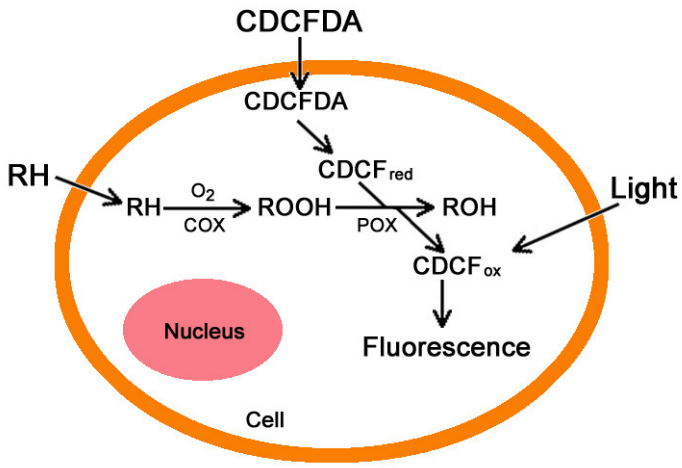
5.2.7.4 COX fluorescence assay

During a cellular assay the peroxidase substrate, 5- (and 6)-carboxy-2‘,7’-dichlorodihydrofluorescein (CDCF) was passively incorporated into cells as the acetoxymethyl ester, 5- (and 6)- carboxy-2',7'-dichlorcdihydrofluorescein diacetate (CDCFDA). Following partitioning into the cell, cytoplasmic esterases cleave the methyl esters and trap the resultant charged CDCF. CDCF serves as a reducing substrate for the peroxidase activities of cyclooxygenase-1 and cyclooxygenase-2. PGG2 generated by the cyclooxygenase activity upon the addition of arachidonate is reduced to PGH2 by the peroxidase activity with resultant oxidation of CDCF to a fluorescent product that can be detected by fluorescence microscopy (Figure 21) [145].

5.2.8 Sensitivity of in vitro and in vivo assays

Different sensitivity of substances in *in vivo* compared to *in vitro* tests may be related to metabolic activities. The metabolic capacity of a living organism could differ from the metabolic activity of single cells. Dependent on this difference an *in vitro* test system could have an enhanced sensitivity over the *in vivo* test system. It is also possible that a metabolite from a substance (e.g. the endocrine disrupting chemical flutamide) exert a higher binding activity on the mammalian hormone receptor as the substance itself. The binding constant of a substance to its biological target molecule influences the lowest observed effect concentration in an *in vitro* assay system. The detection limit of much pharmaceuticals in *in vitro* assays is in the range of nM to µM concentration due to the corresponding IC_{50} concentrations of 10^{-6} to 10^{-9} mol/L. However, when accumulation occurs in an animal, *in vitro* test systems can be less sensitive.

Figure 21: COX fluorescence assay.



Fluorescence assay for cyclooxygenase activity in living cells. After loading of the cells with CDCFDA arachidonate (RH) is added and oxidised. Peroxides are reduced by COX peroxidase activity with resultant oxidation of CDCF to a fluorescent product that can be detected by fluorescence [145].

5.2.8.1 Comparative in vivo / in vitro analysis of endocrine disrupting chemicals

Endocrine disrupting chemicals are defined as exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body. A variety of developmental and reproductive disorders observed in wildlife species have clearly been linked to the exposure to endocrine disrupting chemicals. The importance of identifying potential endocrine disruption has been recognized by regulatory bodies. In order to consider potential endocrine disrupting effects the US Environmental Protection Agency established the Endocrine Disruptor Screening Program. This program employs an approach including a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens for identifying and characterizing endocrine effects of pesticides, industrial substances, and environmental contaminants. Part of the tier 1 screening is a Fish Short-Term Reproduction test and an Amphibian Metamorphosis Assay. These *in vivo* tests are used to identify potential endocrine disrupting chemicals. Because this regulatory approaches focus on screening assays that use animals and are several weeks in duration shorter-term *in vitro* tests that reduce the number of animals and time are required.

