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Final report

Further development of test methods for hormonally active pharmaceuticals and other substances

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Abstract: Further development of test methods for hormonally active pharmaceuticals and other substances

We developed sensor cell lines to efficiently monitor rapid non-genomic signaling cascades influenced by environmentally relevant endocrine-active substances. These sensor cell lines were created by genetically modifying G-Protein coupled estrogen receptor (GPER1) expressing cells, essentially turning them into artificial fluorescent signalosomes. Sensor cells were equipped with reporter units allowing the monitoring of cellular cAMP or Ca^{2+} intracellular concentration changes. Using this sensor cell lines we developed fluorescence-based in vitro assays, enabling us to measure cAMP or Ca^{2+} modulation with high time resolution. We characterized the GPER1 sensor cells using both agonists and antagonist compounds. Our newly developed sensor cell-based in vitro assays were then employed to investigate potentially endocrine-disrupting compounds. Additionally, we used these assays to evaluate the influents and effluents from advanced purification units in wastewater treatment plants.

Kurzbeschreibung: Weiterentwicklung von Testmethoden für hormonell wirkende Arzneimittel und Substanzen

Sensorzelllinien wurden entwickelt, um rasche nicht-genomische Signalkaskaden zu monitoren, die durch umweltrelevante, endokrin aktive Substanzen moduliert werden. Zellen, die den G-Protein-gekoppelten Östrogenrezeptor (GPER1) exprimieren, wurden genetisch verändert, um als künstliche fluoreszierende Signalosomen zu fungieren. Die Sensorzellen wurden mit Reporter-Einheiten ausgestattet, die zeitaufgelöste Bestimmung intrazellulärer cAMP- oder Ca^{2+} Konzentrationsänderungen ermöglichen. Fluoreszenzbasierte in-vitro-Tests wurden unter Verwenden der neuen Sensorzellen entwickelt. Die GPER1-Sensorzellen wurden mit Hilfe von beschriebenen Agonisten und Antagonisten charakterisiert. Die neu entwickelten in-vitro-Tests wurden für die Untersuchung potenziell endokrin aktiver Einzelverbindungen sowie für die Untersuchung von möglichen schädigenden endokrinen Wirkpotentialen in Zu- und Abläufen der vierten Reinigungsstufe verschiedener Kläranlagen eingesetzt.

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List of abbreviations

Abbreviation	Explanation
4-MBC	4-methylbenzylidene camphor
α-estradiol	17α-estradiol
AC	Adenylate cyclase
ß-estradiol	17ß-estradiol
BBP	Butylbenzyl phthalate
BOD	Biochemical oxygen demand
BP-3	Benzophenone-3
BPA	Bisphenol A
BPAF	Bisphenol AF
BPF	Bisphenol F
BPS	Bisphenol S
BSA	Bovine serum albumin
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CaM	Calmodulin
CAS	Chemical abstract service
CEPAC	cAMP Exchange Protein Activated by cAMP
COD	Chemical oxygen demand
COX	Cyclooxigenase
cAMP	cyclic adenosinemonophosphat
DAG	Diacylglycerol
DEHP	Di(2-ethylhexyl)-phthalate
DicEQ	Diclofenac equivalents
DMSO	Dimethyl sulfoxide
DP	Decylphenol
DW	Drinking water
DWTP	Drinking water treatment plants
E1	Estrone
E2	17ß-Estradiol
E3	Estriol
EC50	Half maximal effect concentration
EDC	Endocrine disrupting chemical
EE2	17α-ethinylestradiol
ER	Estrogen receptor
ESI	Electrospray ionization mode
EU	European Union
FID	Flame ionization detection
GAC	Granular activated carbon
GC	Gas chromatography
GPER1	G-Protein coupled estrogen receptor 1
GW	Ground water
HPLC	High-performance liquid chromatography
HWWE	Hospital wastewater effluent
IP3	Inositol 1,4,5-triphosphate

Abbreviation	Explanation
Kd	Equilibrium dissociation constant
LC	Liquid chromatography
LLE	Liquid-liquid-extraction
LOD	Limit of detection
LogD	n-octanol/water distribution coefficient
LOQ	Limit of quantitation
Mass spectrometry	MS
mER(α)	Plasma membrane associated ER(α)
mESR1	Membrane bound estrogen receptor 1
nESR1	Nuclear estrogen receptor 1
MRM	Multiple reaction monitoring
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NP	Nonylphenol
OH	Hydroxyl group
OP	Octylphenol
OPEO	Octylphenol ethoxylates
PAC	Powder activated carbon
PFAS	Per- and polyfluoroalkyl substances
PFBA	Perfluorobutyric acid
PFPrA	Perfluoropropylamine
PNEC	Predicted no effect concentration
PIP2	4,5-Bisphosphate
PKC	Protein kinase C
PTP	Permeability transition pore multi ion channel
PVC	Polyvinyl chloride
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
REACH	Europäische Chemikalienverordnung REACH
RP	Reversed-phased
SPE	Solid phase extraction
SPME	Solid phase microextraction
SW	Surface water
Tandem Mass spectrometry	MS/MS
TBBPA	Tetrabromobisphenol A
TBBPS	Tetrabromobisphenol S
TCBPA	Tetrachlorobisphenol A
TFA	Trifluoroacetic acid
TOF	Time-of-flight
TSS	Total suspended solids
TW	Treated wastewater
WWTP	Waste water treatment plant
WWTP-O	Wastewater treatment plant outflow
YAAS	Yeast anti androgen screen

Summary

Background

The aquatic environment faces continuous contamination from anthropogenic sources. This contamination stems from the usage of various substances, including pharmaceuticals, personal care products, cleaning agents, pesticides, and their transformation products, all of which find their way into wastewater. Although most of these chemicals are present in low concentrations, their persistence, potential for bioaccumulation, and toxicity raise significant concerns regarding their environmental and health impacts.

Conventional wastewater treatment processes provide only partial removal of many of these substances. Therefore, more advanced treatment methods are essential for the effective removal of these pollutants.

Due to the absence of international safety and environmental standards, a significant number of micropollutants and their metabolites persist in wastewater and are subsequently released into aquatic ecosystems. Consequently, increasing quantities of these compounds enter rivers and lakes, often serving as sources of drinking water in industrialized nations. This has raised significant concern, particularly within the European Union, regarding environmental contamination by micropollutants.

Of particular concern are Endocrine Disrupting Chemicals (EDCs), which can exert harmful effects even at low concentrations by modulating central cellular signaling pathways. Out of over 22,000 chemicals marketed in the EU, as well as naturally occurring substances, more than 1,000 are currently being assessed for their endocrine activity. The German Society of Endocrinology, in alignment with other endocrinological professional societies worldwide, advocates for the removal of endocrine-active substances from circulation. Endocrinologists also call for the development of new testing methods to rapidly and reliably detect the effects of endocrine-active substances (aerzteblatt, 2019) .

Hence, the detection and identification of endocrine active substances in wastewater treatment plant (WWTP) effluents play a crucial role in monitoring their release into the environment and ensuring their removal. To accomplish this, bioassays and in vitro assays serve as valuable tools for assessing the effects of mixtures of these substances at the cellular level. These include highly sensitive in vitro test systems that measure the activation of nuclear receptors through the induction of reporter proteins in recombinant cells. Various available bioassays measure endocrine-disruptive effects, including glucocorticoid receptor (Sonneveld *et al.*, 2005), androgen receptor (Satoh *et al.*, 2004), and estrogen receptor reporter gene assays (Rogers and Denison, 2000).

Most studies on EDCs have primarily focused on the genomic effects mediated by nuclear estrogen receptors. However, there is an increasing body of research being conducted to explore potential EDC effects mediated by membrane-bound estrogen receptors.

In fact, some endocrine-active compounds have been described that likely exert a more potent signaling effect through the activation of membrane-bound estrogen receptors (such as mESR1) than through the activation of nuclear estrogen receptors (like nESR1) (Kousteni *et al.*, 2002). Various alternative estrogen receptors are known, including a G-protein coupled estrogen receptor 1 (GPER1), which is located in the cell membrane and endoplasmic reticulum, binds to β-estradiol, and induces rapid cellular signaling cascades. The GPER1 signaling cascade is believed to play a significant role in modulating signal processes in nerve cells (Ding *et al.*, 2019).

While the structure of some EDCs resembles that of natural hormones, others exhibit no structural similarities. As a result, EDCs cannot be defined based on their chemical structure alone but rather by their biological effects in organisms or populations.

GPER1 signaling is a component of an intricate network of G protein-coupled receptor (GPCR) activities. GPCRs are present in all living cells, and often, multiple GPCRs are activated simultaneously on the cell membrane. These activations initiate controlled signaling cascades that regulate the functions of existing proteins within localized regions referred to as signalosomes and influence downstream gene expression. The composition of these signalosomes can differ from one cell to another. Upon activation, these receptors trigger signaling cascades involving calcium mobilization, cAMP generation, and other second messengers (Schwartz *et al.*, 2016).

Objective

The aim of this project is to establish a fluorescence-based in vitro assay that can monitor rapid non-genomic signaling cascades modulated by environmentally relevant endocrine-active substances (estrogens). To achieve this, sensor cell lines will be developed, genetically modified to function as artificial signalosomes. These cell lines will express various proteins, which, together with cellular components, will form functional signal-generating units at the cell membrane, known as signalosomes. Thes signalosomes will contain recombinant membrane-bound estrogen receptors. Additionally, the cells will be equipped with a reporter unit, which will convert the specific signal generated at the signalosome upon binding of an endocrine-active compound into a quantifiable fluorescence signal.

These sensor cell-based new in vitro bioassays will be developed to evaluate the impacts of endocrin-active substances. The specificity and sensitivity of these assays will be tested with selected substances. To assess practical feasibility, we will develop and characterize sample processing techniques for wastewater treatment plant influents and effluents. An important aspect of this effort will be the evaluation of the purification efficiency of advanced treatment units in various wastewater treatment plants. The resulting data will demonstrate the practical utility of these bioassays in assessing the performance of wastewater treatment plants.

Results

Literature search

To evaluate the current methodological basis we conferred a literature search on:

- Membrane bound estrogen receptor involved signaling cascades
- In vitro systems monitoring nongenomic fast estrogen signaling
- Membrane bound estrogen receptor agonist and antagonist compounds
- Potentially relevant chemicals modulating estrogen receptors, corresponding analytical methods and occurrence in surface water and wastewater treatment plant effluents

Development of Ca^{2+} sensor cell lines expressing GPER1

To assess the modulation of the Ca^{2+} signalosome, we genetically modified the GPER1-expressing skin cancer cell line A431 and breast cancer cell line SKBR3, as well as the GPER1 non-expressing oral cancer cell line HSC, by transduction of the calcium sensor protein GCaMP5. Cell lines with endogenous GPER1 expression exhibited Ca^{2+} mobilization when exposed to 50 µM ß-estradiol. Conversely, the HSC cell line transduced with the Ca^{2+} sensor alone did not display ß-estradiol-dependent Ca^{2+} mobilization. The **Ca^{2+} mobilization was specific to ß-estradiol**, as α-estradiol did not induce an increase in cytoplasmic Ca^{2+} concentration. The half-

maximal Ca^{2+} mobilization concentration (EC50) for ß-estradiol in SKBR3 cells was determined to be 2.5 µM. This value is three orders of magnitude higher than the peak concentrations of ß-estradiol typically observed in women's blood. The ß-estradiol concentrations used for measuring Ca^{2+} mobility are in the upper range of water-soluble concentrations.

In natural conditions, ß-estradiol is bound to serum proteins such as sex hormone-binding globulin, which affects its availability. However, our measurements were conducted in a serum-free buffer without proteins or interfering molecules that could affect fluorescence measurements. Therefore, higher hormone concentrations appear to be required to observe an effect. The **GPER1-mediated cytoplasmic calcium rise is attributed to the release of intracellular stored calcium** because Ca^{2+} mobilization was observed in both calcium-free and calcium-containing buffers. We chose the oral cancer cell line HSC for GPER1 transduction to develop a GPER1 sensor cell line because this cell line did not exhibit ß-estrogen-dependent Ca^{2+} mobilization. After transduction of both GCaMP5 and GPER1 genes the resulting HSC-GCaMP5-GPER1 cell line displayed the expected significant Ca^{2+} mobilization response upon the addition of ß-estradiol and showed no Ca^{2+} mobilization in response to α-estradiol. **Bisphenol A activates GPER1**. When exposed to 20 µM of the endocrine-disrupting compound bisphenol A, HSC-GCaMP5-GPER1 cells exhibited Ca^{2+} mobilization comparable to that induced by 20 µM ß-estradiol. This response was not observed in the control cell line HSC-GCaMP5. **The activity of GPER1 is localized to the cellular membrane**. When a 0.5 mg/ml BSA-ß-estradiol conjugate was added, equivalent to an approximate concentration of 180 µM ß-estradiol, it led to a specific increase in cytoplasmic Ca^{2+} concentration only in HSC-GCaMP5-GPER1 cells. In contrast, HSC-GCaMP5 cells did not exhibit any Ca^{2+} mobilization in response to conjugate addition. **The GPER1 agonist G1 induces Ca^{2+} mobilization in HSC-GCaMP5-GPER1 cells** at a concentration of 10 µM. This mobilization was not seen in HSC-GCaMP5 control cells. **The GPER1 antagonist G15 inhibits ß-estradiol induced Ca^{2+} mobilization**. Preincubation with 10 µM of G15 inhibited the ß-estradiol-specific Ca^{2+} mobilization in HSC-GCaMP5-GPER1 cells.

Development of cAMP sensor cell lines expressing GPER1
To investigate the modulation of the cAMP signalosome, we genetically modified the GPER1-expressing breast cancer cell line SKBR3 by transduction of the cAMP sensor CEPAC. Additionally, we modified the HSC cell line by introducing both the CEPAC and GPER1 genes. The resulting cell lines displayed the characteristic fluorescence of CEPAC. However, even after the addition of ß-estradiol at concentrations up to 10 µM, no change in intracellular cAMP concentration was observed in any of the cell lines. It appears **that the investigated cells lack functional GPER1 or ß-estradiol-dependent cAMP signaling pathways.**

Examination of potentially endocrine-disrupting compounds
For the assessment of potentially endocrine disrupting activity we selected the following compounds:

- Halogenated bisphenol flame retardants: tetrabromobisphenol A, tetrachlorobisphenol A, tetrachlorobisphenol S
- synthetic endocrine disruptor compounds: nonylphenol, mestranol, bisphenol A, bisphenol S, octylphenol, norethindron
- neonicotinoid insecticides: clothianidin, acetamiprid, dinotefuran

The halogenated bisphenol A flame retardants tetrabromobisphenol A and tetrachlorobisphenol A modify both, the Ca^{2+} and the cAMP signalosomes independently of GPER1 at low µM concentrations. In contrast tetrabromobisphenol S (TBBPS) showed a significant lower activity. The flame retardants tetrabromobisphenol A (TBBPA) and

tetrachlorobisphenol A (TCBPA), modulating both, the Ca^{2+} and cAMP signalosome, appear to interact with a shared domain among different G protein-coupled receptors (GPCRs). This suggests the possibility for both agonistic and antagonistic interactions.

Unlike TCBPA and TBBPS, preincubation with 100 µM TBBPA inhibited the isoproterenol-induced activation of the ß1-adrenoceptor, indicating that **TBBPA exhibits antagonistic activity against the GPCR ß1-adrenoceptor**.

The synthetic endocrine disruptor compounds nonylphenol, mestranol octylphenol and norethindron did not modify the Ca^{2+} or cAMP signalosomes of all tested cell lines. In contrast to published data (Li *et al.*, 2022) the neonicotinoid compounds clothianidin, acetamiprid and dinotefuran did not modulate Ca^{2+} signaling in GPER expressing cell lines.

Assessing the practical applicability of the developed in vitro bioassays
To assess the practical applicability of the developed sensor cell lines and corresponding in vitro assays, we conducted a characterization of influents and effluents in the advanced treatment processes of five wastewater treatment plants (WWTPs) in Germany. Our analysis included samples from wastewater treatment plants employing granular activated carbon absorption, powder activated carbon absorption, or ozonation as advanced treatment methods. In comparative studies, these influents and effluents were analyzed using our developed effect-based cellular assays. These tests aimed to evaluate cAMP modulation for compounds like halogenated flame retardants, monitor changes in Ca^{2+} concentration to detect potential Ca^{2+} modulating substances and detect cyclooxygenase inhibition as an indicator of nonsteroidal anti-inflammatory drugs. To prepare the samples, we developed a vacuum concentration process at 30°C. The final concentration applied to the tested cell lines was 18-fold.

- cAMP modulation
  none of the WWTP samples demonstrated the ability to modulate cAMP in the cAMP sensor cells.

- Ca^{2+} modulation
  The 18-fold concentrated effluent sample from one of the five WWTPs induced a significant Ca^{2+} mobilization in several Ca^{2+} sensor cell lines. This effect was observed in both the influent and effluent of the wastewater treatment plant unit that used carbon activation as an advanced treatment method. It appears that the compounds responsible for this mobilization do not bind to activated carbon and are not effectively removed from the wastewater.
  These compounds are polar and resistant to oxidation. The Ca^{2+} mobilization activity in the influents and effluents of the WWTP remained unchanged even after additional ozone treatment.

- Cyclooxigenase inhibition
  All effluents from the advanced purification units of the five tested WWTPs exhibited a decrease in cyclooxygenase inhibition activity compared to their corresponding influents. However, the purification performance varied significantly from 70 to 95%.

Conclusions

Cell-based in vitro assays are valuable for characterizing the modulation of central signal pathways. Living sensor cells can be used to characterize cAMP and Ca^{2+} signalosomes by using cAMP and Ca^{2+} sensor proteins. However, due to the complexity of compound mixtures in WWTP effluents and the presence of numerous different GPCRs that can modify cAMP and Ca^{2+} concentrations within the sensor cells, it is challenging to attribute an effect specifically to the

GPER1 receptor. To analyze GPER1-specific signaling, it seems necessary to separate the mixture into several fractions. For this purpose both hydrophobic resin and ion exchange resin based solid phase extraction should be used. Polar and non polar compound fractions should than be used for the assessment of cAMP and Ca^{2+} mobilization effects using the new developed sensor cell lines. Fractions showing an activity could then be used in non-target analysis. The combination of fractionation of WWTP effluents, effect-based in vitro activity assays with non-target analysis will be a powerful method for the analysis of mircopollutants which are not eliminated by WWTP advanced treatment methods.

Zusammenfassung

Hintergrund

Das aquatische Umfeld ist kontinuierlicher Kontamination durch anthropogene Quellen ausgesetzt. Diese Kontamination resultiert aus der Verwendung verschiedener Substanzen, einschließlich Arzneimitteln, Körperpflegeprodukten, Reinigungsmitteln, Pestiziden und ihren Transformationsprodukten, die alle ihren Weg in das Abwasser finden. Obwohl die meisten dieser Chemikalien in geringen Konzentrationen vorhanden sind, verursachen ihre Persistenz, ihr Potenzial zur Bioakkumulation und ihre Toxizität erhebliche Bedenken hinsichtlich ihrer Auswirkungen auf die Umwelt und die Gesundheit.

Konventionelle Abwasserbehandlungsverfahren entfernen viele dieser Substanzen nur teilweise. Daher sind fortschrittlichere Behandlungsmethoden für die effektive Entfernung dieser Schadstoffe unerlässlich. Aufgrund des Fehlens internationaler Sicherheits- und Umweltstandards persistieren eine erhebliche Anzahl von Mikroschadstoffen und ihren Metaboliten im Abwasser und werden in aquatische Ökosysteme freigesetzt. Infolgedessen gelangen zunehmende Mengen dieser Verbindungen in Flüsse und Seen, die oft als Trinkwasserquelle in industrialisierten Ländern dienen. Dies hat insbesondere innerhalb der Europäischen Union erhebliche Besorgnis hinsichtlich der Umweltverschmutzung durch Mikroschadstoffe ausgelöst.

Besondere Sorge bereiten endokrine Disruptoren (EDCs), die selbst in geringen Konzentrationen schädliche Wirkungen besitzen können, da sie zentrale zelluläre Signalwege beeinflussen. Von über 22.000 in der EU vermarkteten Chemikalien sowie natürlich vorkommenden Substanzen werden derzeit mehr als 1.000 auf ihre endokrine Aktivität hin untersucht. Die Deutsche Gesellschaft für Endokrinologie, in Übereinstimmung mit anderen endokrinologischen Fachgesellschaften weltweit, setzt sich für die Entfernung endokrin aktiver Substanzen ein. Endokrinologen fordern die zusätzliche Entwicklung neuer Testmethoden zur schnellen und zuverlässigen Erkennung der Wirkungen endokrin aktiver Substanzen (aerzteblatt, 2019).

Daher spielen die Erkennung und Identifizierung von endokrin aktiven Substanzen in den Abläufen von Kläranlagen eine entscheidende Rolle bei der Überwachung ihrer Freisetzung in sowie ihrer Entfernung aus der Umwelt. Hierfür sind Bioassays und in-vitro-Tests wertvolle Werkzeuge, da sie eine Beurteilung der Wirkungen dieser Substanzgemische auf zellulärer Ebene ermöglichen, ohne die genaue Struktur der Schadstoffe zu kennen. Hierzu gehören hochsensible in-vitro-Testsysteme, die die Aktivierung nukleärer Rezeptoren durch die Induktion von Reporterproteinen in rekombinanten Zellen messen. Verschiedene verfügbare Bioassays messen endokrin-disruptive Effekte wie zum Beispiel Geninduktionstests für den Glukokortikoid-Rezeptor (Sonneveld *et al.*, 2005), Androgen-Rezeptor (Satoh *et al.*, 2004) oder Östrogen-Rezeptor (Rogers and Denison, 2000).

Die meisten Studien zu EDCs haben sich hauptsächlich auf die genomischen Effekte konzentriert, die durch nukleäre Östrogenrezeptoren vermittelt werden. Es gibt jedoch eine zunehmende Anzahl von Forschungsarbeiten, die potenzielle Effekte infolge der Aktivierung membrangebundener Östrogenrezeptoren untersuchen. Tatsächlich wurden einige Verbindungen beschrieben, die wahrscheinlich eine stärkere Signalwirkung durch die Aktivierung membrangebundener Östrogenrezeptoren (wie mESR1) (Kousteni *et al.*, 2002) ausüben als durch die Aktivierung nukleärer Östrogenrezeptoren (wie nESR1). Verschiedene alternative Östrogenrezeptoren sind bekannt, darunter ein G-Protein-gekoppelter Östrogenrezeptor 1 (GPER1). Dieser befindet sich in der Zellmembran und im endoplasmatischen Retikulum, bindet 17β-Östradiol und induziert schnelle intrazelluläre

Signalwege. Die GPER1-Signalwirkung wird als wesentlich für die Modulation von Signalprozessen in Nervenzellen angesehen (Ding *et al.*, 2019).

Die Struktur einiger EDCs ähnelt natürlichen Hormonen, während andere keine strukturelle Ähnlichkeit aufweisen. Daher können EDCs nicht allein anhand ihrer chemischen Struktur definiert werden, sondern allein anhand ihrer biologischen Wirkung in Organismen.

Die GPER1-Signalgebung ist Teil eines komplexen Netzwerks von G-Protein-gekoppelten Rezeptoraktivitäten. G-Protein-gekoppelte Rezeptoren (GPCRs) sind in allen lebenden Zellen vorhanden, und oft werden gleichzeitig mehrere membranständige GPCRs aktiviert. Diese Aktivierungen initiieren zentrale Signalwege, die die Funktionen bestehender Proteine innerhalb lokal begrenzter Regionen, sogenannter Signalosomen, regulieren und häufig eine nachgelagerte Genexpression beeinflussen. Die Zusammensetzung dieser Signalosomen variiert zellspezifisch. Bei Aktivierung lösen diese Rezeptoren Signalkaskaden aus, die die Mobilisierung von Calcium, die Generierung von cAMP und andere sekundäre Botenstoffe verursachen (Schwartz *et al.*, 2016).

Zielsetzung

Das Ziel dieses Projekts ist die Entwicklung eines fluoreszenzbasierten in-vitro-Assays, der schnelle nicht-genomische Signalwege, die durch umweltrelevante endokrin aktive Substanzen beeinflusst werden, monitoren kann. Um dies zu erreichen, werden Sensorzelllinien entwickelt, die genetisch so verändert sind, dass sie künstliche Signalosomen ausbilden. Diese Zelllinien werden verschiedene Proteine exprimieren, die zusammen mit zellulären Komponenten funktionelle Signal-erzeugende Einheiten an der Zellmembran bilden, die auch als artifizielle Signalosomen bezeichnet werden. Diese Signalosomen werden rekombinante membrangebundene Östrogenrezeptoren enthalten. Darüber hinaus werden die Zellen mit einer Reportereinheit ausgestattet, die das spezifische Signal, das beim Binden einer endokrin aktiven Verbindung am Signalosom erzeugt wird, in ein quantifizierbares Fluoreszenzsignal umwandelt.

Diese neuen Sensorzell-basierten in-vitro-Bioassays werden entwickelt, um die Auswirkungen von endokrin aktiven Substanzen einzuschätzen. Die Spezifität und Sensitivität dieser Assays werden anhand ausgewählter Substanzen bewertet. Um die praktische Anwendbarkeit zu prüfen, sollen Techniken zur Probenaufarbeitung von Kläranlagen Zu- und Abläufen entwickelt und charakterisiert werden. In einem Anwendungstest wird die Reinigungseffizienz der vierten Reinigungsstufe unterschiedlicher Kläranlagen untersucht. Die erhobenen Daten sollen die Effektivität dieser neuartigen in-vitro-Bioassays bei der Bewertung der Reinigungsleistung von Kläranlagen in der Praxis untersuchen.

Ergebnisse

Literaturrecherche

Zwecks Sichtung der aktuellen methodischen Grundlagen wurde eine Literaturrecherche zu folgenden Themen durchgeführt:

- Signalwege des membranständigen Östrogenrezeptors
- In-vitro-Systeme für das Monitoren nicht-genomischer Östrogen-induzierter Signalwege
- Agonisten und Antagonisten membranständiger Östrogenrezeptoren
- Potenziell relevante Chemikalien, die Östrogenrezeptoren modulieren, deren Vorkommen in Oberflächenwasser und Abwasserreinigungsanlagen sowie diesbezügliche analytische Methoden

Entwicklung von Ca^{2+} Sensorzelllinien, die GPER1 exprimieren
Um die Modulation des Ca^{2+}-Signalosoms zu verfolgen, wurde die Hautkrebszelllinie A431 und die Brustkrebszelllinie SKBR3, die beide GPER1 exprimieren, sowie die orale Krebszelllinie HSC, die GPER1 nicht exprimiert, durch Transduktion des Calciumsensorproteins GCaMP5 genetisch verändert. Die beiden Zelllinien mit endogener GPER1-Expression zeigten eine intrazelluläre Ca^{2+}-Mobilisierung nach Inkubation mit 50 µM ß-Estradiol. Im Gegensatz dazu zeigte die HSC-Zelllinie, die nur mit dem Ca^{2+}-Sensor transduziert wurde, keine ß-Estradiol abhängige Ca^{2+}-Mobilisierung. Die Ca^{2+}-Mobilisierung war ß-Estradiol spezifisch, da α-Estradiol keine Erhöhung der cytoplasmatischen Ca^{2+}-Konzentration bewirkte. Die halbmaximale Ca^{2+}-Mobilisierung erfolgte bei einer Konzentration (EC50) für ß-Estradiol in SKBR3-Zellen von 2,5 µM. Dieser Wert liegt drei Größenordnungen über den üblicherweise im Blut von Frauen beobachteten höchsten ß-Estradiolkonzentrationen. Die Ca^{2+} mobilisierenden ß-Estradiolkonzentration liegen hierbei im oberen Bereich der Wasserlöslichkeit.

Unter natürlichen Bedingungen ist ß-Estradiol an Serumproteine wie das Sexualhormon-bindende Globulin gebunden, was seine biologische Verfügbarkeit beeinflusst. Unsere Messungen wurden in einem serumfreien Puffer ohne Proteine durchgeführt, die die Fluoreszenzmessungen beeinflussen könnten. Daher scheinen höhere Hormonkonzentrationen erforderlich zu sein, um eine Wirkung zu erzielen. **Der Anstieg des cytoplasmatischen Calciums, der durch GPER1 vermittelt wird, ist auf die Freisetzung von intrazellulärem gespeichertem Calcium zurückzuführen**, da die Ca^{2+}-Mobilisierung sowohl in calciumfreien als auch in calciumhaltigen Puffern beobachtet wurde. Für die Entwicklung einer weiteren GPER1 Sensorzelllinie wählten wir die orale Tumorzelllinie HSC aus, da diese keine ß-Östrogen-abhängige Ca^{2+}-Mobilisierung zeigte. Diese wurde hierfür mit dem Ca^{2+} Sensor GCaMP5 und GPER1 gemeinsam tranduziert. Nach der Transduktion zeigte die resultierende HSC-GCaMP5-GPER1-Zelllinie die erwartete signifikante Ca^{2+}-Mobilisierungsreaktion nach Zugabe von ß-Estradiol. Die Inkubation mit α-Estradiol führte zu keiner Ca^{2+} Mobilisierung. **Bisphenol A aktiviert GPER1.** Nach Zugabe von 20 µM des endokrin aktiven Wirkstoffs Bisphenol A zeigten HSC-GCaMP5-GPER1-Zellen eine Ca^{2+} Mobilisierung, die mit der von 20 µM ß-Estradiol induzierten Mobilisierung vergleichbar war. Diese Reaktion wurde in der Kontrollzelllinie HSC-GCaMP5 nicht beobachtet. **Die Aktivität von GPER1 befindet sich auf der äußeren Zellmembran**. Bei Zugabe von 0,5 mg/ml eines BSA-ß-Estradiol-Konjugates, was einer ungefähren Konzentration von 180 µM ß-Estradiol entspricht, kam es nur in HSC-GCaMP5-GPER1-Zellen zu einem spezifischen Anstieg der cytoplasmatischen Ca^{2+}-Konzentration. Im Gegensatz dazu zeigten HSC-GCaMP5-Zellen keine Ca^{2+} Mobilisierung nach Konjugatzugabe. **Der GPER1-Agonist G1 induziert eine Ca^{2+} Mobilisierung in HSC-GCaMP5-GPER1-Zellen** bei einer Konzentration von 10 µM. Diese Mobilisierung wurde in HSC-GCaMP5-Kontrollzellen nicht beobachtet. **Der GPER1-Antagonist G15 hemmt die ß-Estradiol-induzierte Ca^{2+} Mobilisierung**. Die Vorinkubation mit 10 µM G15 hemmte die ß-Estradiol-spezifische Ca^{2+} Mobilisierung in HSC-GCaMP5-GPER1-Zellen.