In vitro screening for endocrine disruption could address many different hormone systems, such as reproductive hormones (e.g. estrogens, androgens, progesterons), or thyroidal hormones, corticosteroids, growth hormone and their associated hypothalamus/pituitary releasing and stimulating hormones. To date, most alternative assays for detecting potential disruption of estrogen, androgen and thyroid pathway regulation evaluate receptor binding/transactivation, receptor mediated gene/protein expression or hormone synthesis.

For a comparative analysis published *in vivo* data on reproductive effects and metamorphosis were compared with published data of *in vitro* receptor binding or reporter gene assays [146]. For nearly every compound, for which *in vivo* data were available, some alternative *in vitro* assays showed a sensitivity similar to or greater than these *in vivo* data. However, the correlation to *in vivo* data was biased *by* using mainly *in vivo* data for compounds with affinity to the estrogen receptor [146]. Comparative *in vivo* and *in vitro* data for substances with other mode of actions (e.g. COX-inhibitors, β-adrenoceptor blockers) are still lacking.

5.2.9 Evaluation

5.2.9.1 Selection of substances with high priority

There have been many instances in which pharmaceuticals and their metabolites have been identified in water effluents. For developing an *in vitro* assay measuring biological activity of a group of pharmaceuticals first a target molecule has to be defined. An assay could then be developed where the target molecule itself or a downstream signalling event leads to a signal read out after binding of a pharmaceutical to its biological target.

Pharmaceuticals with high priority according to UBA are the β-blocker Atenolol, Bisoprolol, Metoprolol and Propranolol. All this pharmaceuticals as well as metabolites having similar biological activity could be measured by an *in vitro* assay monitoring inhibition of the β-1 adrenoceptor.

Another group with high priority according to UBA are analgesic drugs including Diclofenac, Ibuprofen, Naproxen, Metamizole and 4-N-Methylaminoantipyrin. All these substances as well as the lower priority drugs Indometacin and Mesalazine inhibit the activity of the cycloxygenase 1 and/or 2 (Cox1, Cox2). Therefore an *in vitro* assay measuring inhibition of Cox activity could be used for monitoring a biological effect caused by one or more pharmaceuticals characterized by this mode of action.

5.2.9.2 Evaluation of published in vitro assays

After selection of β-blocker and analgesic drugs for *in vitro* assay development, corresponding published *in vitro* assays are evaluated in the following chapter. A practicable solution for *in vitro* assay systems is a cell based assay system using a stable transfected cell line expressing its recombinant gene of interest in an inducible fashion. In such a case false positive results could be excluded by using the non-induced cell line in parallel. Most of the published cell based *in vitro* assays use cell lines expressing the recombinant protein continuously. There are several inducing expression systems published and it should be possible to use an inducible expressing system for the development of cell line dependent *in vitro* assay systems.

β-adrenoceptor compatible in vitro assays

Biosensors for β1-adrenoceptor activation/inactivation are published for direct and coupled downstream activities (Table 9)

The most specific β-1 adrenoceptor (β1-AR) inhibition assay measures the β1-AR conformation change by a conformation based FRET assay (β1-AR sensor, 5.2.6.4). Concentration-response curves of β1-AR sensor activation for norepinephrine and isoproterenol yielded half-maximal effective concentration (EC_{50}) of 1.800 ± 200 nM and 230 ± 40 nM. Due to a direct conformational effect in transiently transfected cells the FRET signal (∆FRET) was only 5%. This signal was reversible by addition of the antagonist propranolol (10µM).

Downstream effects of β-1 AR activation like cAMP generation or gene activation can be utilized to monitor β-1 AR inhibition. These assays would have the advantage of higher signal read out due to signal amplification. One have to have in mind that levels of intracellular cAMP are tightly regulated, with degradation controlled via the cAMP phosphodiesterase enzymes. When cAMP is produced it binds to protein kinases within the cell, initiating phosphorylation events that regulate target enzymes and transcription factors. There are a variety of cAMP phosphodiesterase enzyme isoforms, which are generally activated by cAMP dependent protein kinases, thus providing an important negative feedback system on the receptor-mediated signalling cascade and regulating the extent of changes in intracellular cAMP concentrations.

Most sensitive cAMP immunoassays (5.2.4) are competitive assays and share the disadvantage that increases in cAMP produce a decrease in signal, making them liable to false positives.