Entwicklung von GPER1 exprimierenden cAMP-Sensorzelllinien

Um die Modulation des cAMP-Signalosoms zu untersuchen, wurde die Brustkrebszelllinie SKBR3, die GPER1 endogen exprimiert, durch Transduktion des cAMP-Sensors CEPAC genetisch verändert. Ebenso wurde die HSC-Zelllinie durch Transduktion der Gene für CEPAC und GPER1 modifiziert. Die resultierenden Zelllinien zeigten die charakteristische CEPAC Fluoreszenz. Allerdings konnte nach Zugabe von ß-Estradiol in Konzentrationen von bis zu 10 µM keine Veränderung der intrazellulären cAMP-Konzentration in den Zelllinien beobachtet werden. Offensichtlich fehlen den untersuchten Zelllinien funktionelle cAMP-Signalosome, die durch GPER1 oder ß-Estradiol induziert werden.

Untersuchung potenziell endokrin wirkender Verbindungen

Zur Bewertung einer potenziell endokrin disruptiven Aktivität wurden folgende Verbindungen ausgewählt:

- Halogenierte Bisphenol A Flammschutzmittel: Tetrabromobisphenol A, Tetrachlorobisphenol A, Tetrachlorobisphenol S
- endokrin wirkende synthetische Verbindungen: Nonylphenol, Mestranol, Bisphenol A, Bisphenol S, Octylphenol, Norethindron
- Neonicotinoid Insektizide: Clothianidin, Acetamiprid, Dinotefuran

Die halogenierten Bisphenol A Flammschutzmittel Tetrabromobisphenol A (TBBPA) und Tetrachlorobisphenol A (TCBPA) modifizieren in niedrigen µM-Konzentrationsbereich sowohl das Ca^{2+} als auch das cAMP Signalosom unabhängig von GPER1. Im Gegensatz hierzu zeigte Tetrabromobisphenol S (TBBPS) eine signifikant geringere Aktivität. Da die Flammschutzmittel TBBPA und TCBPA sowohl das Ca^{2+} als auch das cAMP Signalosom modulieren, scheinen diese mit einer gemeinsamen Domäne verschiedener G-Protein-gekoppelter Rezeptoren (GPCRs) zu interagieren. Dies legt die Möglichkeit sowohl agonistischer als auch antagonistischer Wechselwirkungen nahe.

Im Gegensatz zu TCBPA und TBBPS hemmte die Vorinkubation mit 100 µM TBBPA die durch Isoproterenol ausgelöste Aktivierung des ß1-Adrenorezeptors. **Dies deutet darauf hin, dass TBBPA antagonistische Aktivität gegenüber dem GPCR ß1-Adrenorezeptor aufweist**.

Die synthetischen endokrinen Disruptoren Nonylphenol, Mestranol, Octylphenol und Norethindron zeigten keine Modulation der Ca^{2+} oder cAMP-Signalosome aller getesteten Zelllinien. Im Gegensatz zu veröffentlichten Daten (Li *et al.*, 2022) modulierten die Neonicotinoide Clothianidin, Acetamiprid und Dinotefuran die Ca^{2+}-Signalgebung in GPER-expressierenden Zelllinien nicht.

Bewertung einer praktischen Anwendbarkeit der entwickelten in vitro Bioassays

Um die praktische Anwendbarkeit der entwickelten Sensorzelllinien und entsprechenden in vitro Bioassays zu bewerten, wurden Zu- und Abläufe der vierten Reinigungsstufe von fünf Kläranlagen untersucht. Je nach Klärwerk umfasste die vierte Reinigungsstufe granulierte Aktivkohleabsorption, pulverisierte Aktivkohleabsorption oder Ozonung. In vergleichenden Studien wurden die Zu- und Abläufe mithilfe unserer entwickelten effektbasierten zellulären Assays charakterisiert. Diese Tests bestimmten eine cAMP-Modulation zwecks Nachweis von Wirkungen ähnlich den halogenierten Flammschutzmitteln, eine Variation der intrazellulären Ca^{2+} Konzentration, um potenzielle Ca^{2+} modulierende Substanzen zu erkennen und die Cyclooxygenase-Inhibition als Indikator für nichtsteroidale entzündungshemmende Medikamente. Zur Probenvorbereitung wurde hierfür eine Vakuumkonzentrationsverfahren bei 30°C entwickelt. Die Proben wurden in den zellbasierten Assays 18-fach konzentriert eingesetzt.

- cAMP-Modulation
  Keine der Proben aus den Kläranlagen modulierte die cAMP Konzentration in den cAMP-Sensorzellen
- Ca^{2+}-Modulation
  Die 18-fache konzentrierte Ablaufprobe einer der fünf Kläranlagen induzierte eine signifikante Ca^{2+} Mobilisierung in mehreren Ca^{2+}-Sensorzelllinien im Unterschied zu allen anderen Kläranlagenproben. Dieser Effekt wurde sowohl in dem Zulauf als auch im Ablauf

der Pulveraktivkohle-Reinigungsstufe der Kläranlage beobachtet. Diese für diese Mobilisierung verantwortlichen Verbindungen binden nicht an Aktivkohle und werden daher nicht aus dem Abwasser entfernt. Diese Verbindungen sind polar und oxidationsbeständig. Die beobachtete Ca^{2+}-Mobilisierungsaktivität der Zu und Abläufe der Kläranlage blieb auch nach einer zusätzlichen Ozonbehandlung unverändert nachweisbar.

- Cyclooxygenase-Inhibition
  Alle Abläufe der vierten Reinigungsstufen der fünf getesteten Kläranlagen zeigten eine Abnahme der Cyclooxygenase-Hemmung im Vergleich zu den entsprechenden Zuläufen. Die Reinigungsleistung variierte jedoch signifikant im Bereich von 70 bis 95%.

Fazit

Zellbasierte in vitro Bioassays sind wertvolle Werkzeuge zur Charakterisierung der Modulation zentraler Signalwege. Lebende Sensorzellen können verwendet werden, um cAMP und Ca^{2+} Signalosomen mithilfe von cAMP und Ca^{2+} Sensorproteinen zu charakterisieren. Aufgrund der Komplexität von Verbindungsmischungen in den Abläufen von Kläranlagen und der Vielzahl verschiedener G-Protein-gekoppelter Rezeptoren (GPCRs), die cAMP und Ca^{2+} Konzentrationen in den Sensorzellen parallel modulieren, ist es nicht möglich, einen Effekt dem GPER1-Rezeptor eindeutig und spezifisch zuzuschreiben. Um die GPER1-spezifische Signalgebung zu analysieren, ist es notwendig, die Mischung in einzelne Fraktionen aufzutrennen. Zu diesem Zweck sollten sowohl hydrophobe Trägermaterialien als auch Ionenaustauscher-basierte Festphasen-extraktionen eingesetzt werden. Polare und unpolare Fraktionen sollten dann zum Monitoren der cAMP- und Ca^{2+}-Mobilisierungseffekte unter Einsatz der neu entwickelten Sensorzelllinien analysiert werden. Fraktionen, die eine Aktivität enthalten, können dann in non-target Analysen eingesetzt werden. Die Kombination aus der Fraktionierung von Kläranlagen-Abläufen, effektbasierten in vitro Assays und non-target Analysen stellt eine leistungsfähige Methode dar, Mikroschadstoffe zu analysieren, die nicht durch etablierte Reinigungsverfahren von Kläranlagen entfernt werden können.

1 Introduction

Due to demographic changes the consumption rates of pharmaceuticals are expected to dramatically increase in the future. Beside pharmaceuticals, increasing amount of personal care products, cleaning agents, residues of pesticides as well as transformation products pose an increasing problem since a lot of this compounds are not completely removed by conventional wastewater treatment and rising concentrations of micropollutants are expected to occur in the water cycle. These micropollutants are found in trace amounts but can be persistent and harmful even at low concentrations. They pose challenges for ecotoxicological studies due to their complex mixtures and long-term exposure effects. The recent influx of environmental contaminants into wastewater treatment plants (WWTP) has reached critical levels. These substances and their transformation products, are still not fully understood in terms of their characteristics and environmental impacts.

Endocrine active substances such as estrogens, androgens, glucocorticoids, and their metabolites are introduced into aquatic environments predominantly through urban or industrial wastewater. The treatment processes like activated sludge and ozonation further transform these into various byproducts.

Detecting and identifying Endocrine Disrupting Chemicals (EDCs) in WWTP effluents plays a pivotal role in monitoring their release into the environment and ensuring their removal. Bioassays and in vitro assays are valuable tools for assessing the effects of these chemical mixtures at the cellular level. Various available bioassays measure endocrine-disruptive effects, including glucocorticoid receptor (Sonneveld *et al.*, 2005), androgen receptor (Satoh *et al.*, 2004), and estrogen receptor reporter gene assays (Rogers and Denison, 2000). In addition to these classical hormone receptors, which are found in the cytosol and act as transcription factors upon activation, there exists a non-nuclear rapid signaling response that occurs within seconds to minutes. This response is not delayed by transcription inhibition and stems from the activation of receptors primarily located in the cell membrane. These membrane-bound receptors function as G protein-coupled receptors, initiating signaling cascades that regulate the functions of existing proteins and downstream gene expression. Upon activation, these receptors trigger signaling cascades involving calcium mobilization, cAMP generation, and other second messengers (Schwartz *et al.*, 2016).

Conventional wastewater treatment often fails to completely remove endocrine active substances and micropollutants and advanced treatment methods are necessary for more effective removal of pollutants. Advanced wastewater treatment techniques like ozonation, and activated carbon absorption using powder activated carbon (PAC) or granular activated carbon (GAC) are effective in reducing micropollutants. Ozonation degrades most micropollutants through oxidation but can create harmful by-products, requiring additional filtration. GAC and PAC, with their high surface area, are efficient in adsorbing micropollutants, but their effectiveness depends on the frequency of regeneration. While activated carbon absorption excels in removing non-polar compounds, it is less effective against polar micropollutants. Therefore, assessing the life cycle impacts and optimizing these advanced treatment technologies is imperative for effective environmental contaminant management.

2 Evaluation of the current methodological basis

Estrogen receptors (ER) ERα and ERβ were discovered through their regulation of gene expression after binding to β-estradiol (E2). ER have been thought to signal through the regulation of transcription by binding to special promotor regions in the genome. This "classical" ER signaling pathway is initiated by the formation of ERα and ERβ steroid binding hetero and homodimers, which then translocate to the nucleus of the cell. In addition there also exists a rapid E2-mediated membrane-initiated signaling independent of the classic nuclear signaling pathway. Membrane-mediated E2 signaling triggers a lot of different intracellular cascade pathways leading to regulation of ion channels, protein phosphorylation and gene transcription.

Identified membrane estrogen receptors (mER) include ER-X (Toran-Allerand *et al.*, 2002) , Gq-coupled membrane ER (Gq-mER) (Qiu *et al.*, 2003) and the G protein-coupled estrogen receptor 1 (GPER1) (Revankar *et al.*, 2005). Although several signaling activities are known, the ER-X and Gq-mER genes could not yet be characterized. GPER1 is expressed ubiquitously and has become recognized as the major transmitter of estrogen induced rapid cellular effects.

Estrogen signal transduction is very complex because ERα and ERβ can be palmitoylated, resulting in the localization of the receptors at cell membranes (Li, Haynes and Bender, 2003). ERα appears in three isoforms termed ERα-66, ERα-46 and ERα-36, derived from alternative splicing (Flouriot *et al.*, 1998). ERα-36 differs from the ER-α66 by lacking C- and N-terminal transcriptional activation domains but retaining the central dimerization and DNA-binding domains, and partial ligand-binding domains. An extra, unique 27 amino acid domain replaces the last 138 amino acids of the ER-α66 (Wang and Yin, 2015). Binding to estrogen causes rapid receptor dimerization. Palmitoylation, a reversible post-translational modification important for membrane anchoring (Acconcia *et al.*, 2004), occurs only on receptor monomers. In the case of estrogen binding to ER-α66 this result in rapid dimerization of 90% of receptors limiting the size of the ER-α66 monomeric pool. Only around 5% of ER-α66 receptors is found at the membrane (Razandi *et al.*, 2004). All three isoforms contain the cysteine residue for S-palmitoylation. ERα-46 and ERα-36 isoforms have been reported at the membrane in cancer cell lines (Li, Haynes and Bender, 2003) (Wang *et al.*, 2006). The importance of the ERα-36 isoform for normal or abnormal biology is not yet clear. The low abundance of this endogenous receptor at the membrane suggests a rather limited role in oestrogen signaling from the membrane. In contrast the full length ER-α66 has been identified at the plasma membrane in multiple cell and animal models, with evidence indicating that **ER-α66 mediates most of the rapid actions of estrogen** (Levin and Hammes, 2016).

GPER1 undergoes trafficking from the plasma membrane to the endoplasmatic reticulum and its localization at the plasma membrane is controlled by cellular factors (Gaudet *et al.*, 2015). The mER are expressed in cell-type specific manners resulting in different signaling pathways (Vrtačnik *et al.*, 2014) (Heldring *et al.*, 2007). Therefore, the binding of mER agonists lead to different *in vitro* effects depending on the cell type tested. Finally, direct interactions between estrogen receptors have been described. For example GPER1 forms a complex with the splice variant ER-α36 and other protein components (Pelekanou *et al.*, 2016).

2.1 mER signaling cascades

GPER1 is part of the family of G-protein coupled receptors (GPCRs), one of the largest protein superfamilies. Like GPCRs, plasma membrane associated ERα (mERα) binds to trimeric G-proteins also. Estrogen binding to mERα is critically involved in nongenomic steroid hormone signaling. Through direct interactions with Gαi and Gβγ, mERα mediates a novel mechanism of G protein activation (Kumar et al., 2007).

Ligand binding to GPCRs lead to binding by particular heterotrimeric G-protein complexes, each consisting of Gα, Gβ, and Gγ subunits, where distinct Gα subunits specify both GPCR interactions and the transduction of particular downstream signaling events (Wettschureck and Offermanns, 2005). The Gα subunit is the primary signaling molecule but the Gβγ protein dimer is also involved in downstream signaling (Smrcka, 2008). GPCRs are involved in many diverse signaling events (Kristiansen, 2004), using a variety of pathways that include modulation of adenylyl cyclase, phospholipase C, the mitogen activated protein kinases (MAPKs), extracellular signal regulated kinase (ERK) c-Jun-NH2-terminal kinase (JNK) and p38 MAPK. The general function of the Gα-s subunit (Gs) is to activate adenylate cyclase (Tesmer *et al.*, 1997), which in turn produces cyclic-AMP (cAMP), leading to the activation of cAMP-dependent protein kinases (often referred to collectively as Protein Kinase A). The signal from the ligand-stimulated GPCR is amplified because the receptor can activate several Gαs heterotrimers before it is inactivated. The classic signaling route for Gαq (Gq) is activation of phospholipase C beta, leading to phosphoinositide hydrolysis, calcium mobilization and protein kinase C activation. This provides a path to calcium-regulated kinases and phosphatases and many other proteins. Approximately 400 GPCR genes are encoded in the human genome, and the characterization of signal transduction has been performed only at the subfamily-level signaling outcome (e.g., Ca^{2+}, cAMP, inositol phosphate, Rho activation) and not at the level of G-specific binding events (Thomsen, Frazer and Unett, 2005).

Estrogen induced mERα activation generates both, cAMP and calcium ion influx into the cytoplasm, resulting in enzyme activation and the production of secondary signaling messengers. This results from interactions between mERα and components of multiprotein signalosome complexes that vary by cell type and are currently poorly defined. In vitro experiments with recombinant ERα variants show the formation of ERα – Gαi and ERα – Gβγ complexes. The results suggest that the mechanism of estrogen induced activation of ERα and G proteins is more complex than simple regulation of the Gαi guanine nucleotide switch, thus differing from known GPCR-induced signaling (Kumar *et al.*, 2007). There is emerging evidence that a cross talk between the mERα and nuclear ERα (nERα) pool exist influencing the binding of nERα to promotor and enhancer elements in the nucleus.

GPER1 associates with Gαs protein in a tumor cell line (Thomas *et al.*, 2005). After estrogen binding and consequent Gαs activation, this results in stimulation of adenylyl cyclase activity and increased cAMP production. Both the human and fish GPER1 are directly coupled to the same G protein (Gαs) and activate the same signaling pathways (Peyton and Thomas, 2011). These comparative studies suggest the membrane ER activity of GPER1 is its fundamental, conserved primary physiological function in vertebrates. In the neuroblastoma cell line SH-SY5Y estrogen induced GPER1 activation is followed by rapid Ca^{2+} influx from intracellular stores (Ding *et al.*, 2019). Here the signaling cascade involved Gαq-mediated PLC activation resulting in the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). DAG can activate protein kinase C (PKC) to augment adenylyl cyclase activity generating cAMP. IP3 binds to the IP3 receptor localized at the membrane of the endoplasmic reticulum promoting the release of intracellular calcium ions. Upon increase in calcium concentration, calmodulin (CaM) is activated. Adenylate cyclase (AC) is responsive to calcium and calmodulin. One important physiological role for Calmodulin is the regulation of adenylylcyclases. Four of the ten known ACs are calcium sensitive, in particular type 8 (AC8) (Cooper, Mons and Karpen, 1995). Calcium ion sensitive ACs orchestrate a dynamic interplay between calcium ions and cAMP that is a crucial feature of cellular homeostasis. Defined calcium ion microdomains surrounding AC and associating with specific signaling proteins allow discrete compartmentalized calcium ion signals to arise from specific cell type dependent signalosomes (Willoughby *et al.*, 2010).

2.2 In vitro systems monitoring nongenomic fast estrogen signaling

Originally reported was the rapid cAMP increase in ovariectomized rat as response to β-estradiol (Szego and Davis, 1967). Rapid nongenomic membrane-initiated steroid signals have been characterized in a variety of cell lines. Physiological doses of estrogen immediately stimulated NO release from human endothelial cells through activation of a cell-surface estrogen receptor that was coupled to an increase in intracellular calcium (Stefano *et al.*, 2000).

2.2.1 Estrogen-mediated intracellular Ca^{2+} mobilization

The monkey kidney fibroblast-like cell line **COS7** expressing GPER1 or ERα was stimulated with β-estradiol which resulted in a mobilization of intracellular calcium ions. In contrast the inactive α-estradiol did not induce a calcium response. Estrogen-mediated mobilization of intracellular Ca^{2+} was observed at β-estradiol concentrations below 0.1 nM, with an EC_{50} value of approximately 0.5 nM (Revankar *et al.*, 2005).

The human neuroblastoma cell line **SH-SY5Y** expresses endogenously GPER1. After activation GPER1 rapidly stimulates PLC/IP3-dependent store Ca^{2+} release with subsequent PKC activation (Ding *et al.*, 2019).

ERα-positive breast cancer cells (**MCF7** cells) and ERα-negative breast cancer cells (**SKBR3** cells) both expressing endogenous GPER1 showed agonistic effects of three organophosphate flame retardants on GPER1-mediated calcium mobilization (Ji *et al.*, 2020).

Ca^{2+} mobilization is usually measured using fluorescent Ca^{2+} binding dyes like Fura-3. After incubation of the cells with a cell permeable ester this ester is cleaved in the cell and fluorescent Ca^{2+} complexes are detected by fluorescent microscopy life cell imaging.

2.2.2 Estrogen-mediated stimulation of adenylyl cyclase

GPER1 acts independently of ERα to promote estrogen-mediated stimulation of adenylyl cyclase. The human breast cancer cell line **MDA-MB-231** that express ERβ but not ERα generated cAMP only after transfection with GPER1 (Filardo *et al.*, 2002).

A375 human melanoma cells expressing GPER1 which promotes melanin production. Incubation of the cells with the GPER1 agonist G1 resulted in increasing intracellular cAMP levels. The UV-induced increase in PKA pathway activation was inhibited by the GPER1 antagonist G15. The authors conclude that GPER1 promotes melanin synthesis via the cAMP-PKA-CREB pathway (Sun *et al.*, 2017).

Stable overexpression of human and Atlantic croaker GPER1 in ERα and ERβ negative **HEK293** cells was shown to similar activate a stimulatory G protein (Gαs) resulting in increased cAMP production (Pang, Dong and Thomas, 2008).This finding that estrogen membrane signaling through GPER1 has been conserved for such a long period in two distantly-related vertebrate groups, mammals and fish, suggests that this is a fundamental function of GPER1 in vertebrates.

The breast cancer cell line **MCF7** was separated into mERα-enriched and mERα-depleted subpopulations. Estrogen stimulation resulted in a rapid and transient cAMP increase, which was significantly enhanced in mERα-enriched compared to mERα-depleted subpopulations (Zivadinovic, Gametchu and Watson, 2005). Because MCF7 cells express GPER1 also a GPER1 dependent effect could not be excluded.

The GPER1 agonist G1 or the estrogen E2 stimulated intracellular cAMP levels in GPER1-overexpressing **MCF7** cells (Ji *et al.*, 2020).

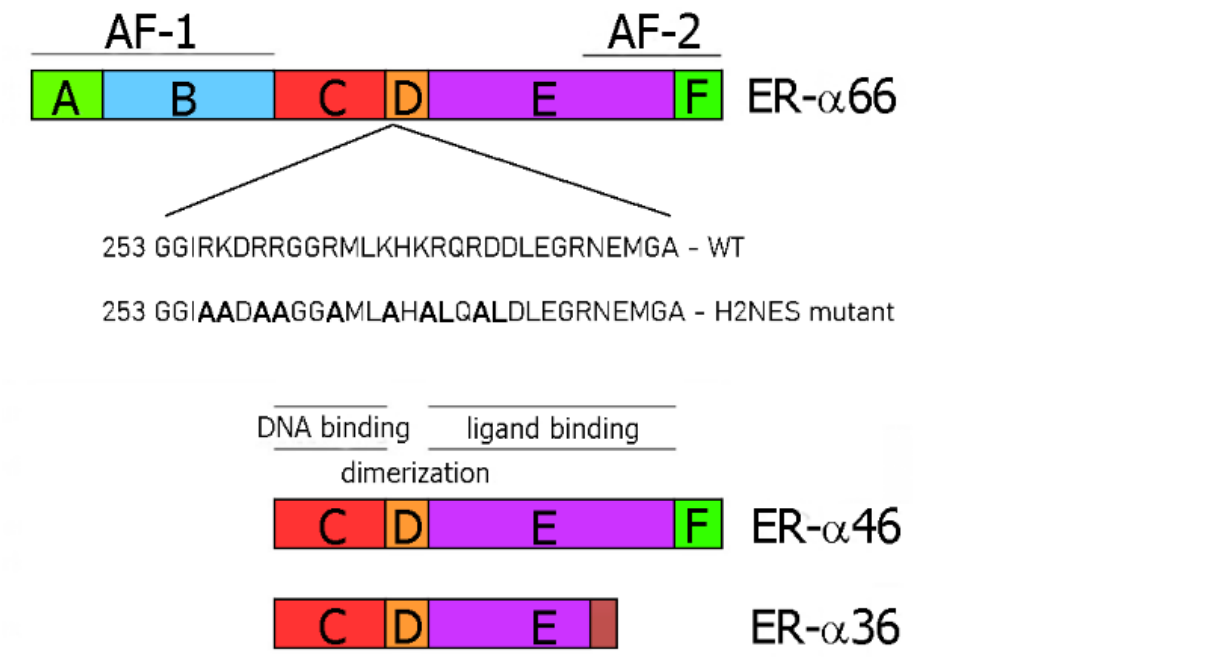
For the quantification of cAMP antibody based ELISA kits were used. cAMP is measured after cell lysis in the supernatant.

2.2.3 ERα variants

For studying the physiological signaling of a mERα directly, Pedram et al. (Pedram *et al.*, 2009) developed a mouse expressing a transgenic human ligand binding ERα E-domain (Figure 1) in an ERα knockout background. In this membrane only ERα mouse model E2 could activate extracellular-signal-regulated kinase and Phosphoinositide 3-kinase in the liver cells isolated from MOER mice, in contrast to the liver cells isolated from ERα knockout mice. This mouse model, while effective in modeling effects of ERα at the membrane, is limited by the fact that only the E-domain of the receptor is present. Other domains that may play significant roles in protein interaction are no longer present.

Another model was developed to study the effects of non-genomic, non-nuclear ERα to account for its action in both the plasma membrane and the cytoplasm. Burns et al. (Burns *et al.*, 2011) created the H2NES ERα mutant, which has point mutations in the putative nuclear localization signal and an incorporated nuclear export signal sequence in the D-domain (Figure 1) This domain is known as the hinge region because it is a flexible linker between the DNA-binding domain and the ligand binding domain, but is also involved in transcriptional regulation and contains putative nuclear localization signals. It is also the site of several post-translational modifications including methylation, phosphorylation, acetylation, ubiquitination and sumoylation. In H2NES ERα transfected HeLa cells, an early increase in phospho-p44/42 MAPK level was observed by 5 minutes, which suggested that H2NES ERα maintains rapid action responses in the cytoplasm. The possible absence of post-transcriptional modification that contributes to the normal ERα function is a possible limitation in studying the non-nuclear ERα actions of this mutant.

Figure 1: ERα variants



The D-domain or hinge region of ERα harbours the putative nuclear localization signal (K-K/R-X-K/R). To force exclusion of the ERα from the nucleus the NES ERα mutant was engineered to have a nuclear export signal (LXXXLXXLXL) in the same region as the putative nuclear localization signal.

The splice variant ER-α36 lacks intrinsic transcription activity and acts as negative regulator of ER-α66.
AF-1 and AF-2: domains mediating genomic estrogen signaling; C domain represents the DNA binding domain; D domain contains the nuclear localization signal; E domain corresponds to the ligand binding domain and the transcriptional activation function of AF-2.
Source: (Burns *et al.*, 2011) (Wang and Yin, 2015)

2.3 mER agonist and antagonist compounds

Several GPER1 and ERα agonists and antagonists have been published. Most compounds bind to ERα and GPER1 some with different activity. Until today no ERα specific antagonist is known, that does not function as a GPER1 antagonist. Raloxifene, a selective ERα modulator, also elicited cellular response via GPER1 in ERα-deficient endometrial carcinoma Hec50 cells (Petrie *et al.*, 2013). In addition, numerous synthetic estrogenic compounds have been shown to bind and/or activate GPER1, including zearalonone, nonylphenol, kepone, p, p′-DDT, o, p′-DDE, 2, 2′, 5′, -PCB-4-OH (Thomas and Dong, 2006), and bisphenol A (Dong, Terasaka and Kiyama, 2011) (Chevalier, Bouskine and Fenichel, 2012). Finally, several lines of research have demonstrated the agonistic actions of some plant-derived polyphenolic compounds toward GPER1, including genistein (Vivacqua *et al.*, 2006), quercetin (Maggiolini *et al.*, 2004), equol (Rowlands *et al.*, 2011), resveratrol (Dong *et al.*, 2013), oleuropein, hydroxytyrosol (Chimento *et al.*, 2014), and daidzein (Kajta *et al.*, 2013).

2.3.1 Simultaneous agonist and antagonist compounds

A widely used selective ERα/β antagonist, fulvestrant (ICI182,780), was shown to bind to GPER1 (Thomas *et al.*, 2005) and activate this receptor(Filardo *et al.*, 2000). Fulvestrant is used for an anti-hormone therapy for advanced breast cancer in women.

2.3.2 Agonist compounds

4-hydroxytamoxifen, the active metabolite of tamoxifen, failed to activate PI3K in ERα positive cells but did activate PI3K in GPER1 expressing cells (Prossnitz, Arterburn and Sklar, 2007).

A highly selective GPER1 agonist, G-1, was synthesized in 2006 (Bologa *et al.*, 2006) and studies of GPER1 action are greatly facilitated by this compound. G-1 showed high binding affinity for GPER1 (Kd = 10 nM) without binding to ERα/β at concentrations as high as 10μM.

Two novel GPER1 specific agonists, GPER1-L1 and GPER1-L2, were synthesized in 2012 with binding affinities of ~100 nM (Lappano *et al.*, 2012).

2.3.3 Antagonist compounds

G15 was identified as a highly selective GPER1 antagonist with a similar structure as G-1 but lacking the ethanone moiety, which displayed a minimal binding to ERα/β (Kd >10μM) (Arterburn *et al.*, 2009). Another GPER1 specific antagonist G36 was generated to restore the steric bulk of G-1 and the ER counter selectivity (Dennis *et al.*, 2011). These selective modulators of GPER1 have been used in over 200 studies to evaluate GPER1 actions in a variety of cellular and animal models.