Table 9: β1-adrenoceptor biosensors. Published biosensors for β1-adrenoceptor and coupled downstream activities

Biosensor	Signal	Advantages/Disadvantages	Ref
β1-adrenoceptor	FRET	Highly specific	[131]
cAMP luminescent biosensor	Luminescence	cAMP EC50= 6µM, large signal to noise window / patent pending	[138]
cyclic nucleotide-gated channels CNGA2 HCN2-camps	 Ca^{2+} current FRET	 cAMP EC50= 1µM, conversion of cAMP into Ca^{2+} signal, rapid transient signal, complicated read out. cAMP EC50= 6µM, good for high basal cellular cAMP	 [140] [141]
PKA based cAMP sensor R-CFP, C-YFP PKA-camps	 FRET FRET	 cAMP EC50= 0,5-0,9 µM, multimeric cAMP EC50= 1,9µM, single chain (faster kinetics)	 [149] [122]
Epac based cAMP sensor Epac1/2-camps Epac2-camp300 CFP-Epac-YFP ICUE1/2	 FRET FRET FRET FRET	 cAMP EC50= 2,4 / 0,9 µM, single chain cAMP EC50= 0,3 µM, single chain, high sensitivity cAMP EC50= 50 µM, low sensitivity cAMP EC50= 10-50µM, low sensitivity	 [122] [150] [125] [124]
cAMP Reporter gene CRE EGFP CRE Luciferase	 Fluorescence Luminescence	 basal CRE signal activity essential for cell viability reporter accumulation	 [139] [139]

A very sensitive *in vitro* assay utilizes reporter genes that contain a cAMP response element that regulates the transcription of an enzyme or a fluorescent/bioluminescent protein (5.2.6.3). Synthetic promoters made up of multiple copies of these sequences are routinely employed in reporter genes and have been extensively used to study GPCRs. The reporter protein needs to have a short half-life to minimize basal accumulation of reporter proteins that can restrict the sensitivity of the final readout. In the case of GFP, this can be achieved by creating a destabilized version of GFP by fusing a degradation domain from mouse ornithine decarboxylase to the C-terminal of GFP. As a result of substantial signal amplification between ligand binding and the final measured response ligands with partial agonist activity are likely to manifest themselves as full agonists in most reporter gene *in vitro* assays. This results in the observation that many β-blockers in common clinical practice produce substantial agonist effects at β1- and β2-adrenoceptors when measured at the level of gene expression.

Kinetic studies of the time course of agonist-stimulated gene expression have been undertaken using reporter genes. These studies have shown that a minimum of 30 min of agonist exposure is required to detect a measureable change in reporter gene activity and that it is the duration of cAMP elevation rather than the total quantity of cAMP produced that is the major determinant

of the final response. The resulting requirement for sustained stimulation needs to be taken into account when designing an inhibitory assay and the potential for receptor desensitization with highly efficacious agonists during the time course of the assay means that lower efficacy agonists should be employed.

Direct cAMP detection is possible using firefly luciferase-based biosensors. Genetic manipulation of firefly luciferase into a reversible biosensor of cAMP generation (5.2.6.2) resulted in broad linearity coupled with high sensitivity of intracellular cAMP concentration determination. The firefly luciferase based cAMP biosensor has a pEC_{50} for cAMP of 6.3 and a large signal-to-noise window of approximately 70-fold. When expressed in HEK293 cells, the addition of 10 µM forskolin, a direct activator of adenylyl cyclase, can mediate a 25-fold increase in the luminescent signal within 3.5 min. This sensor represents a powerful method to detect the kinetics of cAMP generation. This cAMP luminescent biosensor assay is covered by a patent application (EP2281046). However, when interpreting kinetics of the luminescent signal, consideration must be given towards the potential for a delay between the real-time cAMP dynamics and the generation of the active form of firefly luciferase.

Another assay type for detecting intracellular cAMP concentration uses FRET based cAMP Sensors. These sensors are used for investigating the spatial and temporal characteristics of cAMP signalling at a single cell level.

PKA and EPAC-based FRET sensors have a similar affinity for cAMP of approximately 0.3–3 µM and a dynamic range of approximately 0.1-10 µM. As a consequence of their relatively high sensitivity, these biosensors are likely to be quickly saturated in cell types that have particularly high concentrations of cAMP. When expressed in HEK cells, the EPAC-based cAMP probe had a uniform, cytosolic distribution. However, the compartmentalization of cAMP responses results in cAMP signalling targeted to discrete microcellular domains. Generally, the activation kinetics of plasma membrane targeted cAMP sensors are more rapid and of greater amplitude than those of their cytosolically distributed equivalents. This may reflect the fact that the plasma membrane is the site of cAMP production and/or the restriction of cAMP diffusion within microdomains located near the plasma membrane.

cAMP gated ion channels like the rat oCNG channel (5.2.6.5) can induce a Ca^{2+} influx as a result of local increase of cAMP concentration [140]. The Ca^{2+} current was measured by patch clamp recording and fura-2 fluorescence measurement.

Cyclooxygenase compatible *in vitro* assays

From published Cyclooxygenase assays (Table 10) only the COX fluorescence assay is suitable for application in a homogeneous assay system without the need of using purified protein preparations.

The cyclooxygenase reaction is part of an enzymatic cycle after generation of a tyrosyl radical in the cyclooxygenase active side. This enzymatic reaction converts arachidonic acid into prostaglandin G2. The lipid hydroperoxide prostaglandin G2 is then reduced to prostaglandin H2 (Figure 22). Leakage of the peroxyl radical from the cyclooxygenase active side leaves the enzyme in a catalytically inactive form. Reactivation of the cyclooxygenase activity requires reaction of the heme prosthetic group with another molecule of hydroperoxide explaining the need for continued presence of hydroperoxide in cyclooxygenase-arachidonic acid reactions [147]. The reduction of hydroperoxide prostaglandin G2 to prostaglandin H2 needs a cellular electron donor. It is likely that other cellular enzymes take part in the reduction of prostaglandin G2. A possible candidate enzyme is thioredoxin reductase. The mammalian thioredoxin reductase shows reducing activity towards a variety of substrates. It was shown that

mammalian thioredoxin reductase reduces arachidonic acid hydroperoxides [148]. Thioredoxins are small (Mw 12.000) disulfide-containing redox proteins known to be present in all eukaryotic and prokaryotic organisms. Thioredoxins are members of the ubiquitous thiol-disulfide oxidoreductase family representing the major ubiquitous disulfide reductases responsible for the maintaining proteins in reduced state in the cytoplasm. Thioredoxins are reduced by electrons from NADPH via thioredoxin reductase.

Table 10: Cyclooxygenase activity assays. Published biosensors for measuring cyclooxygenase activity activities

COX assay	Signal	Advantages/Disadvantages	Ref
Oxygen consumption assay	I (current)	Clark type oxygen electrode, test use purified COX protein	[143]
Peroxidase co-substrate oxidation assay	Photometric (E436)	Guaiacol oxidation assay with purified COX protein	[143]
COX Immunoassay	Photometric (E405)	Competitive PGE2 immunoassay kit, use of purified protein	(5.2.7.3)
COX fluorescence assay	Fluorescence	Cellular assay using stable cell lines, light and oxygen sensitive reagent	[145]