Recently, a small molecule with high binding selectivity to ERα/β over GPER1, termed AB-1, was generated, which may further aid distinguishing the roles of ERα/β and GPER1 in E2 signaling (Revankar *et al.*, 2019). AB-1 possesses a unique functional activity profile as an agonist of transcriptional activity but an antagonist of rapid signaling through ERα. COS7 cells transiently expressing ERα-GFP, ERβ-GFP, or GPER1-GFP showed an intracellular Ca^{2+} mobilization after

activation with estrogen E2. AB-1 inhibited Ca2+ mobilization in ERα and ERβ expressing COS7 cells only.

3 Evaluation of possible test substances and applied analytical methods

3.1 Selection of model substances

The selection of compounds used as model substances for the development and validation of the novel fluorescence-based *in-vitro* bioassay was based on the review on internally available data of potentially relevant chemicals identified by the German Environment Agency. The review included various commercially used synthetic compounds mainly from three compound classes, namely industrial chemicals, pharmaceuticals, and pesticides. The review also contained information on the mode of action (ER agonists/ER antagonists) and biologically active metabolites of the selected compounds. This list of the German Environment Agency was compared with a compilation of so called "in-vitro and in-vivo reference chemicals of ERα", which was recently published by Tan *et al.*, (2020). The authors of this work used a series of computational methodologies to investigate the structural features of EDCs that bind to and activate ERα and androgen receptor (AR) based on more than 4000 compounds. Furthermore, predictions on the potency of ER agonist reference chemicals were made. Many of the potential model substances identified by the German Environment Agency were also present in the list of ERα reference chemicals of Tan *et al.*, (2020). The latter also included natural and synthetic estrogens as well as other natural substances, which are known to exhibit biological effects via the ER, such as the phytoestrogens genistein and daidzein.

In a second step, a literature search was conducted on the basis of the two aforementioned compound lists to gather more detailed information from the scientific literature on the occurrence of potential model substances in surface water bodies, including rivers and standing waters, and wastewater treatment plant effluents. The contamination of groundwater by these substances was not considered, since groundwater is generally less susceptible to pollution from municipal and industrial sources than surface water. Google Scholar was used as the search engine. Since some of the substances already had begun to attract attention as environmental pollutants several years ago, a relatively wide timespan of publication years (2000–recent) was searched for relevant monitoring studies. In Table 1 to Table 4 environmental concentration of selected natural and synthetic estrogens, industrial chemicals, pharmaceuticals, and pesticides are given.

The reviewed literature proves that estrogens are present in wastewater treatment plant effluents and surface waters on a worldwide scale. Most concentrations reported are in the ng/L-range and thus in the same order of magnitude like other organic contaminants. However, pronounced differences between particular compound classes exist. Whereas for the natural estrogens E1, E2 and E3 as well as for the synthetic contraceptive EE2 concentrations in the low ng/L-range have been detected in most cases, maximum concentration of several µg/L for some industrial compounds with estrogenic activity are not unusual. Examples for the latter are concentration of up to 98 µg/L for Bis(2-ethylhexyl) phthalate and other phthalates and 4-methylbenzylidene camphor which is used as an UV blocker in cosmetics and was detected with more than 4,7 µg/L in Australian surface waters. The same also applies to different bisphenols. Maximum concentration for bisphenol A, bisphenol F, and bisphenol S >1µg/L were reported for surface waters in Malaysia, China, Japan, Korea, or India. Although bisphenols are known to be easily biodegradable, some can be considered as pseudo-persistent compounds due their high production volumes and constant discharge into the aquatic environment.

The naturally occurring isoflavones daidzein and genistein also have been reported to act as (phyto)estrogens in mammals. As daidzein and genistein are present in soy beans and many other legumes their occurrence in natural waters can be the result of drainage water from pastures or agricultural fields (Erbs *et al.*, 2007). The contribution of urine and faeces of humans and livestock and discharges from food and wood pulp factories are also considered to be important sources (Kang and Price, 2009).

Table 1: Occurrence of selected natural and synthetic estrogens in surface waters

Analyte	Matrix	Country/ Region	LOQ in ng/L	Concentration in ng/L	Reference
estrone (E1)	sw	Brazil	0.3	<LOQ to 39 (n=5)	Sodré *et al.*, 2010
		USA	NA	<LOQ to 2.62 (n=21)	Velincu *et al.*, 2007
		China	NA	<LOQ to 78.7	Zhao *et al.*, 2011
		China	0.5	<LOQ to 52.3 (mean: 10.3; n=14)	Wang, Ndungu and Wang, 2016
		Germany	0.1	0.86 (n=5)	Zuehlke, Duennbier and Heberer, 2005
		France	NA	1.1 to 3.0	Cargouet *et al.*, 2004
β-estradiol (E2)	sw	Brazil	0.6	<LOQ to 7.3 (n=5)	Sodré *et al.*, 2010
		USA	NA	<LOQ to 5.04 (n=21)	Velincu *et al.*, 2007
		China	NA	<LOQ to 7.72	Zhao *et al.*, 2011
		China	0.2	0.1 to 10.0 (mean: 3.7; n=14)	Wang, Ndungu and Wang, 2016
		Germany	0.2	<LOQ (n=5)	Zuehlke, Duennbier and Heberer, 2005
		France	NA	1.4 to 3.2	Cargouet *et al.*, 2004
17α-ethinylestradiol (EE2)	sw	Brazil	3.1	<LOQ to 25 (n=5)	Sodré *et al.*, 2010
		USA	NA	<LOQ to 7.7 (n=21)	Velincu *et al.*, 2007
		China	NA	<LOQ to 53.5	Zhao *et al.*, 2011
		China	0.2	<LOQ to 35.3 (mean: 7.8; n=14)	Wang, Ndungu and Wang, 2016
		Germany	0.2	<LOQ (n=5)	Zuehlke, Duennbier and Heberer, 2005
		France	NA	1.1 to 2.9	Cargouet *et al.*, 2004
estriol (E3)	sw	Brazil	0.6	<LOQ to 2.3 (n=5)	Sodré *et al.*, 2010
		USA	NA	<LOQ to 19.7 (n=21)	Velincu *et al.*, 2007
		China	0.3	<LOQ to 81.6 (mean: 17.2; n=14)	Wang, Ndungu and Wang, 2016
		France	NA	1.0 to 2.5	Cargouet *et al.*, 2004
equilin	sw	USA	NA	<LOQ to 12.98 (n=21)	Velincu *et al.*, 2007
17α-dihydroequilin	sw	USA	NA	<LOQ to 2.51 (n=21)	Velincu *et al.*, 2007
progesterone	sw	USA	NA	<LOQ to 11.81 (n=21)	Velincu *et al.*, 2007

sw: surface water; NA: not available; LOQ: limit of quantification.

Table 2: Occurrence of selected industrial chemicals and natural substances in surface and wastewaters

Analyte	Matrix	Country/ Region	LOQ in ng/L	Concentration in ng/L	Reference
4-n-nonylphenol	sw	Brazil	0.1	<LOQ (n=5)	Sodré *et al.*, 2010
4-n-octylphenol	sw	Brazil	0.1	<LOQ (n=5)	Sodré *et al.*, 2010
4-nonylphenol		China	0.73	106 to 553 (n=14)	Jin *et al.*, 2004

	sw		NA	64.8 to 20080	Zhao *et al.*, 2011
			1.7	<LOQ to 33.3 (mean: 10.8; n=14)	Wang, Ndungu and Wang, 2016
4-octylphenol	sw	China	0.3	<LOQ to 120 (mean: 32.3; n=14)	Wang, Ndungu and Wang, 2016
4-t-octylphenol	sw	China	0.17	18 to 31.9 (n=14)	Jin *et al.*, 2004
		China	NA	<LOQ to 3150	Zhao *et al.*, 2011
genistein	sw	USA	2.2	<LOQ to 8 (n=75)	Kolpin *et al.*, 2010
daidzein	sw	Australia	3	2 to 33	Kang and Price 2009
	rs	Australia	3	341 to 1688	Kang and Price 2009
	sw	USA	1.8	<LOQ to 41 (n=75)	Kolpin *et al.*, 2010
butylbenzyl phthalate (BBP)	sw	Germany	20	<LOQ to 2950 (n=116)	Fromme *et al.*, 2002
dibutyl phthalate (DBP)	sw	Germany	20	120 to 8800 (n=116)	Fromme *et al.*, 2002
di(2-ethylhexyl)-phthalate (DEHP)	sw	Germany	30	330 to 97800 (n=116)	Fromme *et al.*, 2002
benzophenone-3 (BP-3; oxybenzone)	sw	Czech Republic	3.9	12 to 67 (n=8; rivers) 21 to 550 (n=6; recreational ponds)	Grabicova *et al.*, 2013
		Spain	7	<LOQ to 27 (n=3)	Rodil *et al.*, 2008
		China	4	12.3 to 15.4 (n=2)	Wu, Chen and Ding, 2013
		Germany	5	<LOQ to 47 (n=3)	Wick, Fink and Ternes, 2010
4-methylbenzyliden-camphor (enzacamen)	sw	Switzerland	2	<LOQ to 28 (n=11)	Balmer *et al.*, 2005
	tw	Switzerland	2	60 to 2300 (n=13)	Balmer *et al.*, 2005
	sw	Australia	NA	>4700	O'Malley *et al.*, 2021
bisphenol A	sw	Europe	varying	81 (median; n=848) 470 (95th percentile; n=848)	Klecka *et al.*, 2009
		North America	varying	10 (median; n=1068) 350 (95th percentile; n=1068)	Klecka *et al.*, 2009
		Malaysia	1.3 ng/L	<LOQ to 1278	Santhi *et al.*, 2012
		Japan	varying	3.1 to 120 (n=8)	Yamazaki *et al.*, 2015
		Korea	varying	1 to 213 (n=10)	Yamazaki *et al.*, 2015
		China	5	<LOQ to 98 (n=6)	Yamazaki *et al.*, 2015
		India	varying	<5 to 1950 (n=14)	Yamazaki *et al.*, 2015
		Brazil	1.2	25 to 84 (n=5)	Sodré *et al.*, 2010
		Germany	0.1	0.5 to 410 (n=116)	Fromme *et al.*, 2002
		China	0.17	19.1 to 8300 (n=14)	Jin *et al.*, 2004
		China	NA	0.23 to 324	Zhao *et al.*, 2011
		China	0.2	<LOQ to 50.1 (mean: 26.6; n=14)	Wang, Ndungu and Wang, 2016
bisphenol F	sw	Japan	varying	76 to 2850 (n=8)	Yamazaki *et al.*, 2015
		Korea	5	<LOQ to 1300 (n=10)	Yamazaki *et al.*, 2015
		China	5	<LOQ to 1110 (n=6)	Yamazaki *et al.*, 2015
		India	varying	<5 to 289 (n=14)	Yamazaki *et al.*, 2015
		Germany	0.1	<LOQ to 180 (n=116)	Fromme *et al.*, 2002
bisphenol S	sw	Japan	varying	1.5 to 8.7 (n=8)	Yamazaki *et al.*, 2015
		Korea	5	<LOQ to 42 (n=10)	Yamazaki *et al.*, 2015
		China	5	<LOQ to 135 (n=6)	Yamazaki *et al.*, 2015
		India	varying	<5 to 7200 (n=14)	Yamazaki *et al.*, 2015

sw: surface water;rs: raw sewage; tw: treated wastewater; NA: not available; LOQ: limit of quantification.

Table 3: Occurrence of selected pharmaceuticals in surface and wastewaters

Analyte	Matrix	Country/ Region	LOQ in ng/L	Concentration in ng/L	Reference
clomifene	sw	China	0.5	<LOQ to 0.18 (n=3)	Liu *et al.*, 2010
	hwwe	China	0.5	<LOQ (n=63)	Liu *et al.*, 2010
furosemide	sw	Spain	NA	1.9 to 139 (n=11)	da Silva *et al.*, 2011
		Italy	NA	up to 605	Meffe and de Bustamante, 2014
bezafibrate	sw	Canada		8	Garcia-Ac *et al.*, 2009
diethylstilbestrol	sw	China	NA	<LOQ to 1.66	Zhao *et al.*, 2011
tamoxifen	hwwe/tw	Switzerland	4	1 to <4	Tauxe-Wuersch *et al.*, 2006
	sw	Japan	NA	<LOQ to 533 (mean: 143)	Azuma *et al.*, 2016
4-hydroxytamoxifen (tamoxifen-TP)	sw	Japan	NA	<LOQ	Azuma *et al.*, 2016
endoxifen (N-desmethyl-4-hydroxytamoxifen) (tamoxifen-TP)	no studies available				
raloxifen	no studies available				
bazedoxifen	no studies available				
lasofoxifen	no studies available				
toremifen	no studies available				
chlorotrianisen	no studies available				
ormeloxifen	no studies available				
ethamoxytripetol	no studies available				
ospemifene	no studies available				
enclomiphene	no studies available				
fulvestrant	no studies available				

sw: surface water; hwwe: hospital wastewater effluent; tw: treated wastewater; NA: not available; LOQ: limit of quantification.

Table 4: Occurrence of selected pesticides in surface waters

Analyte	Matrix	Country/ Region	LOQ	Concentration in ng/L	Reference
atrazine	sw	Poland	350	<LOQ	Barchanska *et al.*, 2017
		Canada	100	<LOQ to 3910 (median: 120; n=739)	Byer *et al.*, 2011
		USA	7	up to 7300 (n=75)	Kolpin *et al.*, 2010
2-chloro-4-ethylamino-6-amino-1,3,5-triazine (atrazine-TP)	sw	Poland	40	5000 to 18000	Barchanska *et al.*, 2017

2-hydroxy-4-ethylamino6-isopropylamino-1,3,5- triazine (atrazine-TP)	sw	Poland	140	<LOQ to 35000	Barchanska *et al.*, 2017
total DDT (o,p-DDT; p,p-DDT; o,p-DDD; p,p-DDD; o,p-DDE; p,p-DDE)	sw	Czech Republic	NA	0.8 to 9.5	Kohušová *et al.*, 2011
total DDT (o,p-DDT; p,p-DDT; p,p-DDD p,p-DDE)	sw	China	NA	<LOQ to 176 (n=14)	Chen *et al.*, 2011

sw: surface water; NA: not available; LOQ: limit of quantification.

3.2 Analytical methods for the analysis of prioritized estrogens

The analytical methods applied for the analysis of estrogens use liquid or gas chromatographic separation techniques coupled to (tandem)mass spectrometry. The sensitivity of state-of-the-art mass spectrometers was considerably improved during the last decade and many analytical methods for micropollutants have been published that abstain from time and labour intensive sample enrichment with solid phase extraction. However, for the analysis of micropollutants with estrogenic activity, SPE of rather large sample volumes (often 1 L for surface water) is still common practice to achieve the lowest possible limit of detection (LOD).

There are two main drivers to push the LODs towards the pg/L-range. One is the low biological activity threshold for some estrogens and the second is that the European Commission included some representatives in the surface water Watch Lists issued in 2015 and 2018 under the Water Framework Directive regarding emerging aquatic pollutants and proposed maximum LOD of 0.035 ng/L for EE2 and 0.4 ng/L for E1 and E2.

Even with state-of-the-art analytical instrumentation these LODs still represents a challenge for laboratories and most reported LOD in scientific literature are in the low ng/L-range and depend on the sample volume used for SPE and the water matrix.

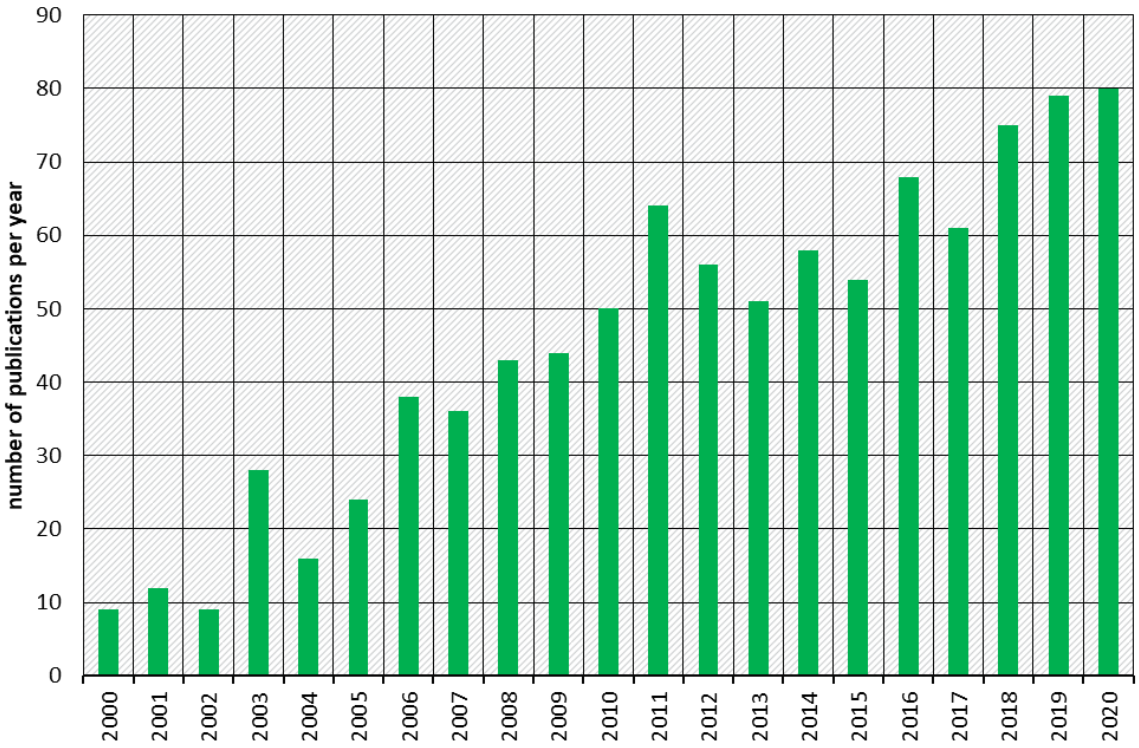
3.2.1 Estradiol

3.2.1.1 General information

β-estradiol (E2) belongs to the group of steroid hormones which have a high endocrine disrupting effect even at very low concentrations. Next to estrone (E1) and estriol (E3), *β*-estradiol is the main natural occurring human (predominantly female) sex hormone which is responsible for the development and function of the reproductive system. It is produced in the ovaries and mainly excreted via urine (Nazari and Suja, 2016). Robles *et al.* (2017) reported daily excretion rates of males and females at single-digit µg-levels. The E2 excretion rates of pregnant women are even greater and are estimated to be as high as 260 µg/day (Johnson, Belfroid and Di Corcia, 2000). Conventional waste water treatment plants (WWTP) are not able to completely remove estrogens from waste waters which ultimately leads to release of estrogens to the environment via WWTP effluent. Additional treatment steps such as membrane filtration can substantially improve the removal of estrogens (Nazari and Suja, 2016; Silva, Otero and Esteves, 2012). The number of studies on E2 regarding its environmental occurrence has grown continuously over the last years. A search on Web of Science limited to the years 2000 until today comprising the terms “estradiol” AND “environment” AND “water” resulted in the results shown in Figure 2.

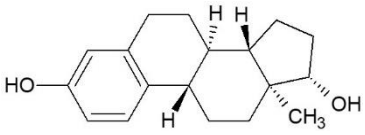
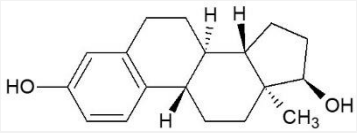
Table 5 lists the structures and physicochemical properties of ß-estradiol and its stereoisomer *α*-estradiol. *α*-estradiol is also a natural occurring hormone, however, with lower reactivity compared to *β*-estradiol (Lewis, 2007).

Figure 2: Number of estradiol publications per year between 2000 and 2020. Search conditions see text.



Source: Web of science

Table 5: Structures and physicochemical properties of β-estradiol and its stereoisomer α-estradiol

Compound name	Structure	CAS No	Formular	Molecular weight	Log*D* (at pH 8)	Log K_{OW}
***β*-estradiol**		50-28-2	$C_{18}H_{24}O_2$	272.4	4.13 [a]	4.01[b]
***α*-estradiol**		57-91-0	$C_{18}H_{24}O_2$	272.4	4.0[b]	4.01[c]

[a](Alturki *et al.*, 2010), [b]PubChem (https://pubchem.ncbi.nlm.nih.gov/,) last access: 18.03.2021, [c](Chimchirian, Suri and Fu, 2007)

3.2.1.2 Analytical methods

In the literature, a large number of various analytical methods for the determination of natural β-estradiol in different types of matrices have already been described. Since β-estradiol normally occurs at trace level concentrations in often complex matrices, many of the analytical methods included sample preparation steps like clean-up or enrichment procedures. However, there are two main methodologies for β-estradiol determination in aqueous matrices which represent the most widely used techniques: gas chromatography coupled with (tandem) mass spectrometry (GC-MS/MS)) and liquid chromatography coupled with tandem mass chromatography (LC-MS/MS). Other techniques, including bio/immunoassays, are not further discussed in this section. Biological samples like animal and human tissues, blood, serum and milk are mainly analyzed in order to understand the effects of estrogens on human health, the interactions of oral contraceptives in combination with other drugs, and the effects of β-estradiol exposure on animals and plants. Waste water treatment sludge, sediments, and different types of water (waste water, surface water, tap water) are evaluated in order to assess the environmental fate of estrogens (Barreiros *et al.*, 2016). However, this study aims to assess estrogenic activity in aqueous samples, especially in selected surface waters. Therefore, sample preparation of biological matrices and extraction methods for other matrices like soils and sludge are not further discussed. The main focus of the herein reviewed literature was laid on the years 2010 until 2020.

LC-MS/MS

Quantitation of β-estradiol in water matrices is challenging since concentrations are generally low and the molecule is not very sensitive in negative ionization mode (Honda, Becerra-Herrera and Richter, 2018). For this reason, direct injection methods are rarely described. Few exceptions can be found in technical application notes (Ferrer, Thurman and Zweigenbaum, 2018); (Baker and Loftus, 2015). In order to enhance sensitivity, 1 mM, respectively 0.15 mM, ammonium fluoride was added to the aqueous mobile phase. Moreover, large injection volumes are used (100 µL or even1200 µL) in order to obtain the low LOQs which are required to meet the demands of the EU directive 2008/105/EC and the associated watch list, which specify the minimum allowable LOQs for E2 among others (EC, 2018). Baker and Loftus (2015) achieved a LOQ of 0.052 ng/L for E2, whereas Ferrer, Thurman and Zweigenbaum (2018) reported a LOD of 5 ng/L. The majority of recently published analytical methods applied a pre-concentration step prior to LC-MS/MS detection. A traditional technique is solid phase extraction (SPE) with different sorbent materials (mostly reversed phase) and eluents (Borrull *et al.*, 2020; Aborkhees, Raina-Fulton and Thirunavokkarasu, 2020; Gonzalez and Cerda, 2020) which provide LOQs in the lower ng/L-range. The popular and successful use of SPE enrichment is also described by (Barreiros *et al.*, 2016) who reviewed analytical methods for the detection of E2 in studies from 2009 to 2014. If the samples contained high loads of particles, filtration with different types of filter materials (glass fiber, regenerated cellulose, PTFE) was applied prior to SPE (Barreiros *et al.*, 2016); Aborkhees, Raina-Fulton and Thirunavokkarasu, 2020; Borrull *et al.*, 2020; Bonfoh *et al.*, 2020). Sweeney *et al.* (2021) replaced the filtration and SPE pre-treatment steps with a – according to the authors - more robust QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method which was originally designed for pesticide extraction from solid matrices by Anastassiades *et al.* (2003). Even though the QuEChERS method was not originally intended to primarily serve as a method for sample enrichment, Sweeney *et al.* (2021) successfully used it for this purpose among others, obtaining a concentration factor of 500 for E2. Thus, the authors achieved a LOQ of 20 ng/L for E2 (matrix: influent of an eel aquaculture system). QuEChERS is basically a liquid-liquid extraction procedure where the addition of salts (MgSO4, NaCl) prevents the miscibility of water and acetonitrile. The target analytes are enriched in the organic phase

which is afterwards cleaned up with dispersive SPE. A similar approach was described by Hassannejad, Alizadeh and Nemati (2019). Other studies included special extraction materials like PEP-PAN@PSF rods (Bonfoh *et al.*, 2020) or derivatization steps prior to LC-MS/MS analysis (van der Berg *et al.*, 2020; Bonfoh *et al.*, 2020; Szterk, Sitkowski and Bocian, 2019). Due to the rather nonpolar character of E2, LC separation is mostly conducted using common C18 columns (Barreiros *et al.*, 2016). The addition of ammonium fluoride to the aqueous phase (up to 1 mM) (Petrie *et al.*, 2016; Fiers *et al.*, 2012) or the derivatization with dansyl chloride are commonly used steps to enhance the signal intensity of E2 in the mass spectrometer. The derivatization leads to the incorporation of a basic secondary nitrogen into the molecule which can be easily ionised in positive ionisation mode, avoiding the less sensitive negative mode (Anari *et al.*, 2002). In order to achieve higher reproducibility, especially for methods which require various sample preparation steps or in samples with complex matrices, isotopically labelled variants of E2 are used as internal standards for quantification (Snow *et al.*, 2013).

GC-MS/MS

While LC-MS/MS often is more prone to matrix effects, but offers lower detection limits and can be applied without derivatization steps, GC-MS, respectively GC-MS/MS is more cost-effective and offers increased chromatographic resolution and no false-positive results (Grover *et al.*, 2009). However, GC-MS necessarily requires derivatization steps for non-volatile compounds like estrogens in order to transfer them from the aqueous to the gaseous phase. Sample enrichment is also required due to lower sensitivity (Honda, Becerra-Herrera and Richter, 2018). GC-MS is the more traditional method for the determination of estrogens and is still successfully applied in recent studies. Various extraction methods exist as pre-concentration steps for aqueous samples prior to or in combination with different derivatization steps. Sample pre-treatment techniques for *β*-E2 analysis in aqueous samples include solid phase (micro) extraction with different sorbent materials (Grover *et al.*, 2009); (Azzouz, Souhail and Ballesteros, 2010); (Vallejo-Rodriguez *et al.*, 2018), (dispersive) liquid-liquid extraction (Gonzalez, Avivar and Cerda, 2015); (Chormey *et al.*, 2017); (Davoodi *et al.*, 2020) and variations thereof like switchable solvent liquid-phase microextraction (Chormey *et al.*, 2018), thin film microextraction (Giordano *et al.*, 2016) and stir bar sorptive extraction (Iparraguirre *et al.*, 2011). Derivatization of estrogens for subsequent analysis by GC-MS was often achieved by substitution of hydrogen atoms at polar functional groups (e.g. hydroxyl groups). Until today, the most common technique is silylation with various derivatization agents like N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), BSTFA with trimethylchlorosilane or N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Gonzalez, Avivar and Cerda, 2015); (Davoodi *et al.*, 2020); (Huang *et al.*, 2010). Fang *et al.* (2010) proposed a simultaneous derivatization of hydroxyl and ketone groups with MSTFA/trimethyliodosilane/Dithioerythreitol (1:2:5, v/v/w) at room temperature for 5 min. With appropriate extraction and derivatization, LODs in the double-digit pg/L-range can be achieved with GC/MS (Davoodi *et al.*, 2020) for E2 even in complex waste water matrices.

In some studies, the chromatographic separation of *β*-estradiol and *α*-estradiol was discussed. As mentioned above, *α*-estradiol is a natural occurring isomer of *β*-estradiol, which, however, is not nearly as reactive as ß-estradiol (Lewis, 2007). The separation of the two isomers can easily be achieved with silylation followed by GC-MS detection, while some published LC-MS/MS applications did not provide proper chromatographic resolution of both compounds (Szarka *et al.*, 2013). If particularly *β*-estradiol is the compound of interest, the separation of both isomers should be addressed during method development.

3.2.1.3 Occurrence

The occurrence of β-estradiol has already been reported for various water compartments. In Table 6 to Table 8 concentrations in surface water, tap water and drinking water as well as in wastewaters are listed exemplarily. Limits of quantitation (LOQs) differed substantially between the studies, which mostly likely affected the reported number of positive detections in studied samples. This becomes apparent in Table 6 where higher LOQs are often related to higher numbers of non-detects. In surface water, β-estradiol concentrations reported by Coelho *et al.* (2020) are by far the highest ones with values up to 1.7 µg/L. According to the authors, this was potentially related to the high degree of urbanization and insufficient waste water treatment in the studied region. Other studies reported estradiol concentrations in the single-digit ng/L-range or below LOQ. The listed values in all tables relate to β-estradiol concentrations. However, the authors of many studies did not provide information if β-estradiol and its natural occurring isomer α-estradiol were identified and quantified separately from each other. Reoyo-Prats *et al.* (2018) differentiated between the isomers but both were detected in their study (LOQ: 5 ng/L). However, the contribution of α-estradiol to the total ß-estradiol concentration might be negligible as the results of Goeury *et al.* (2019) indicate. The authors analysed influents and effluents of WWTPs in the Greater Montreal area in Canada. While β-estradiol was detected in influents and effluents with concentrations up to 0.12 µg/L, α-estradiol was never detected.

Tap water concentrations of β-estradiol are listed in Table 7. Vallejo-Rodriguez *et al.* (2018), who reported β-estradiol concentrations below LOQ in the analysed tap waters, also investigated six bottled drinking waters from Mexico and reported a detection frequency of 100 % for β-estradiol with concentrations up to 0.012 µg/L. The authors suggested different water sources as a possible cause for the discrepancies. High β-estradiol concentrations were found in raw sewage (Table 8). In general, β-estradiol concentrations were lower in WWTP effluent than in influent, indicating at least partial removal during water treatment. Goeury *et al.* (2019) reported significantly higher β-estradiol concentrations in the effluents than in the corresponding influents, without providing potential explanations for this observation. Sorption and biodegradation are the main mechanisms in conventional wastewater treatment which are mainly responsible for the removal of β-estradiol and other steroid hormones from raw wastewater (Ting and Praveena, 2017). However, compared to E1, E3 and EE2, β-estradiol has a relatively low sorption affinity. Biodegradation is very efficient for β-estradiol removal, which is mainly transformed to E1 which is further degraded afterwards. Even if the described techniques are able to remove β-estradiol, most of the studies reviewed by Ting and Praveena (2017) report removal efficiencies from 50 % to 90 %. Better removals (over 90 %) can be achieved with tertiary treatment options like UV, chlorination or membrane filtration and combinations thereof.