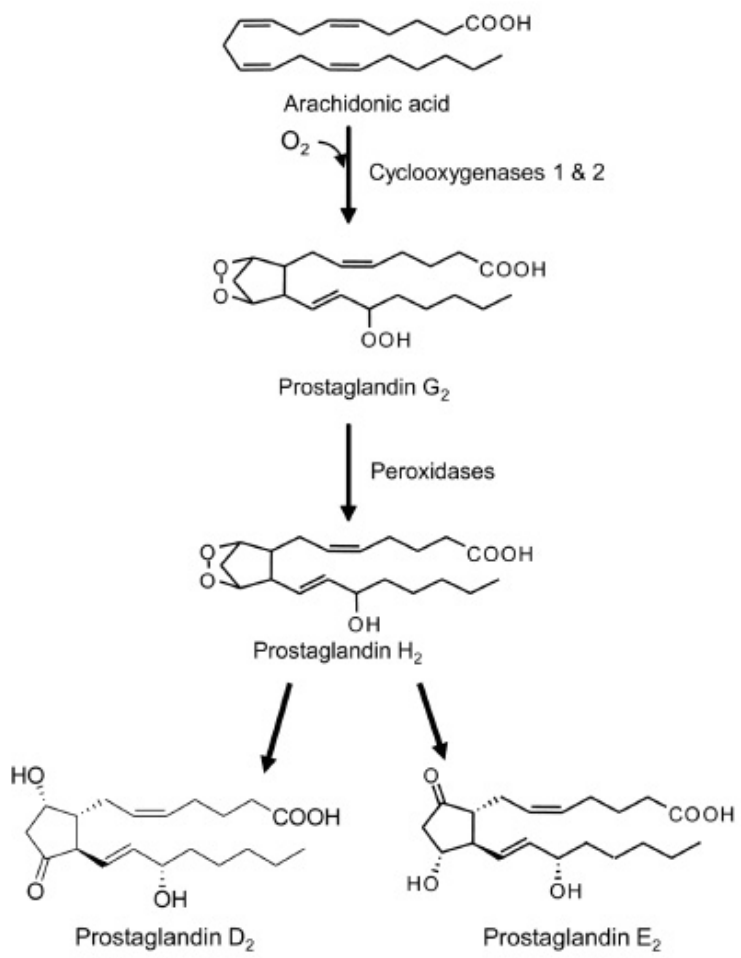
A frequent protein modification as result of oxidative stress is the oxidation of sulfhydryl groups. The redox active protein component cystein would be oxidized completely under normal atmospheric conditions to form a disulfide bond. An unwanted formation of disulfide bonds in the cytoplasm also called “disulfide stress” could be reversed by redox regulation. The simplest scheme of modulation redox state is depicted in Figure 23.

It was shown by Kargman et al. [145] that in CHO cell lines stably expressing cyclooxygenase isoforms exogenous arachidonic acid was metabolized. Cyclooxygenase activity was measured after loading of cells with dichloro-dihydrofluorescein diacetate by fluorescence after converting of the ester into the carboxylic acid by cellular esterases (5.2.7.4). Due to instability of the reagent ester, inhomogeneities in cellular loading and hydrolysis of the reagent into the fluorescent dye, this assay seems to be not convenient for a robust *in vitro* assay method.

5.2.9.3 Suggestion for in vitro assay development

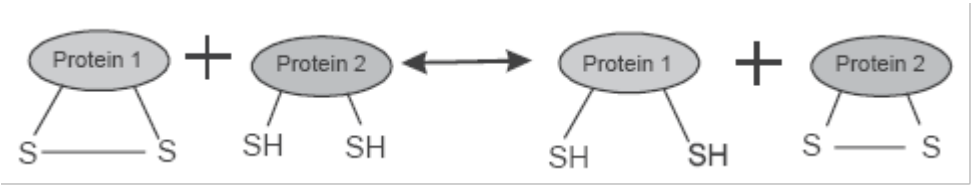
For development of homogenous *in vitro* assay systems with high sensitivity and specificity it is recommended to engineer β-blocker and NSAID biosensor cell lines. The cell lines should stably express their transgenes in an inducible form. The tests should be conducted with induced and non-induced cells in parallel, thus enabling test controls excluding false positive signals. β-blocker biosensor cell lines should monitor the β-adrenoceptor dependent cAMP signal. cAMP should be measured by the FRET based cAMP Sensor and in parallel by the Calcium Sensors GCaMP after converting the cAMP signal into a Ca^{2+} current. NSAID biosensor cell lines should be based on the detection of lipid peroxides. The cyclooxygenase dependent lipid peroxide generation should be monitored by the Redox Potential Sensors roGFP.

Figure 22: Arachidonic acid metabolism through the COX pathway.



COX-1 and COX-2 convert arachidonic acid to the intermediate prostaglandin PGG2 and then to PGH2 which either spontaneously decomposes or is converted by other enzymes to form primarily PGE2 and PGD2 [KEGG pathway]

Figure 23: Modulation of redox regulation.