Table 6: Occurrence of β-estradiol in surface waters

Country	Location	ß-estradiol in ng/L[a]	LOQ in ng/L	Reference
China	Lake Taihu	<LOQ	5.5	Bao et al. 2020
China	East Lake North Lake Han River	<LOQ	12	Bonfoh et al. 2020
Brazil	Rio Grande	<LOQ-1700	100	Coelho et al. 2020
South Africa	Bloukrans River	<LOD-140	0.30 (LOD)	Farounbi and Ngqwala 2020

Country	Location	ß-estradiol in ng/L[a]	LOQ in ng/L	Reference
	Tyhume River Buffalo River Swartikops River			
China	Chaohu Lake	<LOQ-3.5	0.080	Zhang et al. 2020
USA	Shenandoah River	<LOQ-2.4	0.050	Barber et al. 2019
Canada	Chateauguay River Richelieu River Des Prairies River Mille Iles River	<LOQ	2.4	Goeury et al. 2019; Rocha et al. 2019
Portugal	River estuaries	0.20-1.1	0.05	Rocha et al. 2019
Italy	Lamone River Montone River Marecchia River Ronco River Conca River	<LOQ-10	1.5	Pignotti 2017
Romania	Danube River and tributaries	<LOQ	1.6	Galaon et al. 2016

[a] reported minimum and maximum values, LOD: limit of detection; LOQ: limit of quantitation.

Table 7: Occurrence of β-estradiol in tap water

Location	ß-estradiol in ng/L [a]	LOQ in ng/L	Reference
Canada (Quebec)	<LOQ-2.8	1.8	Goeury et al. 2019
Malaysia	<LOQ-6.3	0.58	Wee et al. 2021
Brazil	<LOQ	1.0	Maynard et al. 2019
Mexico (Guadalajara)	<LOQ	3.0	Vallejo-Rodriguez et al. 2018
Portugal (Lisbon)	<LOQ	21	Carvalho et al. 2015
Italy	<LOQ	900	Magi et al. 2010

[a] reported minimum and maximum values, LOQ: limit of quantitation.

Table 8: Occurrence of β-estradiol in waste waters

Location	ß-estradiol in ng/L (influent)[a]	ß-estradiol in ng/L (effluent) [a]	LOQ in ng/L (influent/effluent)	Reference
Greater Montreal area (Canada)	<LOQ -15	31-120	11 / 3	Goeury et al. 2019
Johannesburg (South Africa)	<LOQ	<LOQ	0.32	Mnguni et al. 2018
San Marcos (Texas, USA)	3000[b]	890	61	Oates et al. 2017
Curitiba City, Paraná (Brazil)	6.0	<LOQ	5.0	Liz et al. 2017
Braunschweig (Germany)	36 (ß-estradiol+E1)	1.4	0.50	Ternes et al. 2007
Lisbon (Portugal)	<LOQ	<LOQ	9.9	Carvalho et al. 2016

[a] reported minimum and maximum values, [b] mean value, LOQ: limit of quantitation.

3.2.2 Nonylphenols

3.2.2.1 General information

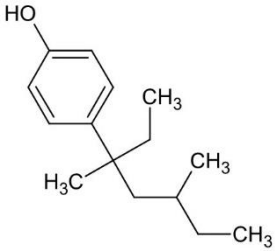
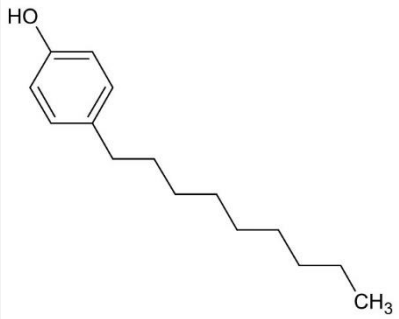
Nonylphenols (NP; molecular formula: C15H24O) refer to a group of isomeric compounds consisting of an alkyl chain with nine carbon atoms attached to a phenol ring. NP are predominantly used in the production of nonylphenol ethoxylates (NPEO), which find broad application as non-ionic surfactants (Eganhouse *et al.*, 2009). NPEO are incompletely degraded to NP during wastewater treatment, which leads to the release of NP into receiving waterbodies (Freeling *et al.*, 2019).

Linear and branched NP have been included to the list of priority hazardous substances under the Water Framework Directive (EC, 2013). NP and NPEO were also added to the SIN-List (ChemSec, 2021), meeting the criteria of substances of very high concern (SVHC) under Article 57 of REACH. On the basis of available ecotoxicological data, the European Commission (EC) has heavily restricted the marketing and the use of NP and NPEO in the European Union (EC, 2003). According to annex XVII to REACH, NP and NPEO shall not be placed on the market, or used, as substances or in mixtures in concentrations equal to or greater than 0,1% by weight for a multitude of purposes, including many industrial and institutional cleaning applications, the manufacturing of pulp and paper or in cosmetic products (ECHA, 2021).

However, NPEO are still utilized in various recently developed countries in a wide variety of industrial applications, for example as cleaning, dyeing and rinsing agents in the textile industry (Ho and Watanabe, 2017). In fact, residues of NP and NPEO in textiles imported from countries outside the EU are believed to be the major source of NP/NPEO to municipal WWTP and therefore the main source of these substances to the environment in Europe (Lassen *et al.*, 2013). As a consequence, according to annex XVII Entry 46a to REACH, textile products with NPEO levels ≥0.01 % by weight shall not be placed on the market after February 3, 2021, if the textile article or each part thereof can be expected to be laundered during the normal life cycle (ECHA, 2021).

The technical synthesis of NP leads to a complex mixture of isomeric compounds that differ in the position of the nonyl moiety attached to the phenol as well as in the branching of the nonyl group, the latter resulting in different lengths of the alkyl side chain. Eganhouse et al. (2009) analyzed the composition of seven technical NP mixtures (CAS No.: 84852-15-3; Table 9) and found that commercial products contain about 85.6–93.7% of 4-NP (synonymously ortho- or o-NP), 2.5–8.9% of 2-NP (synonymously para- or p-NP), 2.2–4.6% of decylphenol (DP), 0.01–2.2% of octylphenol (OP), and 0.01–0.3% of C3–C7 phenols. The most prominent constituent, 4-NP, has 211 constitutional isomers, and when considering stereoisomerism, the theoretical number increases to 550 isomers (Guenther, Kleist and Thiele, 2006). The isomer with a linear alkyl chain, 4-n-NP (CAS No.: 104-40-5; Table 9), is not present in technical NP products (Loos *et al.*, 2007). Consequently, 4-n-NP has already been used in some studies as an internal standard for the quantification of branched 4-NP in environmental samples (Li et al., 2010; Ma and Cheng, 2010). Note that NP technical mixtures are also often referred to as 4-NP technical mixtures, as 4-NP isomers are their main component.

Table 9: Chemical abstract service (CAS) number, molecular formula, molecular weight, and chemical structure of 4-nonylphenol (4-NP) technical mixture and 4-n-nonylphenol (4-n-NP)

Analyte	Structure	CAS No	Formular	Molecular weight
4-Nonylphenol mixture of branched isomers	 4-(3,5dimethylheptan-3-yl)phenol (353-NP;CAS No.: 186825-36-5) is depicted as an example of a branched isomer of 4-NP	84852-15-3	$C_{15}H_{24}O$	220.35
4-*n*-Nonylphenol		104-40-5	$C_{15}H_{24}O$	220.35

3.2.2.2 Analysis

Analysis of individual 4-NP isomers

The analysis of individual isomers of 4-NP is demanding due to the high analytical effort that is required for their separation and identification. Due to the relatively small differences in their physicochemical properties, chromatographic separation of complex mixtures of 4-NP isomers is not feasible using standard GC and HPLC practices. Even efforts to characterize the composition of technical NP mixes using high-resolution capillary GC columns were only able to resolve

roughly 20 major 4-NP isomers (Wheeler *et al.*, 1997; Thiele *et al.*, 2004). Moreover, as there are no isomer-specific mass transitions in MS/MS detection, co-eluting compounds are indistinguishable from each other. Acir, Wüst and Guenther (2016) investigated the separation of eight synthesized 4-NP isomers by five different chiral cyclodextrin GC columns with subsequent flame ionization detection (FID) and using various derivatization approaches (underivatized, methylation, silylation, and acylation). The authors found three of the columns to be suitable for an enantioselective analysis of some chiral NP. The best separation was achieved on a Di-But-γ column, which allowed for the separation of NP10, NP35, NP36, NP112, NP143, NP170, and NP194.

To increase chromatographic separation, two dimensional GC (GC × GC) coupled to MS and time-of-flight MS (TOF-MS) methods have been developed, which allowed for the identification of a much higher number of components of technical NP mixtures (Eganhouse *et al.*, 2009; Ieda *et al.*, 2005; Moeder *et al.*, 2006). In fact, using a non-polar primary column (DB-5) and a polar secondary column (Supelcowax 10), Eganhouse *et al.* (2009) were able to separate and detect 153–204 alkylphenol peaks, 59–66 peaks of which were identified to be 4-NP isomers.

Analysis of total 4-NP

The high analytical effort as well as the limited availability of analytical standards have hindered the analysis and potentially also the regulation of individual 4-NP isomers (Lu and Gan, 2014). As a result, environmental studies often treated NP as a single compound. In many cases, this was achieved by applying HPLC-MS/MS with reversed-phased (RP) columns and using technical 4-NP mixtures as analytical standards (Lee *et al.*, 2013; Loos *et al.*, 2007; Fan *et al.*, 2013; Chen *et al.*, 2013; Padhye *et al.*, 2014).

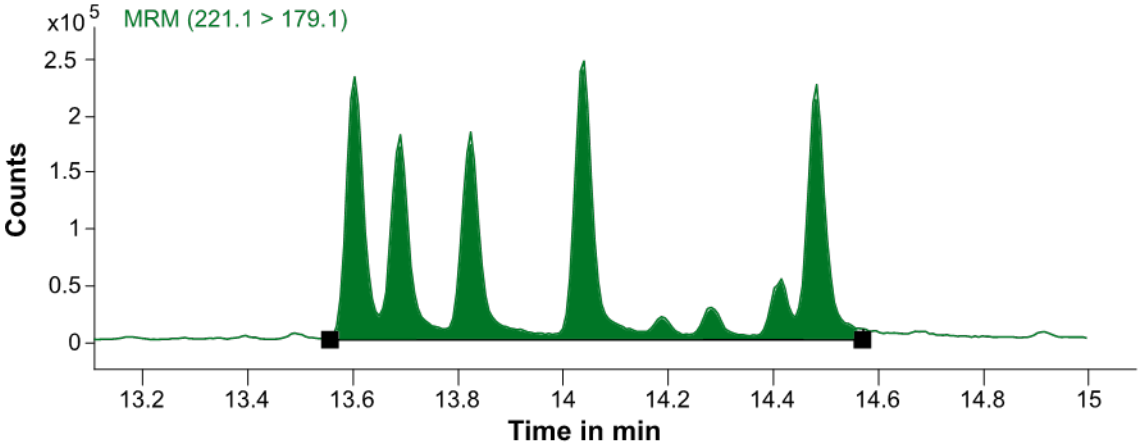
Due to the relatively low chromatographic resolution of these RP-HPLC-methods, 4-NP isomers elute as a single peak, which facilitates peak integration. Thus, only a single concentration value for the sum of all isomers of 4-NP is reported. When HPLC-MS/MS was used for 4-NP analysis of water matrices, SPE was the method of choice for sample purification and pre-concentration. Ultra-sonic extraction and subsequent SPE-HPLC-MS/MS analysis has also been used for the quantification of alcohol ethoxylates and NPEO metabolites, including NP, in sediment samples (Lara-Martín, González-Mazo and Brownawell, 2012). A major advantage of the 4-NP-analysis via HPLC-MS/MS is that a derivatization step is not required.

GC-MS is another technique that is commonly applied for the analysis of total 4-NP in water samples. SPE (Li *et al.*, 2010; Wang *et al.*, 2012), solid phase microextraction (SPME) (Vargas-Berrones *et al.*, 2020; Gong *et al.*, 2015) and liquid-liquid-extraction (LLE) (Ferrey *et al.*, 2015; Jin *et al.*, 2004) have already been used for sample pre-treatment before GC-MS analysis. (Wang *et al.*, 2012) applied ultra-sonic extraction and subsequent GC-MS analysis to quantify 4-NP in sediment samples. Most studies made use of a derivatization step (Li *et al.*, 2010; Ferrey *et al.*, 2015), although derivatization is not a prerequisite for the GC analysis of 4-NP (Gong *et al.*, 2015).

Unfortunately, the chromatographic performance (retention times, peak shapes, etc.) of the applied GC-MS-methods was generally not discussed in the aforementioned studies. It is likely that 4-NP eluted as multiple peaks (each peak representing single or multiple isomers of 4-NP) and that the areas of these peaks were added up for quantification. This procedure is described in ISO 18857-2:2009 (German version: DIN EN ISO 18857-2:2011), which specifies a method for the determination of selected alkylphenols, their ethoxylates and bisphenol A in non-filtered samples of drinking, ground, surface, and waste waters using SPE, derivatization and subsequent analysis by GC-MS. In the example chromatograms provided by DIN EN ISO 18857-2:2011, 4-NP elutes as eight to ten main peaks, which are added up for quantification.

Figure 3 shows the GC-MS/MS multiple reaction monitoring (MRM) chromatogram of a 4-NP analytical standard (CAS No.: 84852-15-3) after derivatization with N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA). Similar to the chromatograms described in DIN EN ISO 18857-2:2011, multiple peaks for 4-NP are discernible.

Figure 3: GC-MS/MS multiple reaction monitoring (MRM) chromatogram of a 4-NP analytical standard (CAS No.: 84852-15-3) after derivatization with MSTFA. For quantification, the area from the first (retention time: approx. 13.6 min) to the last major peak (retention time: approx. 14.5 min) is integrated



Source: own research, TZW Karlsruhe

Modern HPLC-MS/MS and GC-MS(/MS) apparatus, when used in combination with common sample pre-treatment techniques (typical enrichment factors: 102-10^3), usually allow for the quantification of total 4-NP in environmental waters at single- or double-digit ng/L-levels. For example, using their online-SPE-HLPC-MS/MS method with an initial sample volume of 10 mL, Rubirola, Boleda and Galceran (2017) achieved LOQs of 1.6 ng/L, 2.1 ng/L, and 3.7 ng/L of 4-NP in drinking water, surface water, and wastewater effluent, respectively.

Different products have been used to prepare standard solutions for 4-NP analysis, including technical mixtures of branched 4-NP isomers (CAS-No.: 84852-15-3) (Loos et al., 2007; Fan et al., 2013), the 4-NP isomer with a linear nonyl chain (4-n-NP; CAS-No.: 104-40-5) (Ciofi et al., 2016; Prieto, Schrader and Moeder, 2010), the branched 4-NP isomer NP_{112} (CAS-No.: 142731-63-3) (Ciofi *et al.*, 2016) and NP mixtures with CAS-No. 25154-52-3 (Prieto, Schrader and Moeder, 2010; Wang, Zhou and Jiang, 2012). There are also many studies which did not provide any information on the used NP product (Vargas-Berrones *et al.*, 2020; Gong *et al.*, 2015). The usage of the linear isomer in environmental studies is rather surprising, since 4-*n*-NP is not a constituent of technical NP mixtures and should therefore not be present in the environment. This is especially relevant as technical NP produces other precursor-to-product ion transitions than 4-*n*-NP. In HPLC-MS/MS in negative electrospray ionization mode (ESI–) and without derivatization, technical NP has the transitions 219 > 133 and 219 > 147, while the most abundant transition of 4-*n*-NP is 219 > 106 (Loos *et al.*, 2007).

It has to be noted that the use of technical NP mixtures as analytical standards and the integrative (i.e. non-isomer-specific) analysis of 4-NP can lead to inaccurate results. This is mainly due to variability in the isomeric composition of technical mixtures (Eganhouse *et al.*, 2009), dissimilarities between the isomeric composition of commercial NP mixtures and NP present in the environment (KIM *et al.*, 2005), and potential differences in the detector responses of individual isomers. To overcome this issue, Dupuis et al. (2012) only focused on a

single isomer of 4-NP, namely 353-NP (see Table 9), to investigate the fate of 353-NP and its chlorinated derivatives during drinking water treatment. The standardization on single isomers can indeed help to obtain more consistent and comparable results in environmental studies on 4-NP. However, depending on the research question, a single isomer may not be equally well-suited to adequately describe the different properties (i.e., stability, mobility, toxicity) and therefore the environmental behavior and relevance of a mixture of compounds.

3.2.2.3 Occurrence

General Information

The following paragraphs contain information on the occurrence of total 4-NP and individual 4-NP isomers in surface waters, ground waters, wastewater effluents, and drinking waters. Only studies published after the year 2010 were considered. Please note that only a selection of studies is discussed here in order to provide insights on typical concentration ranges of 4-NP in samples from these compartments. For the sake of consistency and enhanced readability, all cited concentration values were rounded off to two significant figures. The compiled concentration data is also summarized in Table 10.

Occurrence of total 4-NP

As previously mentioned, NPEO are incompletely degraded to NP during wastewater treatment (Freeling *et al.*, 2019). Consequently, WWTP effluent is considered the main source of NP to the environment (Soares *et al.*, 2008). A recent study conducted by Zhao *et al.* (2021) in the urbanized Huizhou and Dongguan regions in China showed that the mass loads of certain phenolic compounds, including NP, from "rainfall runoff" (the authors most likely collected mainly Horton overland flow) were up to 62 times higher than those of WWTP effluents. This suggested that the first flush runoff after precipitation events can be an important source of 4-NP to receiving waters in highly urbanized region.

NP was detected in all water and sediment samples collected from three rivers of the Pearl River system in Southern China. Concentrations of 4-NP ranged from 0.065 µg/L to 20 µg/L and 11 µg/kg to 29,000 µg/kg in water and sediment samples, respectively. The highest average and maximum concentrations were detected in the Shijing River, which receives high inputs of treated and untreated industrial and domestic wastewater. Compared to the three rivers, 4-NP concentrations in samples from a water reservoir with only minor anthropogenic activity were considerably lower, ranging from 0.027 µg/L to 0.12 µg/L and 16 µg/kg to 23 µg/kg in water and sediment samples, respectively (Zhao *et al.*, 2011).

Lee *et al.* (2013) investigated the occurrence of NP in water and sediments of 16 major rivers in Taiwan. Concentrations of NP were in the range of 0.02–3.9 µg/L and 6.6–48,000 µg/kg dry weight (dw) in water and sediment samples, respectively. High levels of NP in water and sediments were measured at sampling sites near highly industrialized and urbanized areas. Effluent from NP-related factories appeared to be the major source of NP in the studied waterbodies.

Generally lower concentrations were observed by Wang *et al.* (2012) for the Yellow River in China, with concentrations ranging from 0.17 µg/L to 1.2 µg/L and 17 µg/kg dw to 200 µg/kg dw in water and sediment samples, respectively. The concentrations of 4-NP in dry season (November, winter) were generally higher than those in the wet season, which was explained by lower dilution and less biodegradation of 4-NP in winter than in summer.

Seasonal variation and spatial distribution of OP and NP in river surface water and sediments in the runoff outlets of the Pearl River Delta, China were investigated in the study by Chen *et al.*, (2014a). NP was detected in all water and sediment samples. Average concentrations in surface

water during the dry season ranged from 0.81 to 3.4 µg/L and concentrations in sediments ranged from 14 to 95 µg/kg dw. In surface waters, much higher concentrations were detected in the dry season than in the wet season. In sediments, the concentrations in the dry season were also higher in most cases. Again, high concentrations of NP were found to be related to high levels of domestic and industrial wastewater discharges into the rivers.

Sediments and surface waters of two rivers in the southwestern part of Nigeria were collected and analyzed for 4-NP in a study by Oketola and Fagbemigun (2013). 4-NP concentrations ranged between 0.044 µg/L to 0.079 µg/L and 1.1 µg/kg to 79 µg/kg in water and sediment samples, respectively.

The occurrence and distribution of eight endocrine-disrupting compounds, including 4-NP, were investigated in surface water and sediments of the Three Gorges Reservoir region in China. The mean concentration of 4-NP was 0.011 µg/l and 5 µg/kg dw in surface water and sediments, respectively (Wang, Ndungu and Wang, 2016).

Martín *et al.* (2014) studied the occurrence of various micropollutants, including NP, in surface waters affected by wastewater discharges and in WWTP effluents in Spain. With average concentrations of 13 µg/L and 4.2 µg/L in effluent wastewater and surface water, respectively, NP was among the compounds with the highest concentrations in these compartments. In fact, NP concentrations were above the maximum allowable concentration in inland and other surface waters of 2 µg/L 4-NP according to Directive 2013/39/EU (EC 2013).

Water samples from 50 randomly selected lakes across Minnesota, USA, were analyzed for 125 micropollutants, including 4-NP. 4-NP was one of the most frequently detected micropollutants (detection frequency: 10%) with concentrations ranging from below the reporting limit of 0.003 µg/L to 0.021 µg/L. The highest 4-NP concentrations were found in lakes impacted by shoreline residential development (Ferrey *et al.*, 2015).

Pollution of the Mira River in Portugal by endocrine disruptors, including 4-NP, was investigated by Rocha et al. (2016). 4-NP was detected in all 36 surface water samples with an average concentration of 0.013 µg/L (Rocha *et al.*, 2016).

Rubirola, Boleda and Galceran (2017) analyzed 24 Water Framework Directive priority substances, including 4-NP, in river water and effluent wastewaters in Spain. 4-NP was only detected in two out of 42 surface water samples (0.063 µg/L and 0.14 µg/L). In the effluent of two wastewater treatment plants, 4-NP was always (n=6) below the LOQ of 0.0037 µg/L.

Chen *et al.* (2013) assessed the occurrence of NP, OP, and BPA in raw water (surface water) sources and finished drinking water in Taiwan. All compounds could be identified in most of the sampled raw waters. Higher concentrations of NP were found in raw waters influenced by domestic wastewater. Median NP concentrations in raw water were higher than 0.2 µg/L for five of the eleven studied drinking water treatment plants (DWTP). The highest NP concentrations in raw water was approx. 1 µg/L. Mean concentration of NP in raw water were between 0.12 µg/L and 0.36 µg/L and decreased to average values between 0.068 µg/L and 0.23 µg/L in treated water.

Similar results were obtained by Fan *et al.* (2013) who studied NP and other endocrine-disrupting compounds in 62 DWTPs of 31 major cities across China. 4-NP was detected in all 62 source water samples with a median concentration of 0.027 µg/L (0.0082–0.92 µg/L). Of the 62 source water samples, eight were collected from groundwater, 31 were from reservoirs, and 23 were from river water. The average concentration of 4-NP was the highest in the river water samples (0.25 µg/L), followed by those in reservoir water (0.19 µg/L) and in groundwater

(0.13 µg/L). 4-NP was detected in 55 out of 62 drinking water samples with a median concentration of 0.12 µg/L (<0.0081−0.56 µg/L).

In a year-long evaluation on the occurrence and fate of pharmaceuticals, personal care products, and endocrine disrupting chemicals in an urban DWTP in the southeast United States, 4-NP was detected in all of the collected 24-h composite samples of source water (river water; n=8) and drinking water (n=8). The average 4-NP concentrations in the source and drinking water were 0.11 µg/L (0.053−0.19 µg/L) and 0.025 µg/L (0.0087−0.061 µg/L), respectively (Padhye *et al.*, 2014).

4-*NP* was detected in one out of three groundwater samples (2.2 µg/L; LOQ: 2.0 µg/L) collected at shoreline wells (well depths: 1 to 2 m) of the Great South Bay in NY, USA. The groundwater at the studied site is known to be influenced by discharges from septic systems (Phillips *et al.*, 2015).

Occurrence of individual 4-NP isomers

Several studies showed that 4-NP isomers can differ substantially in their occurrence, mobility, biodegradability, and toxicity, which can be explained by the different branching of the nonyl group. For example, Horii *et al.*, (2004) monitored the occurrence of 13 isomers of 4-NP in water samples from rivers around Tokyo Bay, Japan. The concentrations of 4-NP isomers ranged from 0.6 ng/L to 780 ng/L. The concentration of the most abundant isomer was between 6 to 16 times higher than the concentration of the least abundant isomer in individual samples. However, the authors were not able to identify the structures of these isomers.

Guruge, Horii and Yamashita (2011) also investigated 13 4-NP isomers in surface water samples from Sri Lanka. The concentrations of individual isomers ranged from 3 ng/L to about 230 ng/L. In their study, the concentration of the most abundant isomer was between 2 to 6 times higher than of the least abundant isomer in the respective sample. In most cases, the distribution of 4-NP isomers in environmental samples were similar to the isomeric distribution of a commercial 4-NP mixture. In other studies, 4-NP isomer distribution patterns were found to have been altered after their release to the environment. For example, Kim *et al.* (2005) analyzed NP isomer concentrations at three sites (near the river mouth, 7 km offshore, and 12 km offshore) in the Ariake Sea, Japan. While the total 4-NP isomer concentrations decreased with greater distance of the sampling point from the river mouth, removal ratios of individual 4-NP isomers differed considerably.

The occurrence of twelve selected isomers of 4-NP in reclaimed water from three wastewater treatment plants, surface water, and groundwater was investigated in a long-term sewage irrigation area in Beijing, China. The mean total NP concentrations of the reclaimed water, surface water, and groundwater were 0.47 µg/L, 0.70 µg/L and 0.24 µg/L, respectively. Concentrations ranges of individual 4-NP isomers were as follows: <LOQ to 0.089 µg/L (reclaimed water), <LOQ to 0.12 µg/L (surface water), <LOQ to 0.17 µg/L (groundwater). The LOQs of individual isomers were not specified in the study but ranged from 0.009 µg/L to 0.041 µg/L. Total NP concentration in the groundwater decreased with increasing aquifer depth. Since some isomers were not detected in deeper groundwater monitoring wells, the authors assumed that NP isomers were more or less mobile in the saturated zone (Wang *et al.*, 2015).

From an ecotoxicological standpoint, alteration of the isomeric composition through differences in stability and/or mobility of individual 4-NP isomers is relevant, as their toxicities also differ (KIM *et al.*, 2004). Using ^{14}C- and ^{13}C-ring-labeling, degradation of four branched isomers (4-NP_{38}, 4-NP_{65}, 4-NP_{111}, 4-NP_{112}) and the linear 4-NP_1 isomer (4-*n*-NP) in a rice paddy soil was studied under oxic conditions. The authors showed that 4-NP isomers with higher estrogenicity were more persistent in the rice paddy soil and less prone to microbial degradation. Therefore,

dissimilar degradation of 4-NPs resulted in an increase of specific estrogenicity of the remaining 4-NPs in the soil (Shan *et al.*, 2011).

Table 10: Concentrations of 4-NP in surface water (SW), surface water sediment, reservoir water, ground water (GW), drinking water (i.e. treated raw water; DW) and wastewater treatment plant outflow (WWTP-O). Data is sorted by publication year

Matrix	Country	LOQ in µg/L or µg/kg	Concentration in µg/L or µg/kg	Reference
SW **sediment** **SW (non-polluted site)** **sediment (non-polluted site)**	China	not specified	0.065–20 11–29,000 0.027–12 16–23	(Zhao *et al.*, 2011)
SW **sediment**	China	0.007	0.17–1.2 17–200	(Wang *et al.*, 2012)
SW **DW**	Taiwan	0.095	0.12–0.36 (averages) 0.068–0.23 (averages)	Chen *et al.*, 2013
SW **reservoir water** **GW** **DW**	China	0.0081	0.25 (average) 0.19 (average) 0.13 (average) 0.12 (median)	Fan *et al.*, 2013
SW **sediment**	Nigeria	0.007 0.3	0.044–0.079 1.1–79	(Oketola and Fagbemigun, 2013)
SW **sediment**	Taiwan	0.0021 0.13	0.02–3.9 6.6–48,000	(Lee *et al.*, 2013)
SW **sediment**	China	0.0005 not specified	0.81–3.4 14–95	(Chen *et al.*, 2014a)
WWTP-O **SW**	Spain	0.011 0.0091	13 (average) 4.2 (average)	(Martín *et al.*, 2014)
SW **DW**	USA	not specified	0.053−0.19 0.0087−0.061	(Padhye *et al.*, 2014)
SW	USA	0.0035–0.012	LOQ–0.020	(Ferrey *et al.*, 2015)
GW	USA	2	2.2	(Phillips *et al.*, 2015)
SW	Portugal	not specified	0.013 (average)	(Rocha *et al.*, 2016)
SW **sediment**	China	0.0017 1.5	LOQ–0.033 LOQ–8.5	(Wang, Ndungu and Wang, 2016)
SW **WWTP-O**	Spain	0.0021 0.0037	0.063 and 0.14 <LOQ	Rubirola, Boleda and Galceran, 2017

3.2.3 Octylphenol

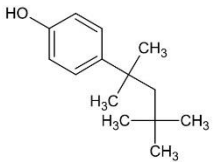
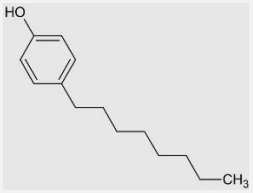
3.2.3.1 General information

Octylphenols (OP; molecular formula: C14H22O) refer to a group of isomeric compounds consisting of an alkyl chain with eight carbon atoms attached to a phenol ring. The octyl moiety can be linear or branched and is located at either the 2-, 3- or 4-position of the phenol (OSPAR 2006). 4-t-OP (synonymously 4-tertiary-OP or 4-tert-OP; CAS-No. 140-66-9; Table 11) is the commercially most important isomer of OP. The linear isomer, 4-n-OP (CAS-No. 1806-26-4; Table 11), is not used for industrial applications and should therefore not be present in the environment (Loos *et al.*, 2008).

4-t-OP is utilized as raw material for a number of industrial applications, including the production of octylphenol ethoxylates (OPEO) surfactants and, more importantly, in the manufacture of phenolic resins (Graca *et al.*, 2016). Furthermore, OP are found as impurities in technical NP mixtures (0.01–2.2%; (Eganhouse *et al.*, 2009)). Since OPEO is partially degraded to 4-t-OP during sewage treatment, 4-t-NP can enter the environment via WWTP effluent (Chen *et al.*, 2013).

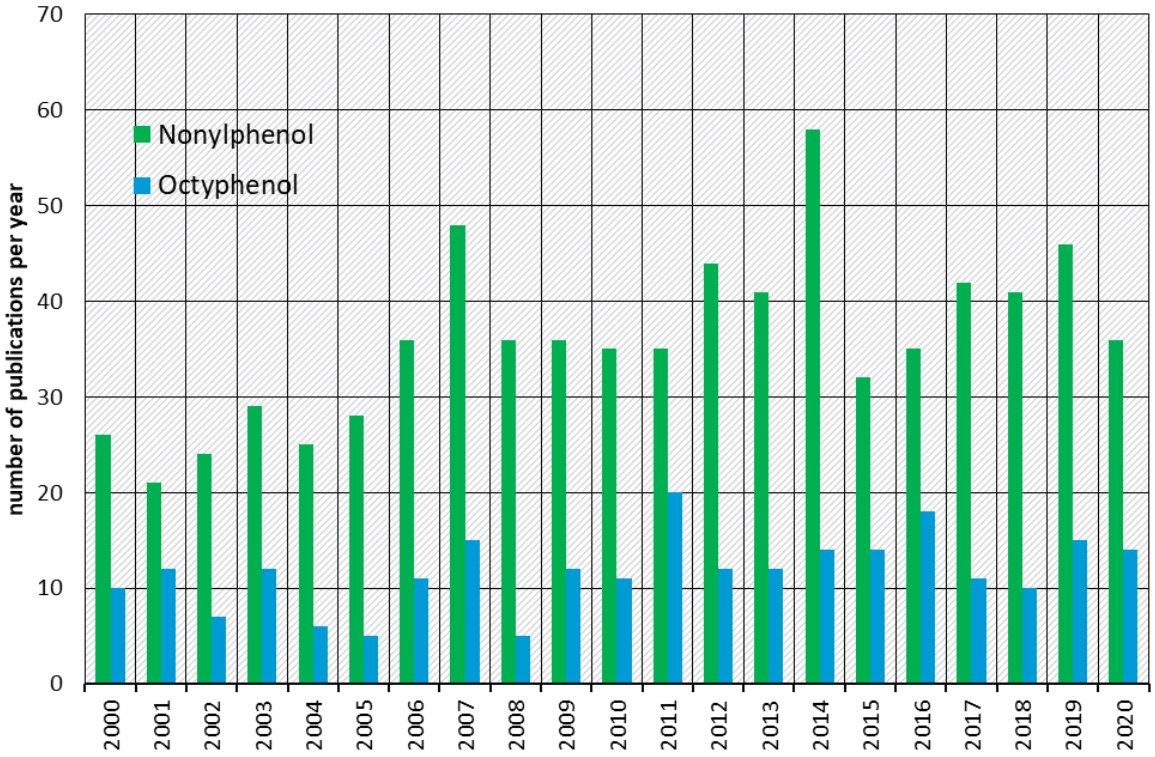
4-n-OP and 4-t-OP are priority substances according to the Water Framework Directive (EC, 2013). 4-t-OP and a substance group called "4-(1,1,3,3-tetramethylbutyl)phenol, ethoxylated", which encompasses various OPEO surfactants, were also included to the list of SVHC under Article 57 of REACH.

Table 11: Chemical abstract service (CAS) number, molecular formula, molecular weight, and chemical structure of 4-t-octylphenol and 4-n-octylphenol

Analyte	CAS No.	Molecular formula	Molecular weight in g/mol	Chemical structure
4-*t*-Octylphenol	140-66-9	$C_{14}H_{22}O$	206.32	
4-*n*-Octylphenol	1806-26-4	$C_{14}H_{22}O$	206.32	

Comparing the results of the literature search on Web of Science for nonylphenol and octylphenol with the ones obtained for estradiol a pronounced difference can be observed. Only about half as many papers were published for nonylphenol as for estradiol. For octylphenol even considerably less. These results are surprising as it seems generally possible to measure all the analytes with the same analytical method.

Figure 4: Number of publications per year between 2000 and 2020. Search conditions "nonylphnol/octylphenol" AND "environment" AND "water".



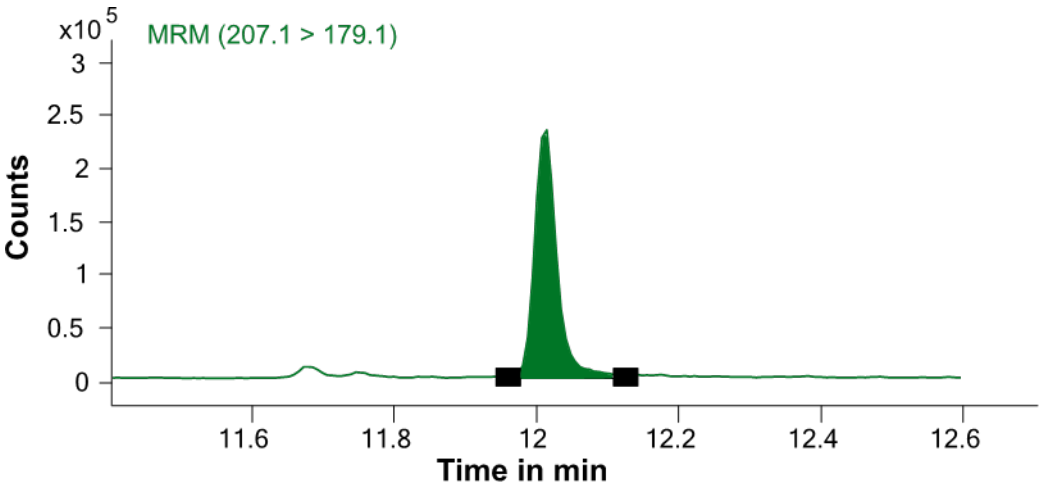
Source: Web of science

3.2.3.2 Analysis

With 4-*t*-OP being the only relevant isomer in industrial applications, analysis of OP in environmental matrices is much less complex than the analysis of NP. As OP and NP both belong to the group of alkyl phenols and only distinguish themselves in a $-CH_2-$ unit in the alkyl chain, they usually can be measured with a single analytical method. In fact, many of the aforementioned studies that analyzed 4-NP in water or sediment samples also screened for residues of 4-*t*-OP, using both HPLC-MS/MS (Loos *et al.*, 2007; Chen *et al.*, 2013; Rubirola, Boleda and Galceran, 2017) and GC-MS (Wang *et al.*, 2012; Gong *et al.*, 2015; Ferrey *et al.*, 2015) for chromatographic separation and quantification. The most commonly applied technique for sample pre-treatment of aqueous and sediment samples was SPE (Loos *et al.*, 2007; Chen *et al.*, 2013; Rubirola, Boleda and Galceran, 2017; Wang *et al.*, 2012). In the named studies, the LOQ for 4-*t*-OP (usually <10 ng/L) was generally lower than the reported LOQ for 4-NP.

Figure 5 shows the GC-MS/MS multiple reaction monitoring (MRM) chromatogram of a 4-*t*-OP analytical standard (CAS No.: 140-66-9) after derivatization with MSTFA.

Figure 5: GC-MS/MS multiple reaction monitoring (MRM) chromatogram of a 4-t-OP analytical standard (CAS No.: 140-66-9) after derivatization with MSTFA



Source: own research, TZW Karlsruhe

Some studies also screened for residues of the linear chain isomer of 4-OP, despite the fact that 4-n-OP should not be present in the environment. Note that 4-t-OP produces other MS/MS transitions than 4-n-OP. In HPLC-MS/MS in negative ESI mode and without derivatization, 4-t-OP has the precursor-to-product ion transitions 205 > 133 and 205 > 134, while the most abundant transition of 4-n-OP is 205 > 106 (Ciofi *et al.*, 2016).

3.2.3.3 Occurrence

General Information

The following paragraphs contain information on the occurrence of 4-t-OP in in surface waters, ground waters, wastewater effluents, and drinking waters. Only studies published after the year 2010 were considered. Again, only a selection of studies is discussed here to provide insights on typical concentration ranges of 4-t-OP in samples from these compartments. All cited concentration values were rounded off to two significant figures. The compiled concentration data is summarized in Table 12.

Table 12: Concentrations of 4-t-OP in surface water (SW), surface water sediment, ground water (GW), wastewater treatment plant inflow (WWTP-I) and outflow (WWTP-O), and drinking water (i.e. treated raw water; DW). Data is sorted by publication year

Matrix	Country	LOQ in µg/L or µg/kg	Concentration in µg/L or µg/kg dry weight	Reference
SW **sediment**	China	0.001 0.9	0.0024–0.015 <LOQ–2.6	Wang et al., 2012b
SW **sediment**	Nigeria	0.001 0.5	0.057–0.069 2.2–24	Oketola and Fagbemigun, 2013
SW **DW**	Taiwan	0.011	0.02–0.075 (averages) <LOQ–0.06 (averages)	Chen et al., 2013
SW	China	0.06	0.0039–0.47	Yang et al., 2013

Matrix	Country	LOQ in µg/L or µg/kg	Concentration in µg/L or µg/kg dry weight	Reference
SW **sediment**	China	0.001 not specified	0.086–0.58 0.4–3	Chen et al., 2014
SW	USA	0.00069–0.0046	LOQ–0.0038	Ferrey et al., 2015
GW	USA	0.14	0.21 and 0.41	Phillips et al., 2015
SW **sediment**	China	0.0003 0.5	LOQ–0.12 LOQ–12.4	Wang et al., 2016
WWTP-I **WWTP-O**	Portugal	0.0034	<LOQ <LOQ	Carvalho et al., 2016
SW	Portugal	not specified	0.010 (average)	Rocha et al., 2016
SW **WWTP-O**	Spain	0.0016 0.0019	<LOQ 0.022	Rubirola et al., 2017
SW	Serbia	not obtainable	0.009 (average) 0.015 (maximum)	Škrbić et al., 2018

Occurrence of total 4-*t*-OP

Different types of surface water samples from Jinan, China were analyzed for residues of 4-t-OP by Yang *et al.* (2013). Concentrations ranged from 0.0039 µg/L to 0.47 µg/L. The sampling site with the highest average concentration (0.21 µg/L) was Xiaoqing River, which was described as being seriously polluted by wastewater discharge from the city of Jinan.

In the study of Chen *et al.* (2013), which assessed the occurrence of 4-t-OP, 4-NP and BPA in raw water (surface water) sources and finished drinking water of eleven DWTP in Taiwan, it was found that 4-t-OP and 4-NP concentrations in studied raw water samples were positively correlated with each other, indicating similar sources (WWTP effluent) for these compounds. However, OP concentrations in raw waters were about ten times lower than the concentrations of NP. Mean concentrations of OP in raw water were between approx. 0.02 µg/L and 0.075 µg/L (values estimated from graph) and decreased to average values between <0.011 µg/L and 0.06 µg/L in treated water.

Much lower concentrations of 4-t-OP compared to 4-NP were also observed by Wang *et al.* (2012) for the Yellow River in China, with concentrations ranging from 0.0024 µg/L to 0.015 µg/L and <0.9 µg/kg dw to 2.6 µg/kg dw in water and sediment samples, respectively. The average OP concentration in surface waters (0.0047 µg/L) was about 120 times lower than the average concentration of 4-NP (0.58 µg/L). For sediment samples, the average OP concentration (0.5 µg/kg dw) was approx. 70 times lower than the average concentration of 4-NP (36 µg/kg dw). Similar to 4-NP, surface water concentrations of 4-t-OP in the dry season were generally higher than those in the wet season, most likely due to higher dilution and less biodegradation in the wet season.

In sediments and surface waters of two rivers in Nigeria, 4-t-OP was detected at concentrations ranging between 0.057 µg/L to 0.069 µg/L and 2.2 µg/kg to 24 µg/kg in water and sediment samples, respectively. The authors attributed the presence of 4-t-OP as well as 4-NP to

discharges of domestic and industrial wastewaters (e.g. from the textile industry) (Oketola and Fagbemigun, 2013).

4-t-OP was detected in all water and sediment samples of the Pearl River Delta in China. Average concentrations of 4-t-OP in surface water and sediment samples during the dry season ranged from 0.086 to 0.58 µg/L and 0.4 to 3 µg/kg dw, respectively. Samples with high levels of 4-t-OP were associated with industrial wastewater discharges. Much higher 4-t-OP concentrations in river water were detected in the dry season than in the wet season. In sediments, the concentrations in the dry season were also higher in most cases (Chen *et al.*, 2014b).

4-t-OP was also included in the study by Wang, Ndungu and Wang (2016), which studied selected endocrine disrupting chemicals in surface water and sediments of the Three Gorges Reservoir region in China. The mean concentration of 4-t-OP was 0.033 µg/l and 5.3 µg/kg dw in surface water and sediments, respectively.

The compound was also detected in 4% of the studied 250 water samples collected from selected lakes across Minnesota, USA. The maximum reported concentration of 4-t-OP was 0.0038 µg/L, which was more than 5-times lower than the maximum of 4-NP in the studied samples (Ferrey *et al.*, 2015).

Much smaller differences in the average surface water concentrations of 4-NP and 4-t-OP were found in the study of Rocha *et al.* (2016). In the Mira River in Portugal the average concentration of 4-t-OP was 0.010 µg/L, while the average for 4-NP was only slightly higher (0.013 µg/L).

Rubirola, Boleda and Galceran (2017) studied the occurrence of 24 Water Framework Directive priority substances, including 4-t-OP, in river water and effluent wastewaters in Spain. In all 42 surface water samples, 4-t-OP was always below its LOQ of 0.0016 µg/L. In the effluent of two WWTP, it was detected only once (n=6) with a concentration of 0.022 µg/L. In a study conducted in Portugal, 4-t-OP was always below the LOQ of 0.0034 µg/L in influent and effluent samples from a single WWTP (Carvalho *et al.*, 2016).

In the surface water samples of four rivers, one irrigation canal system, and two lakes in northern Serbia, 4-t-OP was also very frequently (72%) detected. Average and maximum concentrations were 0.009 µg/L and 0.015 µg/L, respectively (Škrbić, Kadokami and Antić, 2018).

4-t-OP was detected in two out of three groundwater samples (0.21 µg/L and 0.41 µg/L; LOQ: 0.14 µg/L) from the Great South Bay in NY, USA. As already mentioned above, the sampled groundwater was influence by septic tank discharges.

3.2.4 G-1 (GPER1-selective ligand) and AB-1 (ER-selective ligand)

3.2.4.1 General information

G1 (CAS No.: 881639-98-1; Table 13) is a potent and selective GPER1 agonist, which displays no activity towards ERα and ERβ at concentrations from 1 nM to 10 mM (Dennis *et al.*, 2011; Wang *et al.*, 2013). AB-1 (CAS No.: 849721-95-5; Table 13) on the other hand is an ER-selective ligand that lacks GPER1 cross-reactivity. It binds with high affinity to ERα and ERβ, but not to GPER1. Hence, GPER1-mediated signaling pathways are not activated. Moreover, AB-1 initiates ER-mediated transcription but not rapid signaling by ER (Revankar *et al.*, 2019). Due to the special properties of G-1 and AB-1, both compounds were selected as additional test substances in this study.

Table 13: Chemical abstract service (CAS) number, molecular formula, molecular weight, and chemical structure of G-1 and AB-1

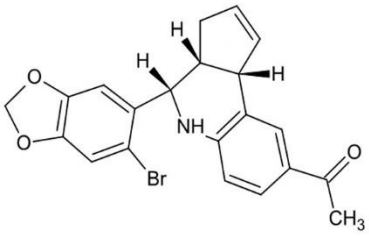
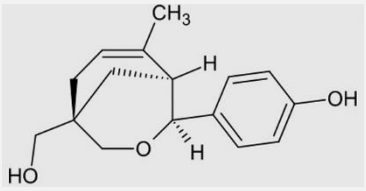
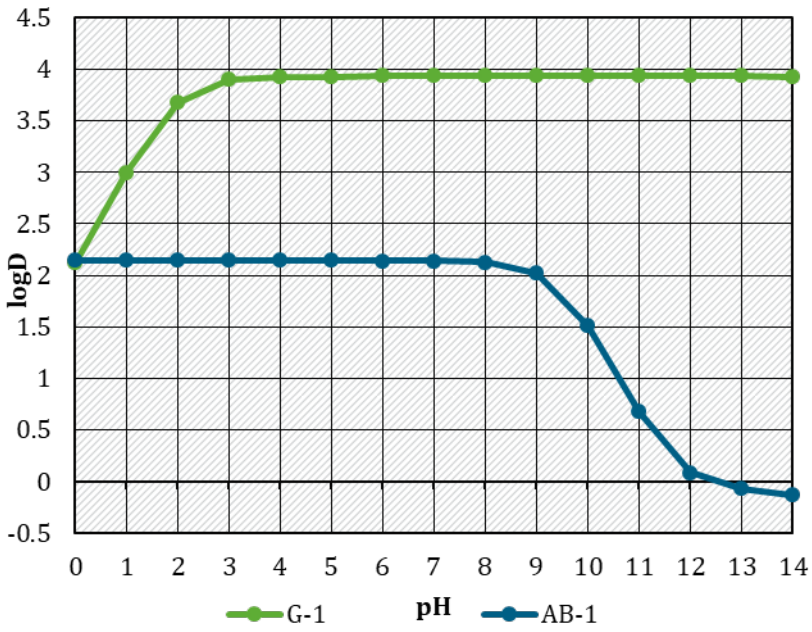
Analyte	CAS No.	Molecular formular	Molecular weight in g/mol	Chemical structure
G-1	881639-98-1	$C_{21}H_{18}BrNO_3$	412.28	
AB-1	849721-95-5	$C_{16}H_{20}O_3$	260.33	

Figure 6: LogD versus pH diagram of G-1 and AB-1. LogD data was generated using the LogD plugin of ChemAxon



Source: own research, TZW Karlsruhe

Figure 6 depicts the modelled logarithm to base 10 of the n-octanol/water distribution coefficient (LogD) at different pH for G-1 and AB-1. The logD is the ratio of the sum of the concentrations of all species of a compound (both neutral and ionized) in each of the two phases. The value is greater than one if the substance is more soluble in the nonpolar solvent n-octanol than in water. Consequently, it is a measure for the relationship between lipophilicity and hydrophilicity of a compound.

3.2.4.2 Analysis

To the best of our knowledge, no analytical methods for the quantification of G-1 and AB-1 in aqueous samples have yet been described in the scientific literature. Hence, the amenability of HPLC/MS-MS and GC-MS/MS for the analysis of G-1 and AB-1 was estimated based on the chemical structures of these compounds. The following paragraphs also include available information on the solubility and storability of G-1 and AB-1 provided by the manufactures of the analytical standards.

G1

From Figure 6 it can be seen that G-1 is fairly nonpolar over a wide pH-range (logD of approx. 4 for pH >3). Due to the elevated hydrophobicity, which is a typical property of organobromine compounds in general, G-1 should be sufficiently well retained by RP-SPE sorbents and RP-HPLC columns. Since G-1 contains a secondary amine in its structure, it potentially can be detected using MS/MS in ESI+ mode.

According to Tocris Bioscience/Bio-Techne GmbH, G-1 is soluble in dimethyl sulfoxide (DMSO) up to concentrations of 100 mM (41.23 g/L). Vo *et al.* (2019) prepared a 5 mM stock solution of G-1 in DMSO and stored it at –20 °C. However, no information on the long-term storage stability of G-1 in DMSO and other solvents is available. Because of the hydrophobic nature of G-1, potential sorption of G-1 on sampling and laboratory equipment (containers, syringes, etc.) should to be evaluated as part of the validation of the analytical method.

AB-1

AB-1 has a similar molecular weight and structure than the estrogen β-estradiol (272.38 g/mol). Due to the phenol- and oxabicyclo-substructures, AB-1 is also relatively nonpolar over a wide pH-range (LogD of approx. 2.2 for pH <9; Figure 6) and should therefore interact with the hydrophobic stationary phases of common RP-SPE sorbents and RP-HPLC columns.

Unlike G-1, AB-1 carries two hydroxyl (OH) groups, which indicates that the compound ionizes in ESI– mode. However, due to its molecular structure, it can be expected that AB-1 does not exhibit high sensitivity in ESI– compared to many other micropollutants such as e.g. acidic pharmaceuticals. This is because deprotonation of the OH group is unfavorable compared to other molecules that have stronger electronegative moieties in their chemical structures. This is also a common issue for estrogens. As a consequence, studies have utilized ammonium fluoride as a novel additive for mobile phases of HPLC, since ammonium fluoride can immensely enhance the sensitivity of estrogens in ESI– mode (Li, Li and Kellermann, 2018; Petrie *et al.*, 2016). Consequently, due to the structural similarity of AB-1 and ß-estradiol, the usage of ammonium fluoride as a mobile phase additive might also bolster the sensitivity of AB-1 in HPLC-ESI-MS/MS analysis.

AB-1 might also be GC-amenable after derivatization. For example, the hydrogen atom of the two OH groups of AB-1 could be substituted with trimethylsilyl groups to enhance the volatility and thermal stability of the analyte. This approach, called silylation, has already been used for the GC-analysis of estrogens and other endocrine active substances (Rocha, Cruzeiro and Rocha, 2013).

According to Sigma Aldrich/Merck KGaA, AB-1 is soluble in DMSO up to concentrations of 7.7 mM (2 g/L) and should be kept at –20 °C for storage. Long-term storage stability as well as potential mass loss in aqueous solutions caused by sorption should also be evaluated for AB-1 during method development.

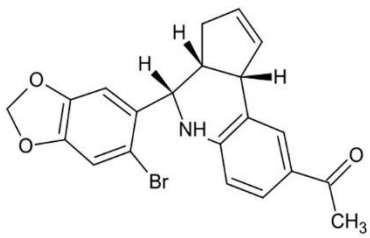
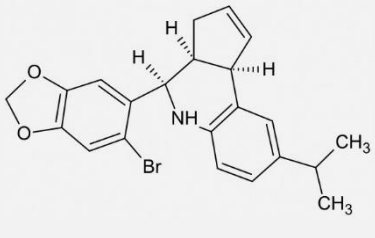
3.2.4.3 Search for compounds with similar chemical structure to G-1

G-1 is a compound specifically designed and synthetized for its use a highly GPER1-selective ligand. During the process of searching suitable test substances for this study, one idea was to search for compounds with chemical structures similar to that of G-1, as they probably have estrogenic properties akin to G-1.

The databases of PubChem® (https://pubchem.ncbi.nlm.nih.gov/) and eMolecules, Inc. (https://www.emolecules.com/) were searched for chemicals with structures similar to G-1. Using a 2-D similar search with a Tanimoto threshold of 90%, 134 compounds were found in the PubChem® database. When using the search of the eMolecules website, 24 compounds (similarity threshold: 80%) were identified. The results of both databases were screened for compounds used for major industrial or domestic applications (e. g., as pharmaceuticals, pesticides, industrial chemicals), as these compounds are usually produced in large amounts and therefore are of potential environmental relevance. Among the gathered compounds with similar structure to G-1, it was not possible to identify a single chemical that, at least to our knowledge, finds major use in industrial or domestic applications.

It should be noted that the list of similar compounds contained the chemical G-36 (CAS No.: 1392487-51-2), which is also GPER1-selective ligand. However, instead of being a GPER1 agonist like G-1, G-36 acts as an antagonist of GPER1. Interestingly, both compounds are structurally extremely similar to each other. The oxygen atom of the ketone group of G-1 is simply substituted by a methyl group (Table 14). This shows that minor differences in the chemical structure of a molecule can lead to very different biological activity.

Table 14: Chemical abstract service (CAS) number, molecular formula, molecular weight, and chemical structure of G-1 and G-36.

Analyte	CAS No.	Molecular formular	Molecular weight in g/mol	Chemical Structure
G-1	881639-98-1	$C_{21}H_{18}BrNO_3$	412.28	
G-36	1392487-51-2	$C_{22}H_{22}BrNO_2$	412.32	

3.2.5 Bisphenol A and bisphenol analogues

3.2.5.1 General information

According to Song *et al.* (2012) bisphenols are a group of chemicals containing two phenolic rings bridged by a carbon group with Bisphenol A (BPA; 2,2bis(4-hydroxyphenyl)propane) as

the most infamous representative (Table 15). Russian chemist A. P. Dianin synthesized BPA for the first time in 1891 by a condensation reaction of two phenol molecules and one acetone molecule at the presence of a catalyst (Rubin and Soto, 2009). Commercial production of BPA began in the United States in 1957 and then in Europe a year later (Corrales *et al.*, 2015). BPA is considered to have one of the highest production volumes of all chemicals on a world wide scale.

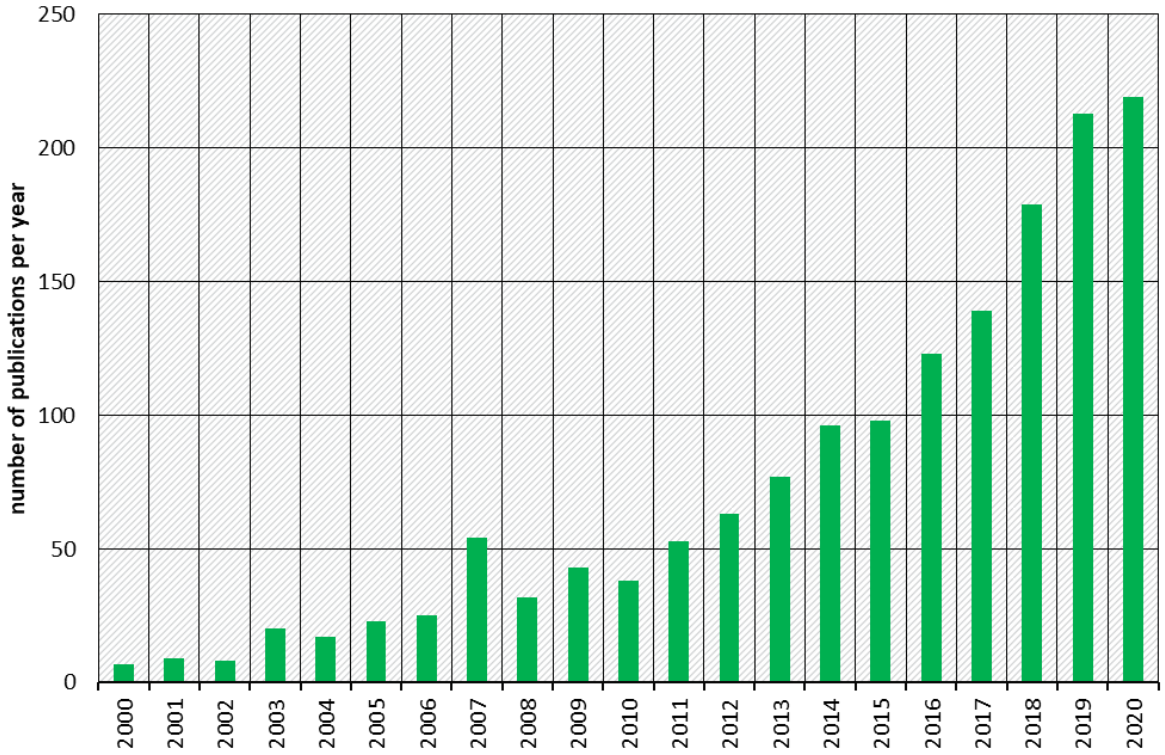
The importance of BPA is also underlined by the number of publications regarding its environmental occurrence, that considerably exceed the number of the aforementioned compounds (Figure 7).

In its main application BPA is used as a monomer in the production of polycarbonate plastics and epoxy resins. Both account for 65% and 28%, respectively, of the total usage of BPA. As lightweight and durable materials have gained increasing popularity also in third world countries over the last decades, BPA production has steadily increased. In scientific literature, there are diverging numbers regarding the global production rate, which is stated by most authors to be over eight million tons per year (Huang *et al.*, 2012; Almeida *et al.*, 2018). Other sources project the global production to reach 10.6 million metric tons by 2022 (Research and Markets, 2016).

When BPA is used in polycarbonates it can be found in products like water bottles, infant feeding bottles, water pipes, toys, thermal paper, household appliances and medical equipment. Whereas epoxy resins are used as protective coatings in food containers (tin linings) for food and beverages, adhesives, paints, electrical and electronic laminates (Abraham and Chakraborty, 2020; Vandenberg *et al.*, 2007), BPA can also be found in polyvinyl chloride (PVC) as an antioxidant additive, toilet paper and napkins produced from highly BPA contaminated recycled papers (Safakhah *et al.*, 2020).

Due to its phenolic structure, BPA is able to interact with estrogen receptors and therefore may exert estrogenic actions (Konieczna, Rutkowska and Rachoń, 2015). The concerns over widespread human exposure and associated health effects have led to regulations on the production and usage of BPA. Especially BPA based plastics in contact with food and beverage are under regulatory surveillance in an increasing number of countries. The use of BPA in food packing products, baby bottles, and toys became gradually illegal in Canada, United States, Japan, and European Union (EU) to name only a few. Canada already prohibited the import and sale of baby bottles containing BPA in 2010. The EU followed this approach to ban BPA in infant feeding bottles in 2011.