β -adrenoceptor *in vitro* assay suggestion

In a first step, two parallel approaches are recommended:

1. A FRET based microscopic assay (S.51). This assay enables detection of space resolved signals. Although new microtiter plate readers with higher sensitivity are commercially available it is not clear whether this sensitivity is sufficient for measuring FRET signals without spatial resolution. cAMP is restricted into microdomains located near the plasma membrane. This could result in a limited signal range. Because of this uncertainty it is suggested to develop a second approach:

2. An assay system where a membrane located cAMP signal is converted into a cytoplasmic Ca^{2+} signal as described in 5.2.6.5. Such a signal could be measured in a microtiter assay format without the need of spatial resolution. This should be possible cloning a mutant of the cAMP gated rat olfactory CNG channel [140]. The Ca^{2+} influx could be measured by a Ca^{2+} dependent fluorescent dye [140] or by a genetically encoded Calcium Sensors.

For development of a homogeneous cell based assay system, a cell line expressing the β-1 adrenoceptor together with an Epac1/2 cAMP sensor in an inducible form should be developed and tested whether the cAMP coupled FRET signal is sufficient for measuring in a microtiter plate assay (approach 1).

In addition, a cell line has to be engineered in such a way that the cell line expresses the β-1 adrenoceptor, a specific mutant of the cyclic nucleotide gated channel CNGA2 and the Ca^{2+} sensor GCaMP3. All proteins should be expressed stably in an inducible form (approach 2).

For saving costs it is recommended to prove in principal functionality on a very early development step for the two *in vitro* test systems and to define appropriate milestones. After reaching the first milestone it has to be decided on the test system which has to be further developed.

If β-blockers are present in the test sample the inhibition of the β-adrenoceptor leads to a reduced cAMP generation and thus to an increase of the Epac FRET signal (approach 1) or a decrease of Ca^{2+} dependent fluorescence (approach 2) both relative to the control.

Cyclooxygenase *in vitro* assays suggestion

For screening of COX inhibitors, a test based on the detection of Redox Potential Sensors is recommended. The inducible expression of cyclooxygenase in a genetically engineered cell line should result in generation of lipid peroxides after addition of arachidonic acid. Due to this inducible peroxide generation the redox state should be influenced resulting in some disulfide stress. This disulfide stress could be monitored by redox sensitive fluorescence protein indicators.

For development of a homogeneous cell based assay system one would have to engineer a cell line in such a way that the cell line expresses both, a cyclooxygenase and a redox sensitive fluorescent GFP protein variant in parallel. Both proteins should be expressed stably in an inducible form.

If COX inhibitors are present in the test sample, a diminished lipid peroxide generation will result in a decreased roGFP fluorescence signal relative to the control.

6 Conclusions

As a basis for the development of an effect-based strategy to biomonitor pharmaceuticals, a literature review has been conducted which revealed the necessity to develop mode of action-based biotests for routine monitoring of distinct pharmaceutical classes. In this context, a strategy to monitor entire classes of pharmaceuticals with the same mode of action is given preference because a monitoring programme comprising different biotests for each single substance would cause unrealistically high costs, and a monitoring programme which focusses on just a few lead substances would drastically underestimate the risk exerted by a plethora of pharmaceuticals excluded from analysis. Consequently, priority should be given to develop effect-oriented *in-vitro* tests for pharmaceutical classes and, within those, to analgesics, β-blockers, and antibiotics. Such *in-vitro* assays need to be evaluated with *in-vivo* test systems in parallel. In view to incorporate living organisms here, the literature review revealed ciliate, fish, and mollusk species to exhibit particular sensitivity to pharmaceuticals. Most sensitive endpoints were behavior (chemotaxis), vitellogenin synthesis, growth, reproduction, histological responses, biochemical stress markers, changes in gene expression profiles, receptor binding, and, with reservation, the heart rate. Numerous pharmaceuticals have also been tested for their impact on parameters that directly influence population development: reproduction, fecundity, ontogeny, mating patterns. Here, most significance has been assigned to pharmaceuticals with endocrine action. In contrast to single substances, effects of mixtures of pharmaceuticals can hardly be assessed reliably, since data are not consistent. Consequently, mode of action-based biotests are necessary to decipher the contribution of single substances to the toxicity exerted by a mixture of pharmaceuticals. Endpoints which may be candidates to track mode of action-specific effects of pharmaceuticals are receptor affinity, vitellogenin induction (for estrogenic hormones), specific induction of gene expression, specific repression of enzymes (e.g. COX) or the formation of biochemical secondary products (e.g. lipid peroxides).