Figure 7: Number of bisphenol A publications per year between 2000 and 2020. Search conditions "bisphenol A" AND "environment" AND "water". Source: Web of Science

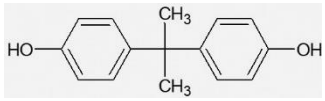
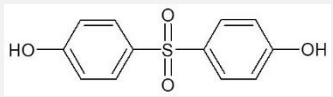
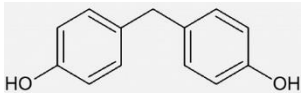
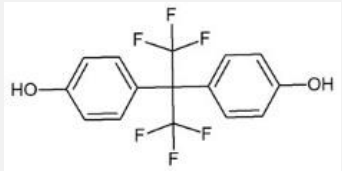
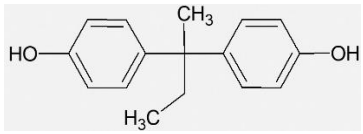
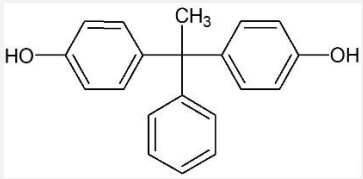
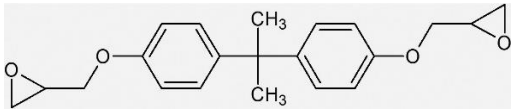
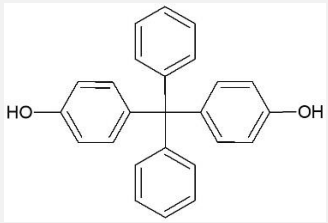
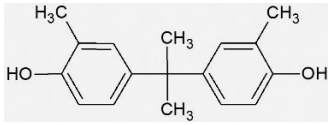
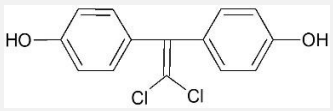
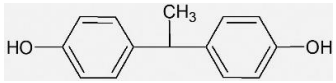


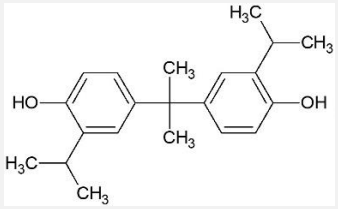
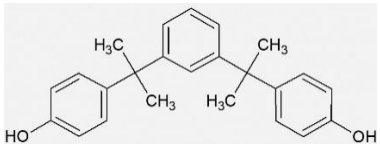
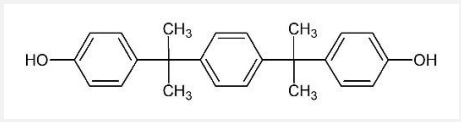
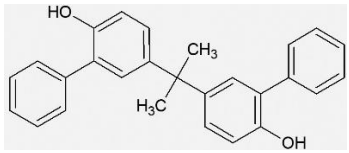
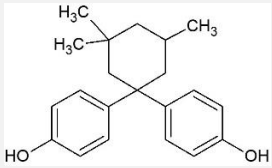
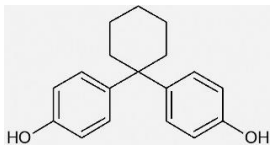
Source: Web of science

The new regulations and increasing concerns about the safety of BPA stimulated the development and production of more sustainable and more environmentally friendly substances that can alternatively replace BPA. Thus, a number of structurally similar chemicals have been developed and are in use since years. All share a common structure of two hydroxyphenyl functionalities and are referred to as bisphenol analogues. Bisphenol F (BPF), Bisphenol S (BPS), and Bisphenol AF (BPAF) are the main substitutes of BPA in the manufacturing of polycarbonate plastics and epoxy resins. Similar to BPA, they share a broad range of applications: BPF is used in lacquers, varnishes, liners, and plastic adhesives, as well as in dental sealants, oral prosthetic devices, tissue substitutes and coatings for food packaging. Lee *et al.* (2015) mentioned that BPF was increasingly applied in water pipes and structures used in water treatment plants. BPS is applied in epoxy glues, tin coatings and thermal papers, and as an additive in dyes and tanning agents. BPAF is used as a cross-linker in fluoroelastomers, electronics and optical fibers, and as a high performance monomer for polyimides, polyamides, polyesters, polycarbonate copolymers and other specialty polymers (Chen *et al.*, 2016).

Chen *et al.* (2016) mentioned that a total number of 16 bisphenols has been documented to be commercially applied so far (Table 15). They also showed that BPAF, BPAP, BPB, BPF, BPP, BPS or BPZ have already been found in human urine, food, indoor dust, sediment, sludge or water.

Table 15: Commercially applied bisphenols with chemical structure, chemical formula, molecular weight and their logP. Source: PubChem (nih.gov)

Compound name	Structure	Molecular formula and molecular weight	logP
BPA		$C_{15}H_{16}O_2$ 228.3 g/mol	3.3
BPS		$C_{12}H_{10}O_4S$ 250.3 g/mol	1.9
BPF		$C_{13}H_{12}O_2$ 200.2 g/mol	2.9
BPAF		$C_{15}H_{10}F_6O_2$ 336.2 g/mol	4.5
BPB		$C_{16}H_{18}O_2$ 242.3 g/mol	3.9
BPAP		$C_{20}H_{18}O_2$ 290.4 g/mol	4.4
BPA-DGE		$C_{21}H_{24}O_4$ 340.4 g/mol	4.0
BPBP		$C_{25}H_{20}O_2$ 352.4 g/mol	5.6
BPC		$C_{17}H_{20}O_2$ 256.3 g/mol	4.7
BPC-dichloride		$C_{14}H_{10}Cl_2O_2$ 281.1 g/mol	5.0
BPE		$C_{14}H_{14}O_2$ 214.3 g/mol	3.9

Compound name	Structure	Molecular formula and molecular weight	logP
BPG		$C_{21}H_{28}O_2$ 312.5 g/mol	6.3
BPM		$C_{24}H_{26}O_2$ 346.5 g/mol	6.1
BPP		$C_{24}H_{26}O_2$ 346.5 g/mol	6.1
BPPH		$C_{27}H_{24}O_2$ 380.5 g/mol	7.3
BP-TMC		$C_{21}H_{26}O_2$ 310.4 g/mol	6.3
BPZ		$C_{18}H_{20}O_2$ 368.4 g/mol	5.4

3.2.5.2 Analysis

BPA has been analyzed in environmental matrices such as air, water, sediment, soil, food, human plasma and urine samples using different analytical methods. Common extraction techniques adopted for the determination of BPA include solvent-based extraction methods such as solid-SPE for water and wastewater samples, soxhlet extraction and microwave assisted extraction for soil and sediment samples (Abraham and Chakraborty, 2020).

In most cases water samples were spiked with internal standards prior to filtration or SPE. Shehab, Jamil and Aris, (2020) and Lan *et al.* (2019) adjusted the samples pH to 5. No explanation was given for that, but due to the physico-chemical properties of BPA (pKa 9.6) no improved extraction efficiency can be expected by sample acidification. After extraction, cartridges were dried and the analytes were eluted with an organic solvent (e. g. ethyl acetate, methanol). Some authors also included a washing step e.g. with methanol/water (30:70, v/v) and ultrapure water before drying the cartridge material to remove interferences (Jin and Zhu, 2016). In the final step of sample preparation, the eluate is dried and redissolved with an appropriate solvent (Huang *et al.*, 2020).

An astonishing number of publications use the Oasis HLB cartridges for the extraction of bisphenols from water samples (Jin and Zhu, 2016; Huang *et al.*, 2020), but other materials

suitable for non-polar extractions like LiChrolut RP-18 have been successfully applied, too Lee *et al.* (2013). A different approach to SPE was applied by Lan *et al.* (2019) who liquid-liquid extracted water samples three times with dichloromethane. The combined extracts were rotary evaporated and redissolved in 1 mL of methanol. For analysis a high resolution Orbitrap Fusion Tribrid mass spectrometry was used for qualitative and quantitative analysis.

Although Corrales *et al.* (2015) stated that GC-MS continues to be the most common method employed, the review of publications published between 2010 to 2020 measuring bisphenol analogues in environmental matrices, showed almost exclusively the application of LC-MS/MS methods. However, Akhbarizadeh *et al.* (2020) only recently published a new analytical method based on GC using a 6890 Agilent gas chromatograph equipped with a Combi-PAL autosampler and a single quadrupole detector with electron ionization for the analysis of BPA, BPB, BPF, BPE, BPAF, BPZ, and BPAP. Chromatographic separation was carried out using a DB-5MS column and the injection was made in splitless mode.

Conditions of liquid-chromatography comprise standard analytical columns with hydrophobic interactions and solvents commonly used in routine laboratories. Interesting details were reported by Owczarek *et al.* (2018) who developed a chromatographic separation of BPA and ten BPA analogues in human blood serum. Two chromatographic methods were applied to determine the analytes. BPC, BPE, BPF, BPG, BPM, BPP, BPZ, BPFL, BPBP were separated using a gradient consisting of ultra-pure water and methanol both modified with 0.01% of ammonia. During the chromatographic separation of BPA and BPS the mobile phase consisted of water and methanol only without any additives. Methanol was used instead of acetonitrile as for the latter peak broadening, peak shape deterioration along with smaller signal response were noted. The testing of different concentrations of ammonium acetate, formic acid, acetic acid and ammonia as modifier showed that the best results in terms of response and peak shapes could be obtained with ammonia, whereas ammonium acetate and acid caused signal suppression. However, in the case of BPS the addition of ammonia resulted in decreased sensitivity and shifting the BPS signal towards the system´s void time. The authors highlighted that a certain initial content of an organic solvent is advantageous and that this organic content has also to be maintained during the whole analytical run in order to avoid enrichment of BPA at the column´s head, leaching from system elements.

The analytical instrumentation used for the analysis of BPA and its analogues were in most cases tandem mass spectrometers of the latest generation. High resolution mass spectrometers are not necessary, but have also been applied. The analytes are generally measured in negative ionization mode when applying LC-MS/MS (Liu *et al.* 2016; Lan *et al.* 2019) . Only Niu *et al.* (2017) reported that they operated a Waters Xevo TQ triple quadrupole mass spectrometer with ESI ion source in positive ionization mode.

Most analytical methods published in recent years still enrich samples volumes of several 100 mL and end up with enrichment factors between 103 and 104. By these means, LOQs in the ng/L- or even sub ng/L-range can be achieved with state-of-the-art mass spectrometers (Lee *et al.*, 2013; Jin and Zhu, 2016).

For quality assurance isotope labelled internal standards are available for BPA (e.g. 13C12 BPA, BPA-d16) and some other bisphenol analogues. As BPA and its analogues are chemicals with widespread applications, care must be taken to avoid sample contamination during sampling, sample transport and sample preparation in the laboratory. It is recommended to wash all glassware with methanol and if plastic cannot be avoided to use only high quality polypropylene to avoid contamination of the samples by bisphenols (Owczarek *et al.*, 2018).

3.2.5.3 Occurrence

The sources of BPA to the environment have been divided in the release from preconsumer and postconsumer sources. Preconsumer sources comprise those attributed to the production processes of BPA and BPA-containing products e.g. due the discharge of effluents from manufacturing plants. Transport and processing of BPA and BPA-containing products are additional sources for its preconsumer release. Postconsumer sources include those associated with disposal or waste including effluent discharge from municipal WWTPs, leaching from landfills, combustion of domestic waste, and degradation of plastics in the environment (Corrales *et al.*, 2015). Especially in developing countries high temperature conditions such as open burning of dumped waste have been identified as an important source to relocate BPA from plastic waste into the environment (Abraham and Chakraborty, 2020).

In general, scientific literature reports fairly good removal rates of bisphenol analogues in WWTPs. For instance, a Greek study revealed that around 90% of BPA was removed on average by conventional processes in six sewage treatment plants, when comparing median influent and effluent concentrations (Stasinakis *et al.*, 2008). However, care has to be taken when evaluating such data as removal efficiency highly depends on the applied treatment techniques. In a Canadian study highly heterogenous BPA removal was reported among the investigated wastewater treatment technologies that ranged from 1% to 77% as medians, with the highest removal rates observed in advanced WWTPs, followed by secondary WWTPs, lagoons, and primary WWTPs (Gewurtz *et al.*, 2021). Due to the structural similarities, it is not surprising that the applied treatment technologies also influence the degradation of other bisphenol analogues as it was reported for four different WWTPs in China. Here Qian *et al.* (2021) investigated nine bisphenol analogues and observed discrepant results regarding the removal efficiencies. For some BPs, such as BPE, BPF, BPZ and BPAF, and BPAP even negative removal rates were found in at least one WWTP. The authors also studied the degradation of the target compounds in a lab-scale continuous flow conventional activated sludge bioreactor. Here removal rates of >94% were found for BPA, BPB, BPE, BPF, BPM and BPS, whereas BPZ, BPAP, BPAF were found to be more recalcitrant.

In a recently published study Chiriac *et al.* (2021) investigated the fate of BPA and five other bisphenol congeners in two municipal WWTPs in Romania. BPF, BPB, and BPC could not be detected in any wastewater sample, whereas BPA was measured with up to 9.1 µg/L, BPS up to 1.7 µg/L and BPE up to 0,74 µg/L in the influent samples. High removal rates for the three bisphenols with positive findings were observed and maximum concentration in effluent samples drastically decreased to 0.075 µg/L for BPA. Concentrations of BPS and BPE were <LOQ in all effluent samples.

Lopez-Velazquez *et al.* (2021) proved that BPA concentrations are associated with the organic matter in the wastewater as they are positively correlated with the chemical oxygen demand (COD), the biochemical oxygen demand (BOD) and total suspended solids (TSS). It was also confirmed that warmer temperatures favored the removal of BPA. In the four studied WWTPs in Mexico BPA concentrations ranged from 0.0005 µg/L to 0.012 µg/L in summer time and from 0.0005 µg/L to 0.45 µg/L in winter time.

Despite the general high removal during wastewater treatment, WWTPs are still an important point source of BPA. Gewurtz *et al.* (2021) proved that BPA concentrations were significantly higher in surface water downstream compared to upstream of WWTPs in three of five urban areas in Canada. Interesting results were also obtained by Zhao *et al.* (2021) who proved that

even higher BPA mass loads can stem from rainfall run-off in urbanized areas than from WWTP discharges.

Only very few studies reported long-term temporal trends of BPA concentrations. But a Canadian research group only recently published results that showed a significant decrease at 10 of 16 monitoring sites evaluated between 2008 and 2018 (Gewurtz *et al.*, 2021). This was interpreted as a proof that governmental actions against the usage of BPA have been generally successful in reducing BPA concentrations in Canada. The authors also highlighted that long-term monitoring programs using surface water are more effective than to track other media for understanding future environmental trends of BPA, which is also an important outcome for the here presented research project.

As it can be seen from Table 15, logP values of bisphenol analogues range from 1.9 to 7.3 with a considerable share of representatives having logP values ≥4, which suggests their tendency to adsorb onto suspended matter or sediments in the aqueous environment. Furthermore, BPA is considered to be rapidly degraded in river water (Safakhah *et al.* (2020) and references therein). Nevertheless, numerous studies have reported the relevance of bisphenol analogues in various aqueous matrices all over the world, with most occurrence data reported for BPA (Table 16).

Nowadays a clear picture about the most important individual bisphenol analogue becomes more and more difficult. Still some publications mention BPA to be the predominant representative, e.g. Jin and Zhu (2016) reported a mean contribution of 75% in two Chinese river systems followed by BPS (approx. 20%) and BPAF (<5%). However, due to the proceeding replacement of BPA in several countries, it can be assumed that BPS can now also ubiquitously be found in the environment on a worldwide scale. Concentration levels of BPS in aquatic environments, have been reported to be almost comparable or equal to that of BPA together with a higher stability of BPS (Wu *et al.*, 2018). Elevated detection frequencies and concentrations of BPAF and BPF have also been found, e.g. BPF was the most abundant bisphenol analogue in surface water from some sites in Japan, Korea, and China, contributing to over 70% of the total concentrations on average (Chen *et al.*, 2016).

A selection of published concentrations of bisphenol analogues in scientific literature can be found in Table 16. For more data about the occurrence of bisphenol analogues in international water bodies the review articles of Chen *et al.* (2016), Jin and Zhu, (2016) and Wu *et al.* (2018) are recommended.

Table 16: Selection of reported occurrence data of BPA and bisphenol analogues in the aqueous environment

Compound name	Reported concentrations	Environmental compartment	Country	Reference
BPAF	up to 15.3 µg/L	river water around manufacturing plant i	China	Song *et al.* (2012)
BPA BPS BPF	<LOQ to 0.43 µg/L 0.0015 to 0.0087 µg/L <LOQ to 2.9 µg/L	3 rivers and Tokyo bay	Japan	Yamazaki *et al.* (2015)
BPA BPS BPF	0.001 to 0.27 µg/L <LOQ to 0.042 µg/L 0.12 to 1.3 µg/L	3 rivers	Korea	Yamazaki *et al.* (2015)

Compound name	Reported concentrations	Environmental compartment	Country	Reference
BPA BPS BPF	<LOQ to 0.098 µg/L <LOQ to 0.14 µg/L <LOQ to 1.1 µg/L	2 rivers	China	Yamazaki *et al.* (2015)
BPA BPS BPF	<LOQ to 2.0 µg/L <LOQ to 7.2 µg/L <LOQ to 0.29 µg/L	5 surface waters	India	Yamazaki *et al.* (2015)
BPA	0.0035 to 0.059 µg/L 0.011 ± 0.0053 µg/L,	tap water drinking water	Malyasia	Santhi *et al*, (2012)
BPA	0.002 to 13 µg/L Up to 0,18 µg/L	surface waters drinking water	Brazil	Montagner *et al.* (2019)
BPA	maximum 2.5 µg/L	wastewater samples from paper production industries	Austria	Fürhacker, Scharf and Weber, (2000)
BPA	0.097 µg/L	lake water	Taihu Lake, China,	Yan *et al.* (2017)
BPA	Max0.19 µg/L	sea water	Baltic Sea	Staniszewska *et al.* (2015)
BPA	0.01 to 45 µg/L	16 rivers	Taiwan	Lee *et al.* (2013)
BPA	0.028 to 0.57 µg/L	Taihu Lake and incoming creeks	China	Liu *et al.* (2016)
BPA	0.0011 to 0.0055 µg/L	surface water	Malaysia	Shehab, Jamil and Aris (2020)
BPS	0.00019 to 0.015 µg/L	120 surface water samples, Yangtze River	China	Wan *et al.* (2018)
BPA BPAF BPS BPF BPB	0.0066−0.075 µg/L 0.0009−0.25 µg/L 0.00029 to 0.019 µg/L <LOQ to 0.0035 µg/L All values <LOQ	surface water, Hangzhou Bay	China	Yang *et al.* (2014)
BPA, BPAF BPB BPE BPF BPS BPA, BPAF BPB BPE BPF BPS	<LOQ to 0.035 µg/L <LOQ to 0.011 µg/L <LOQ to 0.014 µg/L <LOQ to 0.0062 µg/L <LOQ to 0.013 µg/L <LOQ to 0.0052 µg/L <LOQ to 0.0065 µg/L <LOQ to 0.0047 µg/L <LOQ to 0.0032 µg/L <LOQ to 0.0006 µg/L <LOQ to 0.0009 µg/L <LOQ to 0.0016 µg/L	Source water for drinking water drinking water	China	Zhang *et al.* (2019)
BPA	median 0.10 µg/L, max.	surface waters	Canada	Gewurtz *et al.* (2021)

Compound name	Reported concentrations	Environmental compartment	Country	Reference
	6.4 µg/L downstream papermill			
BPA BPS BPE BPF BPB BPC	0.022-0.14 µg/L 0.006-0.008 µg/L <LOQ <LOQ <LOQ <LOQ	river waters (Danube and Jiu river)	Romania	Chiriac *et al.* (2021)

After reviewing the methodological foundations of in vitro test systems for monitoring membrane-bound estrogen receptor signaling pathways (Chapter 2) and the described analytical methods of compounds capable of modulating these pathways (Chapter 3), the subsequent focus was on developing, characterizing, and testing cell-based in vitro test systems for practical application. Details of this process are outlined in Chapters 4-6.

4 Development and Evaluation of cell lines expressing estrogen receptor GPER1

To understand how endocrine active substances affect rapid non-genomic signaling pathways, we need to monitor time-resolved changes in intracellular concentrations of specific second messengers. We focused on G-protein coupled estrogen receptor 1 (GPER1) and its interactions with membrane-bound multiprotein complexes, which can vary among different cell types. Our initial investigation involved various cell lines to observe how ß-estrogen influences Ca^{2+} mobilization or cAMP (cyclic adenosine monophosphate) formation, shedding light on GPER1 involved complex signaling cascades. GPER1 has been found to be expressed endogenously in the breast cancer cell line MCF-7, SKBR (Bologa *et al.*, 2006) and the skin cancer cell line A431 (Bubb *et al.*, 2022). Data on GPER1 expression at the protein level are contradictory, likely because highly specific antibodies are not yet available. In Figure 8Figure 8, a distinct band at around 50 kD is observed only in the HeLa and A431 extracts. Notably, this bands are discernible exclusively when using the GPER1 antibody developed by A. Lupp's team (Bubb *et al.*, 2022). The immunoreactive band of HeLa and A431 cells run beneath the signal of the recombinant GPER1 standard from Abcam. This suggest the presence of a truncated GPER1 or a posttranslationally modified GPER1 form. All other cells including cells stably transfected with the GPER1 gene express minimal GPER1 levels, insufficient for immunological detection in the Western blot. Therefore, we first screened cell lines described to express GPER1 endogenously for ß-estradiol dependent activities focusing on Ca^{2+} mobilization and cAMP formation.

4.1 Ca^{2+} Signalosome

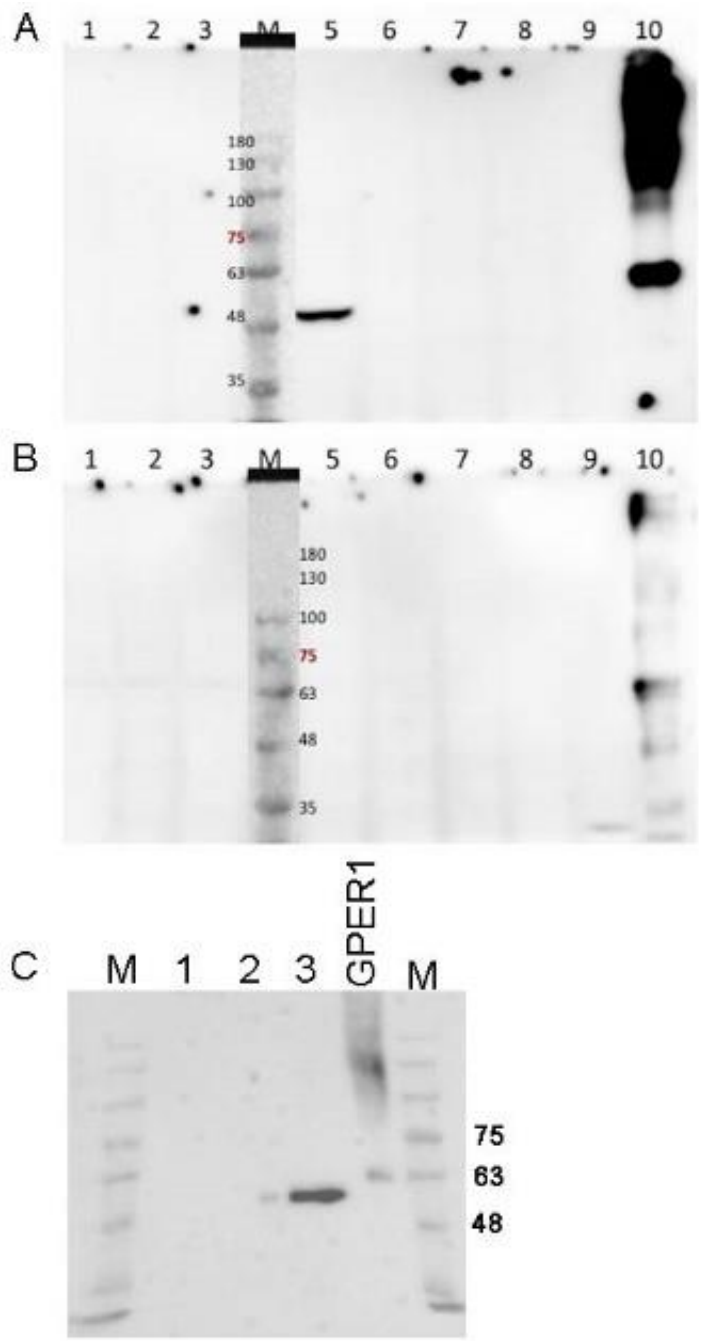
Ca^{2+} is a vital second messenger in cellular processes, operating at low levels within the cytosol to regulate Ca^{2+}-dependent enzymes. Maintaining this low Ca^{2+} concentration is challenging for the cell due to a substantial gradient, approximately 10,000-fold, between cytosolic Ca^{2+} and both intracellular store and extracellular Ca^{2+} concentrations. Ca^{2+} acts as a natural stressor, and its excessive deregulation can deplete endoplasmic reticulum (ER) Ca^{2+}, resulting in elevated cytosolic Ca^{2+}. In cases of mild cytosolic Ca^{2+} increases originating from stressing events, potentially harmful Ca^{2+} is sequestered within mitochondria and released once the stress subsides, with mitochondria playing a pro-survival role. However, if the amount of sequestered Ca^{2+} surpasses mitochondrial capacity, it can lead to collapse due to the opening of the permeability transition pore multi ion channel (PTP). This complex Ca^{2+} signaling system, involving amplification loops driven by the coordinated interplay of ER, IP3 channels, PTP and cytochrome c, can further elevate cytoplasmic Ca^{2+} levels, ultimately resulting in cellular damage and cell death (Cerella, Diederich and Ghibelli, 2010).

GPER1 dependent Ca^{2+} mobilization

A possible ß-estradiol dependent Ca2+ mobilization was assessed in 7 different cell lines (Figure 9Figure 8). Among the tested cell lines, only SKBR, a human breast carcinoma cell line, exhibited significant Ca^{2+} mobilization when exposed to µM concentrations of ß-estradiol. The half-maximal Ca^{2+} mobilization concentration (EC50) for ß-estradiol in SKBR cells was determined to be around 2.5 µM (Figure 10). This value is three order of magnitude higher than ß-estradiol peak concentrations measured in the blood of women. The Ca^{2+} concentrations used for measuring a Ca^{2+} mobility lie in the upper water soluble concentration range. In natural conditions, ß-estradiol is bound to serum proteins like the sex hormone-binding globulin, which affect its bioavailability (Anderson, 1974). However, our measurements were conducted in a

serum-free buffer without proteins and molecules that could interfere with fluorescence measurements. Therefore, higher hormone concentrations are required to observe an effect in this context.

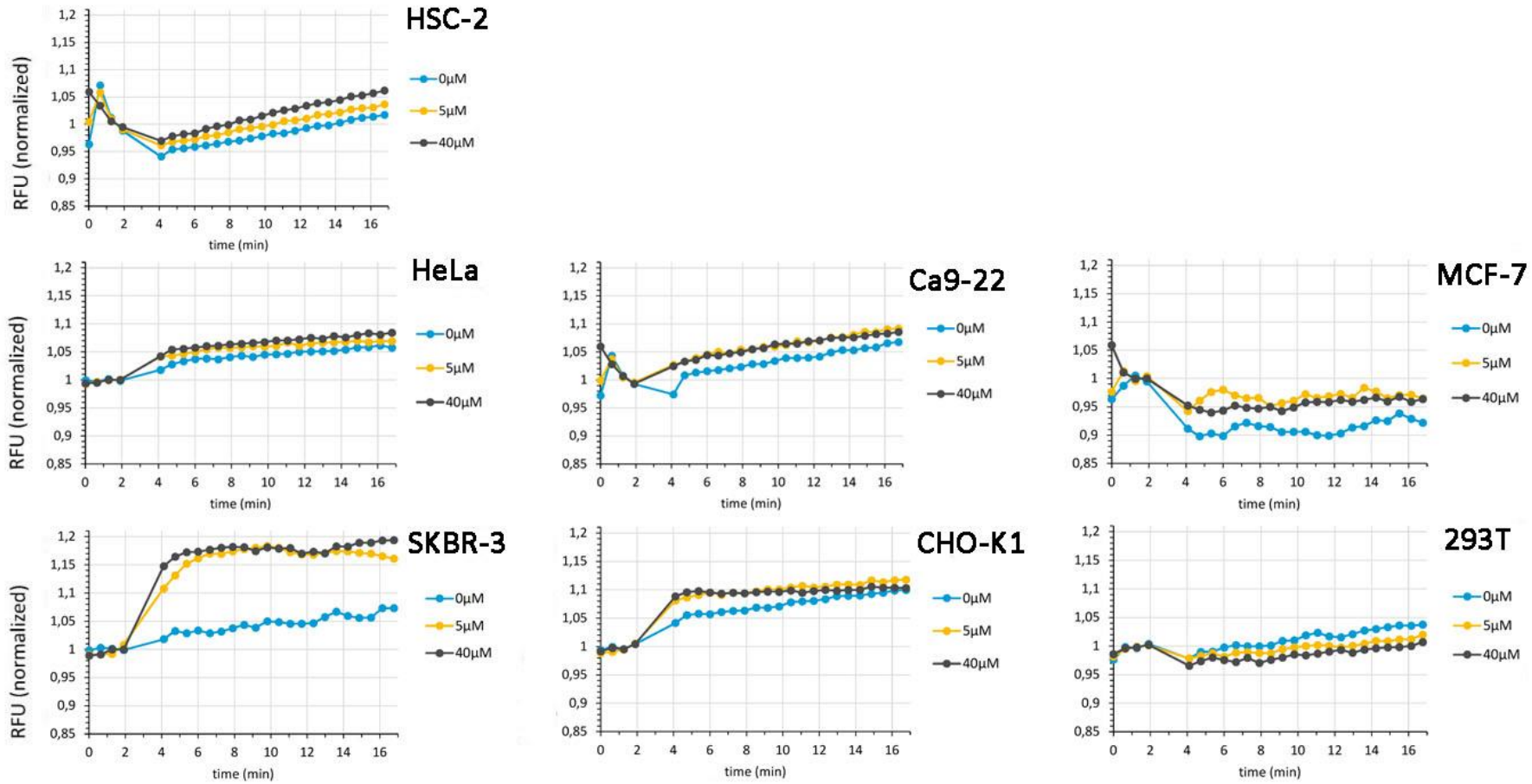
Figure 8: Western blot analysis of whole cell extracts with two GPER1 antibodies



Western blot analysis of whole-cell preparations from different cells using the polyclonal rabbit-anti-GPER1 (Abcam ab39742) (A) or the monoclonal rabbit-anti-GPER1 (A. Lupp) (B and C). Cell extracts from SKBR (1), SKBR stably transfected with CEPAC (2), SKBR stably transfected with GCaMP5 (3), HeLa (5), HSC (6), HSC stably transfected with CEPAC and GPER1 (7), HSC stably transfected with GCaMP5 and GPER1 (8), CHO stably transfected with a GPER1-eGFP fusion protein (9). 200 ng recombinant GPER1 (Abcam; ab152434) (10), M: marker. C: HSC (1), HSC stable transfected with GPER1 and GCaMP5, A431 (3), 200 ng recombinant GPER1 (Abcam; ab152434) (GPER1) M: marker. Samples were separated by 7.5% sodium

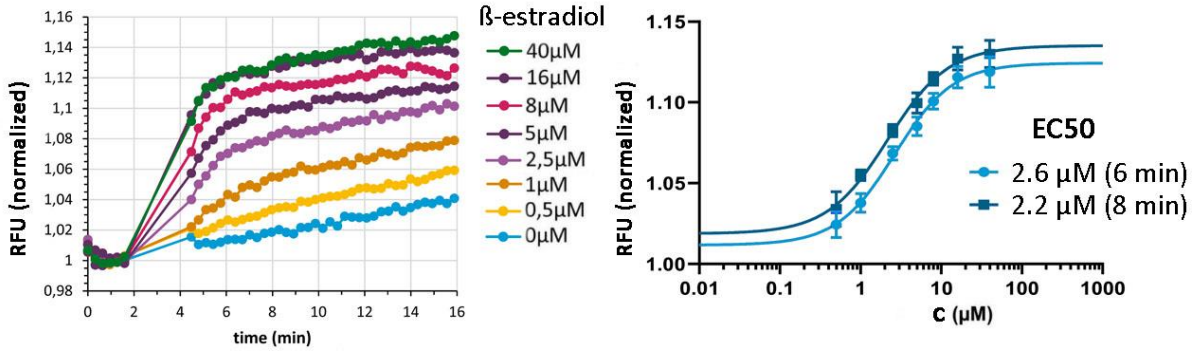
dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. The membranes were then incubated with non fat milk proteins, and the blots were developed using enhanced chemiluminescence. The relative migration of protein molecular weight markers is indicated (in kDa).
Source: own research, SIZ Zellkulturtechnik

Figure 9: ß-estradiol dependent Ca^{2+} mobilization



Intracellular Ca2+ concentrations were measured by using the fluorescent indicator dye Fluo-8-AM (Biomol) the membrane-permanent acetoxymethyl ester. Confluent cell monolayers were incubated with 1,5 µM Fluo-8-AM for 30 minutes at37°C in serum free HEPES buffer (pH7.4). Cells were washed with the same buffer to remove excess dye and incubated for 10 minutes in the microplate reader at 24°C in buffer containing 1% DMSO. Fluorescence (Excitation: 485 nm; Emission: 530 nm) was measured in relative fluorescence units (RFU). ß-estradiol was added in buffer containing 1% DMSO. Tested cell lines: HSC-2 (human oral carcinoma), HeLa (human cervix carcinoma), SKBR (human breast cancer), Ca9-22 (human oral carcinoma), CHO (hamster ovarial carcinoma), MCF-7 (human breast carcinoma), 293T (human kidney carcinoma)
Source: own research, SIZ Zellkulturtechnik

Figure 10: Dose-response curve of ß-estradiol-dependent Ca^{2+} influx in SKBR-3 cells



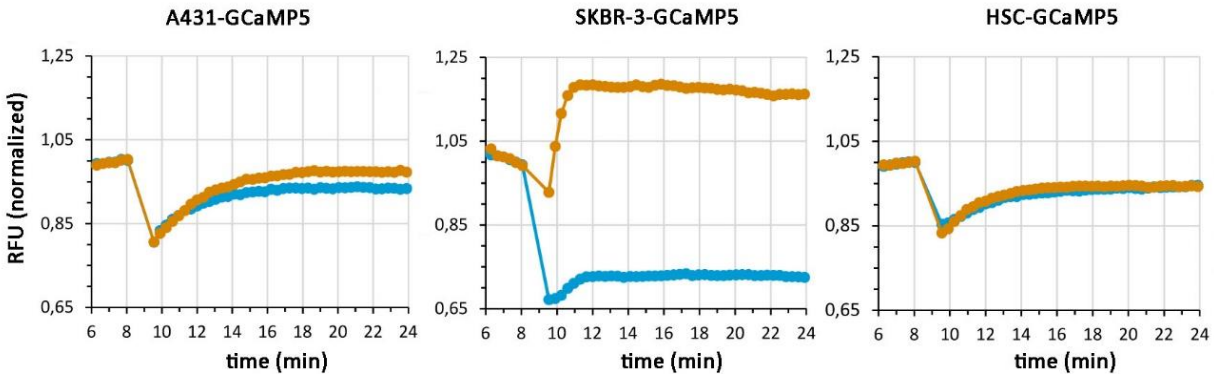
SKBR cells were subjected to varying ß-estradiol concentrations following Fluo-8 staining. Fluorescence measurements at 6 and 8 minutes were utilized to determine the EC50 value

Source: own research, SIZ Zellkulturtechnik

Published data on GPER1-dependent intracellular Ca^{2+} mobilization induced by ß-estradiol are conflicting. Revankar's group reported a ß-estradiol dependent Ca^{2+} mobilization in SKBR cells (Dennis *et al.*, 2009) and COS7 cells expressing a recombinant GPER1-GFP fusion protein (Revankar *et al.*, 2005), while Otto and colleagues (Otto *et al.*, 2008) did not observe ß-estradiol-stimulated Ca^{2+} mobilization in either transiently transfected or endogenously GPER1-expressing cells.

We stably transfected cells that naturally express GPER1 (A431, SKBR) and the oral cancer cell line HSC with the Ca^{2+} sensor protein GCaMP5 (Akerboom *et al.*, 2012). Following selection with G418, all cells exhibited green Ca^{2+} dependent fluorescence. In particular, **both cell lines expressing endogenious GPER1 displayed Ca^{2+} mobilization upon the addition of 50 µM ß-estradiol.** In contrast, the HSC cell line did not exhibit ß-estradiol-dependent Ca^{2+} mobilization (Figure 11). Therefore, we conclude that the cell line HSC does not express a functional GPER1 protein.

Figure 11: Ca^{2+} mobilization of GCaMP5 expressing cell lines



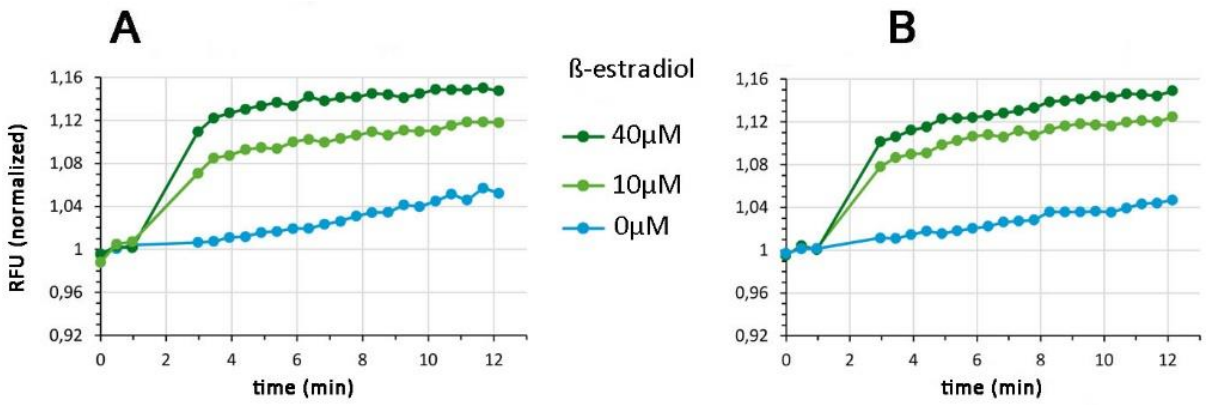
A431, SKBR and HSC cells were stably transfected with the Ca^{2+} sensor GCaMP5. Ca^{2+} fluorescence was measured at 520 nm emission following excitation at 485 nm. Cells cultured in 384 wells-plates were exposed to 50 µM ß-estradiol in HEPES buffer containing 0.2% DMSO. Time-resolved changes in fluorescence were recorded in relative fluorescence units (RFU). A control buffer with 0.2% DMSO was also included.

Source: own research, SIZ Zellkulturtechnik

GPER1 dependent Ca^{2+} mobilization is ß-estradiol specific and results in depletion of intracellular Ca^{2+} stores

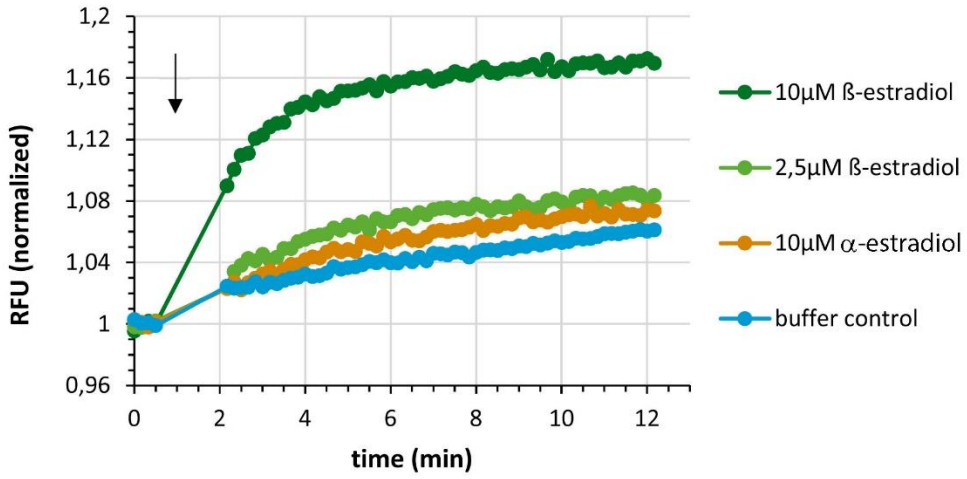
ß-estradiol caused a rapid Ca^{2+} rise in SKBR cells expressing the Ca^{2+} sensor GCaMP5. The GPER1-mediated cytoplasmic calcium rise is due to store calcium release because the Ca^{2+} mobilization was seen in both, Ca^{2+} free and Ca^{2+} containing buffer (Figure 12). The Ca^{2+} mobilization is ß-estradiol-specific, α-estradiol did not cause a Ca^{2+} rise (Figure 13).

Figure 12: ß-estradiol dependent Ca^{2+} mobilization in Ca^{2+} free buffer



SKBR-3 cells expressing the Ca^{2+} sensor GCaMP5 exhibit an identical ß-estradiol-dependent increase in cytoplasmic Ca^{2+} concentration in both Ca^{2+} free (A) and Ca^{2+} containing (B) buffer. Source: own research, SIZ Zellkulturtechnik

Figure 13: ß-estradiol specific Ca^{2+} mobilization



SKBR-3 cells expressing the Ca^{2+} sensor GCaMP5 exhibit an identical ß-estradiol-specific Ca^{2+} mobilization. α-estradiol was not able to cause a Ca^{2+} rise. The compound addition is indicated by an arrow.

Source: own research, SIZ Zellkulturtechnik

Developing a sensor cell line that measures estrogen-induced Ca^{2+} mobilization is challenging due to the commonality of Ca^{2+} mobilization as a pathway among G-protein coupled receptors (GPCRs). GPCRs can be categorized into three main types - Gαq, Gαs, and Gαi/o coupled receptors - each with its distinct cell-specific signal transduction mechanism, potentially leading to cellular Ca^{2+} mobilization(Dhyani *et al.*, 2020). According to Vassilatis et al., there are 367 GPCRs for endogenous ligands in human cells (Vassilatis *et al.*, 2003). Consequently, in order to measure GPER1-specific Ca^{2+} mobilization, it is necessary to compare identical cell lines, with and without recombinant GPER1 expression.

4.1.1 Development of a GPER1 Ca^{2+} sensor cell line

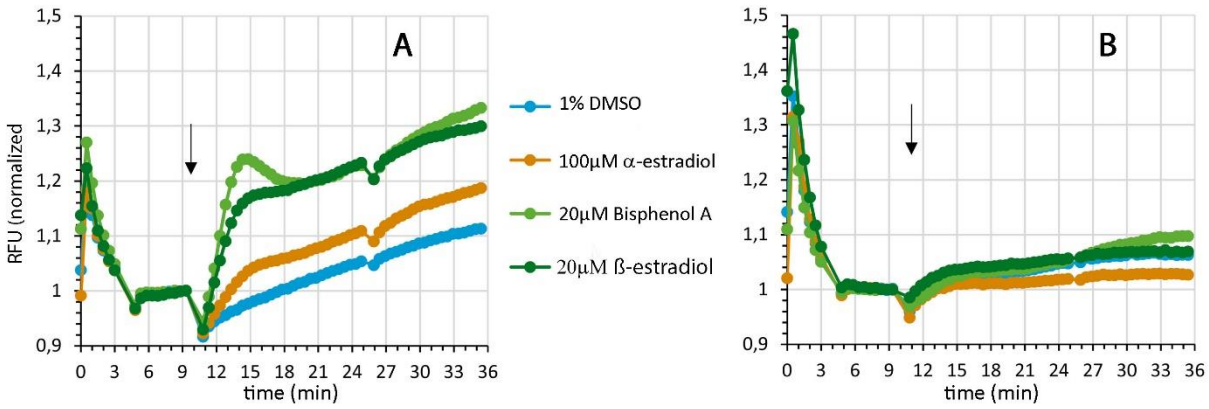
We choose the oral cancer cell line HSC for developing a GPER1 sensor cell line because this cell line did not show a ß-estrogen dependent Ca^{2+} mobilization (Figure 14). Both, the triplet optimized human gen coding for the G-protein coupled estrogen receptor 1 (GPER1) protein (NCBI Reference Sequence: NP_001035055) and the Ca^{2+} sensor GCaMP5 (Akerboom *et al.*, 2012) were cloned into the vector pWPI-IRES-Bla-AK (Addgene #154980). For the transduction of HSC cells lentiviral particles were produced by cotransfection of both, the GPER1 and GCaMP5 coding transferplasmid together with the lentiviral packaging plasmids pCMV-dR8.91 and pMD.G. Blasticidin S selection followed the transduction of HSC cells and resulted in fluorescent cells continuously expressing their transgenes. As a control the HSC cell was transducted with the Ca^{2+} sensor GCaMP5 only.

Upon the addition of ß-estradiol, only the GPER1-expressing HSC cell line displayed a significant Ca^{2+} mobilization response. The control HSC cell line, which was transduced with the Ca^{2+} sensor alone, did not exhibit any Ca^{2+} mobilization following ß-estradiol exposure. Importantly, the Ca^{2+} mobilization was specific to ß-estradiol; the addition of α-estradiol did not produce a significant Ca^{2+} signal (Figure 14).

GPER1 dependent Ca^{2+} mobilization is triggered by the binding of ß-estradiol to GPER1 located in the cellular membrane.

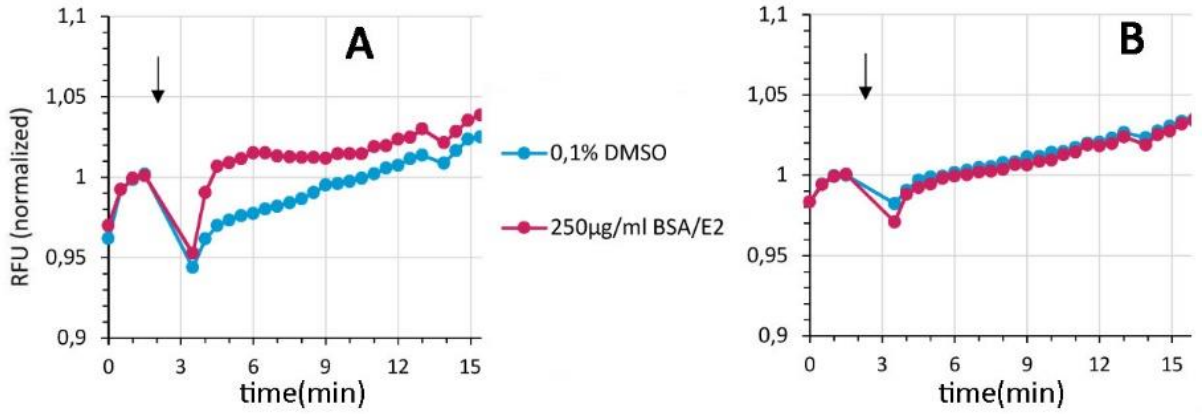
We investigated calcium mobilization following the addition of a BSA-ß-estradiol conjugate to confirm the localization of GPER1 in the membrane of HSC-GCaMP5-GPER1 cells. The addition of 0.5 mg/ml BSA-ß-estradiol conjugate, which corresponds to an approximate concentration of 180 µM ß-estradiol according to the manufacturer, resulted in a specific increase in cytoplasmic Ca^{2+} concentration only within the HSC-GCaMP5-GPER1 cells. HSC-GCaMP5 cells showed no Ca^{2+} mobilization (Figure 15). This observation underscores the presence of GPER1 activity in the outer cell membrane, as the BSA-coupled ß-estradiol cannot diffuse through the cell membrane.

Figure 14: GPER1 dependent Ca^{2+} mobilization



GPER1 expressing HSC Ca^{2+} sensor cells exhibit a Ca^{2+} mobilization following addition of a ß-estradiol or bisphenol A (A). HSC cells expressing only the Ca2+ sensor GCaMP5 showed no ß-estradiol-specific Ca^{2+} mobilization (B). Samples were added in 1% DMSO. Sample addition is indicated by an arrow. Source: own research, SIZ Zellkulturtechnik

Figure 15: GPER1-specific Ca^{2+} mobilization induced by a BSA-E2 conjugate

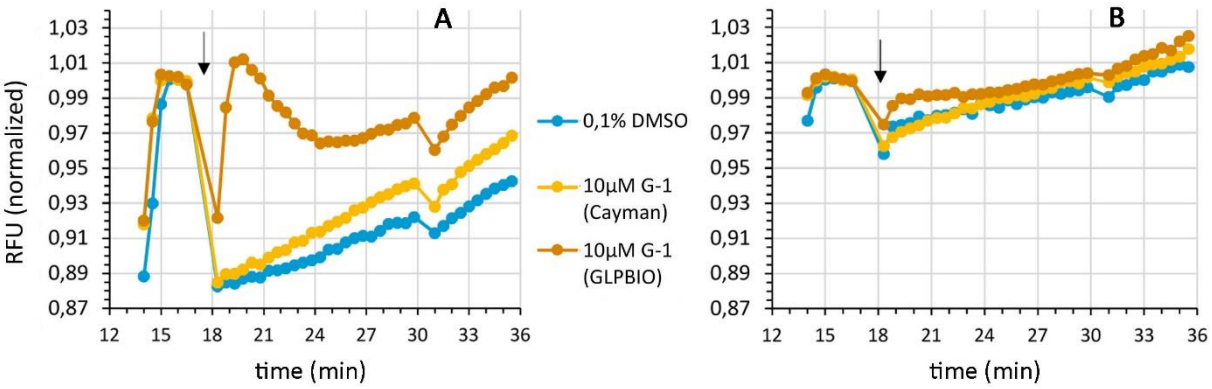


The addition of 0.5 mg/ml BSA-E2 conjugate, which corresponds to an approximate concentration of 180 µM ß-estradiol according to the manufacturer, resulted in a specific increase in cytoplasmic Ca^{2+} concentration only within the HSC-GCaMP5-GPER1 cells (A). HSC cells expressing only the Ca^{2+} sensor GCaMP5 showed no Ca^{2+} mobilization (B).
Source: own research, SIZ Zellkulturtechnik

G1 as GPER1 agonist was able to induce GPER1-specific Ca^{2+} mobilization in HSC-GPER1-GCaMP5 cells.

The addition of G1 (from GLPBIO), an agonist of GPER1 resulted in Ca^{2+} mobilization only within the GPER1 expressing HSC cell (Figure 16A). The HSC-GCaMP5 cell which does not express GPER1 showed no significant concentration change of cytoplasmic Ca^{2+}. Unexpectedly G1 from Cayman Chemicals was not active. Obviously the two G1 products differ in their GPER1 agonist activity.

Figure 16: G1-induced Ca^{2+} mobilization



The addition of G1 (from GLPBIO), an agonist of the classic ß-estrogen receptor resulted in Ca^{2+} mobilization only within the GPER1 expressing HSC cell (A). The HSC-GCaMP5 control cell showed no significant concentration change of cytoplasmic Ca^{2+} (B). Substance addition is marked by an arrow.
Source: own research, SIZ Zellkulturtechnik

The stability of the HSC-GCaMP5-GPER1 cell population is a concern. After just four months, these cells lose their ability to mobilize Ca^{2+} in response to ß-estradiol. This loss of function is presumably due to the stress induced by GPER1 activity, leading to selective pressure for GPER1

inactivation. In a heterogeneous mixed population, cells that no longer express functional GPER1 may outcompete others expressing GPER1, gaining a growth advantage.

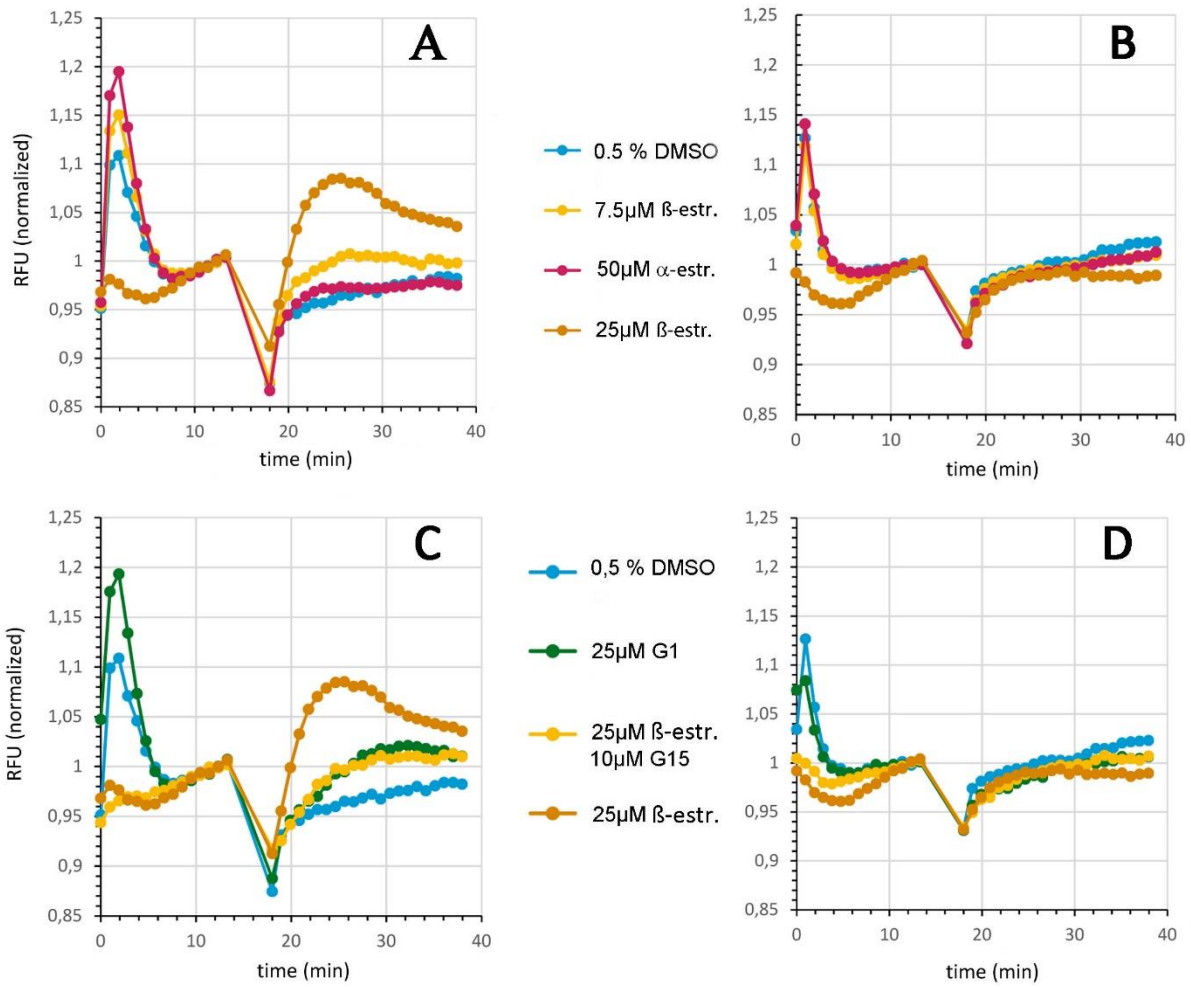
The HSC-GCaMP5-GPER1 subclone 6 shows a ß-estradiol-specific Ca^{2+} mobilization

To establish a stable cell population, we employed a strategy involving the transduction of HSC cells with both GPER1 and GCaMP5. Subsequently, single clones were isolated from the resulting mixed clone through subcloning and screened for GPER1 expression using ß-estradiol-mediated Ca^{2+} mobilization. One of these subclones, HSC-GCaMP5-GPER1 subclone 6 (SK6), exhibited a significant concentration-dependent Ca^{2+} mobilization in response to ß-estradiol.

SK6 exhibit a ß-estradiol-specific Ca^{2+} mobilization (Figure 17A). The HSC-GCaMP5 control cell line showed no ß-estradiol-specific Ca^{2+} mobilization (B). GPER1 within SK6 was inhibited by the GPER1 inhibitor G15 ((3aS*,4R*,9bR*)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3H-cyclopenta[c]quinolone). Preincubation with 10 µM of G15 inhibited the ß-estradiol-specific Ca^{2+} mobilization (Figure 17C). GPER1 within SK6 was activated by the GPER1 agonist G1 (1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c] quinolin-8-yl]-ethanone). 25µM G1 addition resulted in an increase of the cytoplasmic Ca^{2+} concentration (Figure 17C) which was not seen in the HSC-GCaMP5 control cell line (Figure 17 D).

This approach ensures the retention of functional GPER1 expression over time and enhances the stability of the cell population.

Figure 17: ß-estradiol-specific Ca^{2+} mobilization within HSC-GCaMP5-GPER1 subclone 6



The HSC-GCaMP5-GPER1 subclone 6 exhibit a ß-estradiol-specific Ca^{2+} mobilization (A). The HSC-GCaMP5 control cell line showed no ß-estradiol-specific Ca^{2+} mobilization (B). Addition of samples is indicated by an arrow. Samples were added in

0.5% DMSO. Preincubation with 10 µM of the GPER1 G15 inhibitor inhibited the ß-estradiol-specific Ca^{2+} mobilization within the HSC-GCaMP5-GPER1 subclone 6 (C). 25µM G1 addition resulted in an increase of the cytoplasmic Ca^{2+} concentration (C) which was not seen in the HSC-GCaMP5 control cell line (D).
ß-estr: ß-estradiol; α-estr.: α-estradiol; DMSO: dimethylsulfoxid; G1: GPER1 agonist G1; G15: GPER1 antagonist G15
Source: own research, SIZ Zellkulturtechnik

4.2 cAMP Signalosome

In addition to calcium, cAMP serves as a crucial downstream intracellular second messenger for G protein-coupled receptors (GPCRs). With over 200 GPCRs regulating receptor-specific cell functions primarily through cAMP modulation (Inoue *et al.*, 2019), cells face a formidable challenge in distinguishing between inputs from different GPCRs to ensure specific downstream cellular functions. Individual GPCRs signal via receptor-associated, independent cAMP nanodomains, forming self-sufficient, distinct cell signaling units known as cAMP signalosomes. These signalosomes comprise cell-specific, localized associations of receptor and protein components. GPER1 signaling cascades are known to involve the second messenger cAMP in a cell specific manner (see chapter 2.1).