Overall, the constructed database represents an extensive compilation of recently generated data on pharmaceutical effects, which will help researchers orienting in this quickly growing field. Besides the identification of certain promising test species and effect endpoints, there is growing evidence that several pharmaceuticals bear the potential to exhibit effects at environmentally relevant concentrations. They may therefore pose serious risks towards aquatic ecosystems and further studies are urgently needed.

The evaluation also showed that researchers need to put a higher effort into improving the reliability of their reported data; this includes a conclusive experimental design as well as a comprehensibly documentation and interpretation of the results.

7 Identification of shortcomings

The literature review generally revealed a strong heterogeneity of data, frequently resulting in just a single dataset for a species. Furthermore, in fish, most studies have been conducted with model species or with species of only local relevance. Consequently, single studies providing numerous data for a large number of chemicals which have been conducted with a single test organism in a single test run will bias the empirical evidence, particularly if the reliability of this test or study is in question.

In view to the relevance of data for Germany, a major shortcoming is the scarcity of data on ecologically relevant invertebrates (gammarids or biota of the sediment). Thus, the sensitivity of key species of home waters cannot be reliably assessed. Generally, data on sediment toxicity are limited in number. Only few publications report on studies that have used the same endpoint in the same test organism for different pharmaceuticals, and mode of action-specific endpoints are rarely used both in studies on single substances and mixtures. Quite often, chemical analytics supplementing biological tests is missing, and only about 70% of publication reviewed met the reliability criteria of Wright-Walters et al. (2011) to a sufficient extent. In general, a mode of action-based *in vitro* test for non-hormonal pharmaceuticals does not exist.

8 Future perspectives in the biomonitoring of pharmaceuticals

(1) We suggest to coin biotests that are based on mode of action-specific mechanisms and thus are specific for pharmaceutical classes and can be implemented in monitoring programs. Advantages of such biotests would be

- the integration of overall chemicals belonging to an mode of action-specific class of pharmaceuticals, irrespective of their accessibility by chemical analytics which can be limited by constraints posed by methodological detection limits, laboratory capacities, or budgetary limits,
- a pre-adaptation for the monitoring of future pharmaceuticals that exhibit the same mode of action as those the test has been developed for,
- and the integration of combinatory effects of mixtures of pharmaceuticals.

(2) Suitable prototypes implementing this idea would be *in vitro* tests for analgesics, like NSAIDs, and *in vitro* tests for β-blockers.

(3) The development of these *in vitro* biotests must go in line with *in vivo* experiments on ecologically relevant species which represent water and sediment biota in order to validate the sensitivity of the novel *in vitro* tests and to "ecologically calibrate" their signals. In these *in vivo* studies, identical endpoints shall be investigated in the laboratory and in field-relevant exposure systems, both for single pharmaceuticals and their mixtures. This strategy will provide necessary information regarding

- - the relevance of in vitro test signals for the situation in vivo,
- - the necessity to artificially concentrate water samples,
- - the relevance of laboratory studies for the field situation,
- - the significance of mixture toxicity, and
- - differences in the toxicity of pharmaceuticals to water- and sediment-living biota.

These suggestions are completely in line with the postulations of Ankley et al. (2007) who emphasize the impotance of mode of action-based studies for pharmaceutical monitoring, and of Brausch et al. (2012) [6] who stress the necessity of

"(a) chronic toxicity data for individual pharmaceuticals to benthic invertebrates, including bivalves, and fish is lacking;

(b) Effects of pharmaceuticals on threatened or endangered species, which warrant protection at the individual level of biological organization;

(c) MOA-based studies, in which biochemical and histological alterations are investigated or studies in which genetic alterations are monitored in responseto long-term pharmaceutical exposure;

(d) Techniques capable of detecting sensitive endpoints in aquatic organisms, such as in vitro and computational toxicology, for prioritizing chemicals andpathways for future studies;

(e) Data on complex mixtures of pharmaceuticals that are found in WWTP effluents"

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