GPER1 expressing cAMP sensor cell lines lack ß-estradiol-dependent cAMP signaling

To detect estrogen-dependent cAMP changes in cells, we stably transfected breast cancer cell lines MCF-7 (ACC 115, DSMZ) and SKBR 3 (ACC 736, DSMZ) with the cAMP sensor CEPAC (Salonikidis *et al.*, 2011). Resistant mixed clones continuously expressed CEPAC and exhibited CEPAC characteristic fluorescence. Both cell lines have been reported to express GPER1. In addition to GPER1, MCF-7 cells are known to express alpha- and beta-estrogen receptors, whereas SKBR cells do not express α- or ß-estrogen receptors (Ford *et al.*, 2011) (Yang *et al.*, 2019). Therefore, we expected to observe a ß-estradiol-dependent change in intracellular cAMP concentration. However, even after the addition of ß-estradiol at concentrations up to 10 µM, no change in intracellular cAMP concentration was observed. It's possible that the cellular expression of GPER1 is too low to induce a measurable cAMP change. Consequently, we prepared MCF-7 and SKBR cells that continuously expressed the CEPAC sensor and expressed GPER1 by an inducible system. Surprisingly, even with this modified setup, no change in cAMP concentration was observed after the addition of 10 µM ß-estradiol. We then transduced the HSC cell line with GPER1 and CEPAC. Resistant cells expressed the CEPAC sensor but also did not show a ß-estradiol dependent change in cytoplasmic cAMP concentration. It is unlikely that GPER1 activation induced cAMP formation by media components before the addition of ß-estradiol and thus prevented further cAMP formation. Control experiments showed that the addition of forskolin induced cAMP formation in the respective cell lines. Our results correspond to the work of Otto et al. (Otto *et al.*, 2008). It appears that the investigated cells lack functional GPER1 or ß-estradiol-dependent cAMP signaling pathways.

5 Examination of potentially endocrine-disrupting compounds

We selected different compounds to test for potential modification activities of cellular Ca^{2+} and cAMP signalosomes using our new cell-based *in vitro* assays. Initially, we examined analog compounds of bisphenol A due to its GPER1-specific Ca^{2+} mobilization observed in our in vitro assays (see Figure 14).

Tetrabromobisphenol A (TBBPA), which has not yet been regulated but classified as an endocrine disruptor is among the most utilized flame retardant in the industry globally. The total production of TBBPA was approximately 241,352 tons in 2016 and the global market for TBBPA is expected to increase 4% per year (Zhou, Yin and Faiola, 2020). TBBPA accumulates in a variety of aquatic organisms (Yang *et al.*, 2022) and was measured at concentrations up to 0,1 µM in breast milk (Shi *et al.*, 2017). Studies in human and animals suggest a correlation between TBBPA exposure and adverse health outcomes, namely thyroid disorders, neurobehavior and development disorders, reproductive health, immunological, oncological and cardiovascular diseases and metabolic disorders (Feiteiro, Mariana and Cairrão, 2021) (Miao *et al.*, 2023). However, in humans these effects are still poorly understood, once only a few data evaluated the human health effects. Among possible alternative flame retardants tetrachlorobisphenol A (TCBPA) and tetrabromobisphenol S (TBBPS) are discussed.

Halogenated bisphenol A flame retardants TBBPA and TCBPA modify both, the Ca^{2+} and the cAMP signalosomes independently of GPER1.

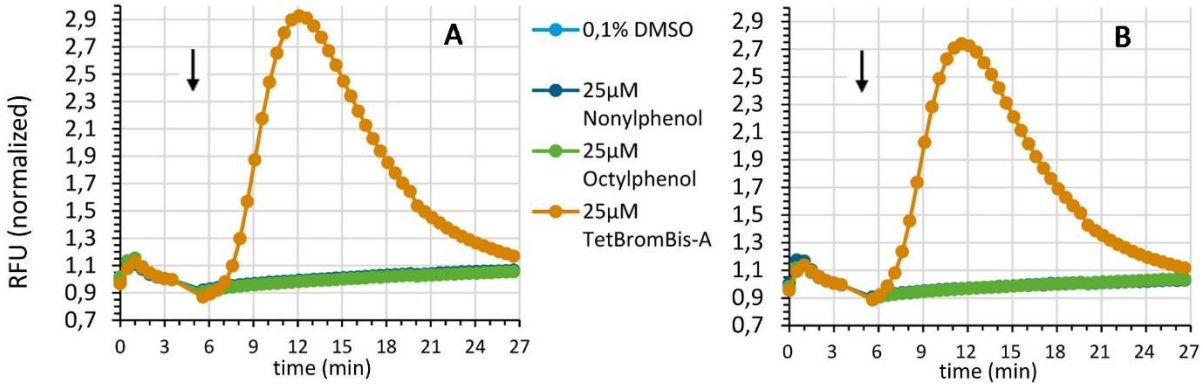
The halogenated flame retardants TBBPA and TCBPA both significantly modified various GPCR-dependent cellular pathways across all tested cell lines. This modulation affected both cAMP and calcium signaling pathways simultaneously, suggesting an interaction of these flame retardants with a common domain shared among different G protein-coupled receptors. An interaction with such a central GPCR domain would have effects on a wide variety of signaling pathways and could explain the various negative effects on human health that have been observed.

Addition of TBBPA to the Ca^{2+} sensor cell lines SKBR-GCaMP5, HSC-GCaMP5, and HSC-GPER1-GCaMP5 at µM concentrations resulted in a significant increase in cytoplasmic Ca^{2+} concentration. Notably, this Ca^{2+} mobilization was not specific to GPER1, as both GPER1-expressing HSC-GPER1-GCaMP5 and non-expressing HSC-GCaMP5 cell lines exhibited the same response.

However, in contrast, endocrine disruptor compounds like 4-nonylphenol and 4-tert.-octylphenol did not elicit any change in cytosolic Ca^{2+} concentration in both cell lines (Figure 18).

Furthermore, unlike TBBPA and TCBPA, tetrabromobisphenol S (TBBPS) did not induce Ca^{2+} mobilization in HSC-GCaMP5 cells (Figure 19).

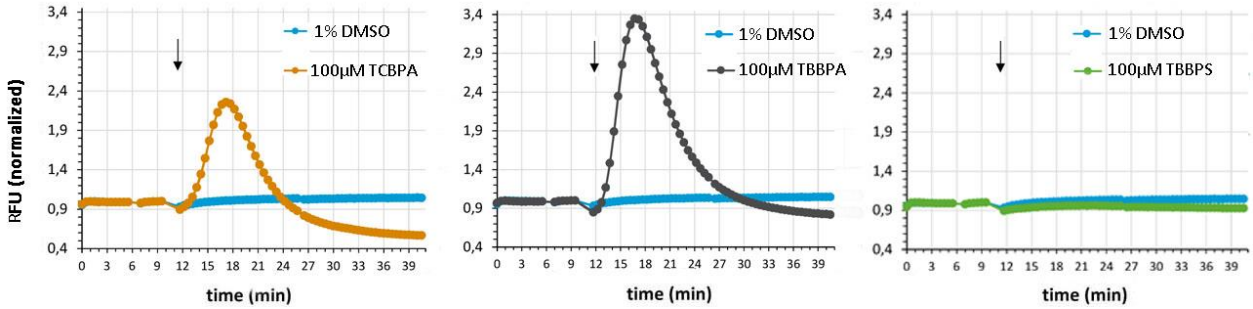
Figure 18: Ca^{2+} mobilization of tetrabromobisphenol A



25 µM tetrabromobisphenol A mobilized Ca^{2+} in both, HSC-GCaMP5 and HSC-GPER1-GCaMP5 cells. 4-tert.-Octylphenol and 4-nonylphenol showed no effect. Addition of compounds is indicated by an arrow.

Source: own research, SIZ Zellkulturtechnik

Figure 19: Ca^{2+} Mobilization by TBBPA, TCBPA and TBBPS in HSC-GCaMP5 cells

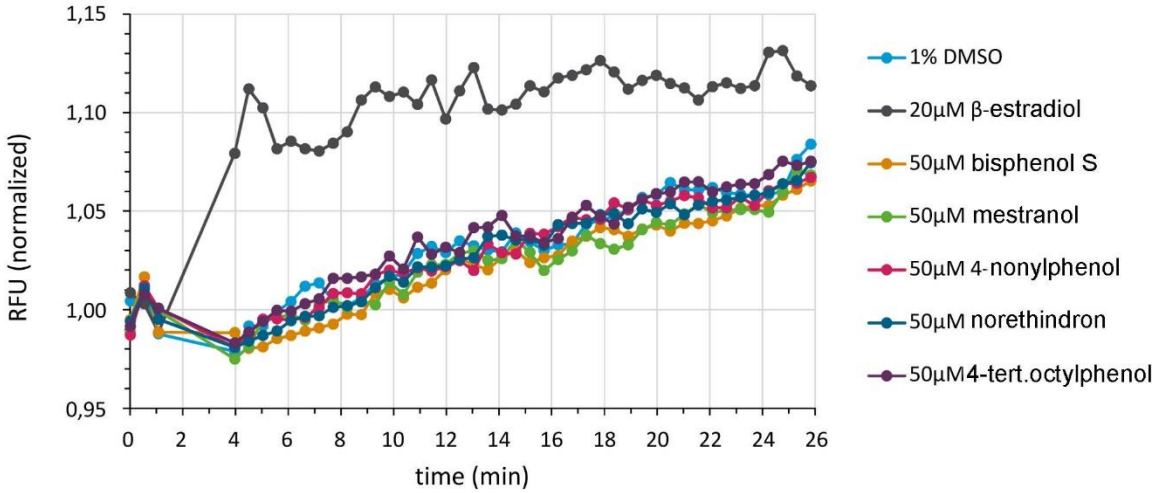


In contrast to TBBPS and TCBPS tetrabromobisphenol S (TBBPS) TBBPS showed no Ca2+ mobilization within HSC-GCaMP5 cells. Addition of compounds is indicated by an arrow.

Source: own research, SIZ Zellkulturtechnik

At a concentration of 50 µM, known endocrine disruptor compounds 4-nonylphenol, 4-tert.-octylphenol, as well as mestranol (a prodrug of ethinylestradiol) and the Bisphenol A substitute Bisphenol S did not induce Ca^{2+} mobilization in SKBR cells (Figure 20).

Figure 20: Characterization of Ca^{2+} mobilization by known endocrine disruptor compounds in SKBR cells



SKBR cells stained with the calcium indicator fluo8 showed no Ca^{2+} modification when incubated with the known endocrine disruptor compounds 4-nonylphenol and 4-tert.-octylphenol as well as mestranol (a prodrug of ethinylestradiol) and the

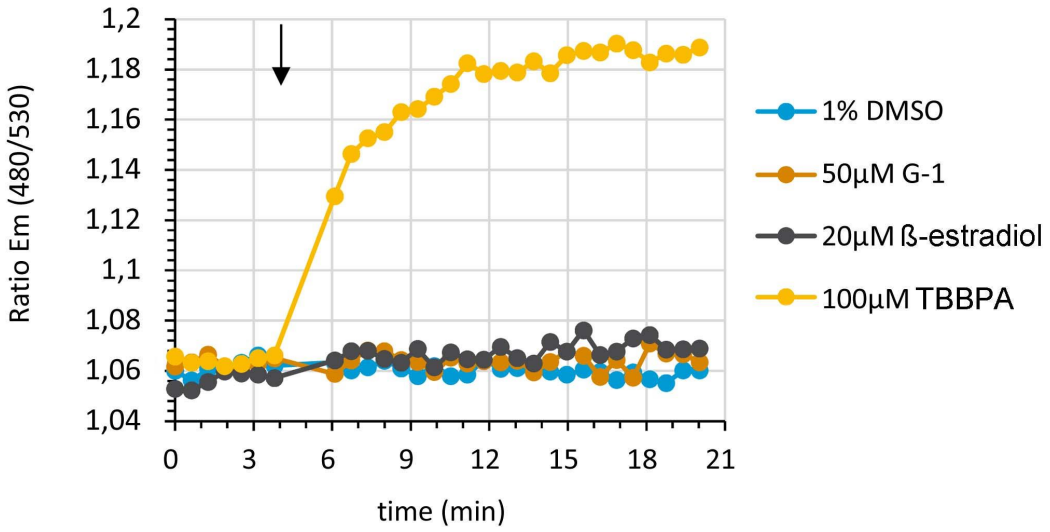
Bisphenol A-substitute Bisphenol S. Positive control: 20µM ß-estradiol

Source: own research, SIZ Zellkulturtechnik

Addition of Tetrabromobisphenol A (TBBPA) induced a rapid increase in cAMP concentration in SKBR-CEPAC cells (Figure 21). Similarly, the addition of Tetrachlorobisphenol A (TCBPA) to SKBR-CEPAC cells led to a concentration-dependent and rapid increase in cellular cAMP levels. TCBPA exhibited a half-maximal effect concentration (EC50) of 5 µM (Figure 22 and Figure 23).

The flame retardants TBBPA and TCBPA, modulating both, the Ca^{2+} and cAMP signalosome, appear to interact with a shared domain among different G protein-coupled receptors. This suggests the possibility of both agonistic and antagonistic interactions. Consequently, we studied the potential antagonistic effect of halogenated bisphenol A derivatives on the b1-adrenoceptor, a crucial receptor involved in blood pressure regulation. For this study, we utilized a CHO cell line expressing the cAMP sensor CEPAC and the human b1-adrenoceptor (Bernhard *et al.*, 2017). In contrast to TCBPA and tetrachlorobisphenol S (TBBPS), preincubation with 100µM TBBPA inhibited the isoproterenol-induced activation of the b1-adrenoceptor (Figure 24).

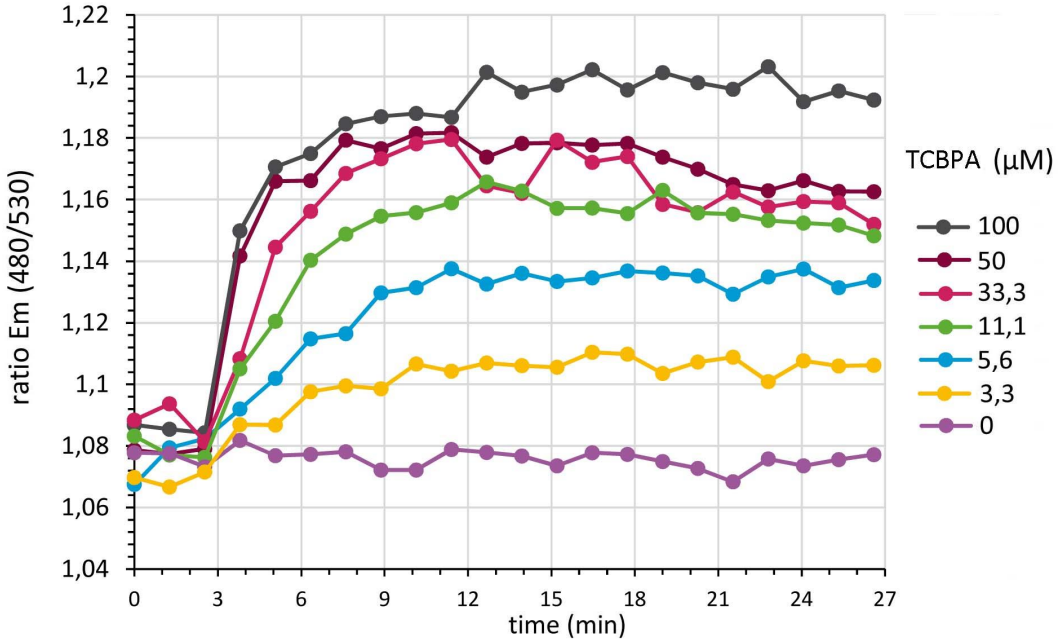
Figure 21: TBBPA initiated cAMP generation in SKBR-CEPAC sensor cells



Tetrabromobisphenol A (TBBPA) addition induced a rapid cAMP concentration increase in SKBR-CEPAC cells. Increasing intracellular cAMP levels result in an increase in the emission fluorescence ratio at 470 nm vs. 535 nm when excited at 420 nm.

Source: own research, SIZ Zellkulturtechnik

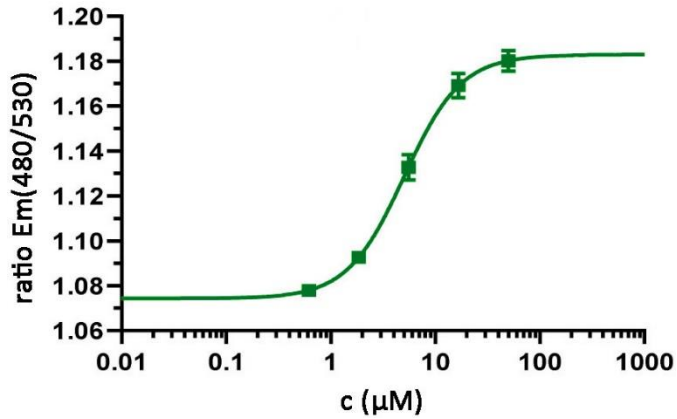
Figure 22: TCBPA-dependent cAMP induction within SKBR cells



Tetrachlorobisphenol A (TCBPA) induced a rapid cAMP concentration increase in SKBR3-CEPAC cells.

Source: own research, SIZ Zellkulturtechnik

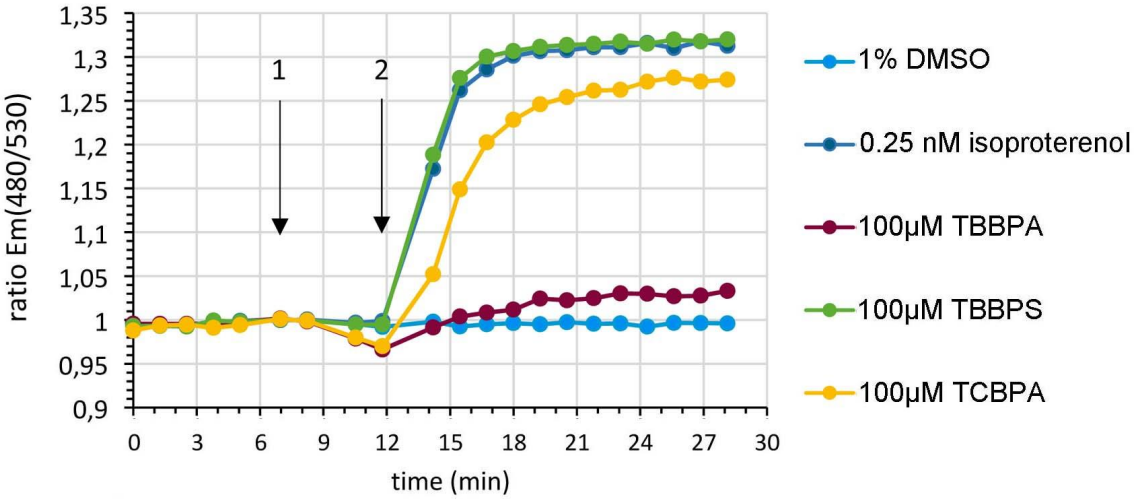
Figure 23: Concentration-response curve of TCBPA



The half maximal effect concentration (EC50) of 5 µM was calculated for TCBPA by fitting the concentration response curve (sigmoidal fitting, 4 parameters, graph pad prism). For the concentration-response curve, the means of three measurements (seven minutes after substance addition) were calculated.

Source: own research, SIZ Zellkulturtechnik

Figure 24: Antagonistic activity of TBBPA on the b1-adrenoceptor



In CHO-K1 cells co-expressing the b1-adrenoceptor and the cAMP sensor CEPAC, pre-incubation with 100 µM TBBPA (1) inhibited isoproterenol-dependent cAMP formation triggered by the addition of 0.25 nM isoproterenol (2).
Source: own research, SIZ Zellkulturtechnik

Interestingly TBBPS also exhibits significantly lower anti-androgenic activity compared to TBBPA. TBBPA has an EC50 of 0.3 µM, whereas TBBPS has a much higher EC50 of 6 µM, as determined by the YAAS assay (Natalie Reininger, University Frankfurt, personal communication).

In a recent paper, Li et al. reported that neonicotinoid insecticides such as clothianidin, acetamiprid, and dinotefuran had an agonistic effect on GPER1 (Li *et al.*, 2022). Li et al. tested these compounds, which are found in the environment, in calcium mobility assays using SKBR cells that naturally express GPER1. The specificity of GPER1 activation was confirmed by the inhibition of effects using GPER1 antagonists.

However, in our GPER1 sensor cell line, HSC-GPER1-GCaMP5, we were unable to detect any potential GPER1 activation. As a result, we cannot confirm the data published by Lis group (Li *et al.*, 2022) and propose that the insecticides clothianidin, acetamiprid, and dinotefuran may not activate GPER1.

The detection of GPER1-specific activation of intracellular signaling was achieved by comparing sensor cell lines expressing a functional GPER1 and respective sensor cell lines expressing no active GPER1. ß-estradiol, the GPER1 agonist G1 and the endocrine disruptor bisphenol A mobilized intracellular Ca^{2+} specifically in the GPER1 expressing sensor cell line HSC-GPER1-GCaMP5. This Ca^{2+} mobilization was exclusive to GPER1, as these compounds had no effect on the GPER1 non-expressing HSC-GCaMP5 control cell line. Conversely, halogenated bisphenol A derivatives TBBPA and TCBPA modulated both, the Ca^{2+} and cAMP signaling in all tested sensor cells, indicating a GPER1-unspecific property. These compounds seemingly interact with a shared domain among different G protein-coupled receptors. Table 17 summarizes selected compounds and their effects on selected sensor cell lines.

Table 17: Selected compounds and their effects on selected sensor cell lines

compound	HSC-GCaMP5	HSC-GPER1-GCaMP5	SKBR3	SKBR3-CEPAC	b1-adrenoceptor
ß-estradiol	no effect	activation	activation	no effect	
Bisphenol A	no effect	activation			
G1	no effect	activation		no effect	
G15	no effect	antagonistic activity			
BSA-E2	no effect	activation			
ß-estradiol	no effect	no effect	no effect		
TBBPA	activation	activation	activation	activation	antagonistic activity
TCBPA	activation	activation	activation	activation	antagonistic activity
TBBPS	no effect		no effect		no effect
mestranol			no effect		
4-nonylphenol			no effect		
norethindron			no effect		
4-tert octylphenol			no effect		
isoproterenol					activation
Insecticides (clothianidin acetamiprid dinotefuran)		no effect			

no effect: compounds do not modify intracellular Ca^{2+} concentrations (HSC-GCaMP5, HSC-GCaMP5-GPER, SKBR3), do not induce cAMP synthesis (SKBR3-CEPAC) and do not modify b1-receptor activation (b1-adrenoceptor)

activation: increases intracellular Ca^{2+} concentration (HSC-GCaMP5, HSC-GCaMP5-GPER, SKBR3), induces intracellular cAMP synthesis (SKBR3-CEPAC, b1-adrenoceptor)

antagonistic activity: inhibits ß-estradiol induced Ca^{2+} mobilization (HSC-GCaMP5-GPER), inhibits isoproterenol induced cAMP synthesis (b1-adrenoceptor)

6 Assessing the practical applicability of the developed *in vitro* bioassays.

In recent decades, the introduction of environmental contaminants into our surroundings has reached alarming levels. These contaminants include trace amounts of emerging pollutants such as hospital wastewater, cosmetics, personal care products, and endocrine active substances, as well as their transformation products. Despite their relatively recent detection, limited information is available regarding their characteristics, effects, and environmental impact. Furthermore, conventional wastewater treatment methods often fail to completely remove endocrine active substances, which can exhibit ecotoxicity even at low concentrations. Consequently, there is a pressing need to enhance wastewater treatment methods through advanced (fourth) treatment stages.

The efficacy of a fourth wastewater treatment step in removing environmental contaminants depends on the treatment technique and the properties of the substances involved, including polarity and oxidizability. Currently, the advanced wastewater treatment methods include ozonation and powder or granular activated carbon absorption. Ozonation has proven effective in degrading a majority of environmental contaminants through oxidation. However, this process requires ozone doses which could result in the formation of potentially harmful transformation products. On the other hand, activated carbon absorption successfully removes non-polar compounds but falls short in eliminating polar micropollutants.

Our newly developed effect-directed cell-based assays, designed to monitor modifications in central GPCR-involved signal cascades, could provide valuable insights for the detection and identification of compounds with toxicological properties in water quality research. Specifically, assays that assess the modulation of the membrane-bound G-protein coupled estrogen receptor have the potential to identify special endocrine disruption activities within complex mixtures such as wastewater treatment effluents. In light of this, we conducted a characterization of influents and effluents in the advanced treatment processes of five wastewater treatment plants in Germany. Our analysis included samples from wastewater treatment plants employing granular activated carbon absorption, powder activated carbon absorption, or ozonation as advanced treatment methods. In comparative studies these influents and effluents were analyzed using effect-based cellular assays. These tests assessed cyclooxygenase inhibition to detect nonsteroidal anti-inflammatory drugs, cAMP monitoring to evaluate cAMP modulating compounds like halogenated flame retardants, and Ca^{2+} concentration changes to monitor possible Ca^{2+} modulating substances.

6.1 Cycloxigenase inhibition

Inflammatory mediators often inhibit cyclooxygenase (COX) and are frequently detected in European surface water at concentrations exceeding the respective Predicted No Effect Concentration (PNEC) (Simon *et al.*, 2022). To detect COX-inhibiting pollutants in wastewater treatment plant effluent, we followed a method similar to Bernhard et al. (Bernhard *et al.*, 2017) We concentrated the wastewater 18-fold through vacuum concentration, extracted the residue using a cell-compatible aqueous buffer, and analyzed it with the COX sensor cell.

Table 18 illustrates diclofenac equivalents (DicEQ) in ng/L for the influents and effluents of the 4th purification stage. It's evident that all effluents from the 4th purification stage showed a reduction in pollutant effect compared to their corresponding influents. However, the purification performance varied significantly.

In three out of the five tested effluents, the pollutant load of diclofenac equivalents (DicEQ) remained significantly above the PNEC threshold which is set at 50 ng/l. Ozonation, utilized in one of the five wastewater treatment plants, achieved the best purification performance, reducing the pollutant effect by 95%.

The advanced treatment employing granulated activated carbon used by wastewater treatment plant 3, 4 and 5 exhibited differences in purification performance, which could be attributed to variations in process management, leading to divergent purification outcomes.

Table 18: Performance of wastewater treatment plants in eliminating of NSAID compounds

wastewater treatment plant	Advanced treatment	Influent DicEQ (ng/L)	Effluent DicEQ (ng/L)	Reduction
1	Powder activated carbon absorption	1570	237	85 %
2	Ozonation	1525	70	95 %
3	Granular activated carbon absorption	607	74	88 %
4	Granular activated carbon absorption	814	237	71 %
5	Granular activated carbon absorption	459	133	71 %

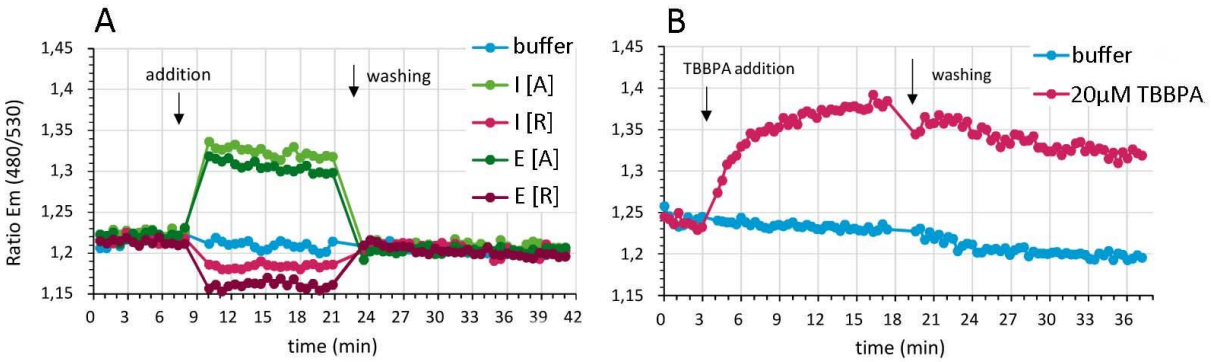
6.2 cAMP modulation

As outlined in section 5, halogenated bisphenol A flame retardants have the potential to induce cAMP modulation within the low µ-molar range. To investigate the potential for cAMP modulation in the influents and effluents of wastewater treatment plant 2, we developed an assay using SKBR3-CEPAC cAMP sensor cells (section 4.2). To prepare the samples for analysis, we subjected the water samples to a 40-fold concentration through vacuum concentration.

For the cell-based assay, we separated the samples into two fractions: those soluble in acetonitrile and those insoluble. The residues from the vacuum concentration were initially extracted with acetonitrile. After removing the acetonitril, we dissolved the resulting pellet in aqueous buffer, samples denoted as [A] in Figure 25A. The acetonitrile-insoluble residue was similarly dissolved in aqueous buffer, represented as [R] in Figure 25A. Following sensor cell incubation with the compound containing buffer, we eliminated fluorescent compounds which are present in the incubation buffer leading to a cAMP unspecific signal increase in [A] and an unspecific signal decrease in [R] by washing the cells with fresh buffer. This washing step did not change the intracellular cAMP concentration (Figure 25B).

Notably, none of the wastewater treatment plant samples demonstrated the ability to modulate cAMP in the cAMP sensor cells, as depicted in Figure 25A. To serve as a positive control for cAMP mobilization in the SKBR3-CEPAC cAMP assay, we utilized the flame retardant tetrabromobisphenol A (TBBPA), as illustrated in Figure 25B.

Figure 25: cAMP mobilization assay induced by wastewater treatment plant samples



A: cAMP mobilization effects in influent (I) and corresponding effluent (E) of ozone advanced treatment stage. We examined both acetonitrile extract [A] and acetonitrile insoluble fraction [R]. B: Positive control using the flame retardant tetrabromobisphenol A (TBBPA). Buffer: buffer control. A buffer washing step was conducted 15 minutes after addition of the samples to SKBR sensor cells.

Source: own research, SIZ Zellkulturtechnik

6.3 Ca^{2+} modulation

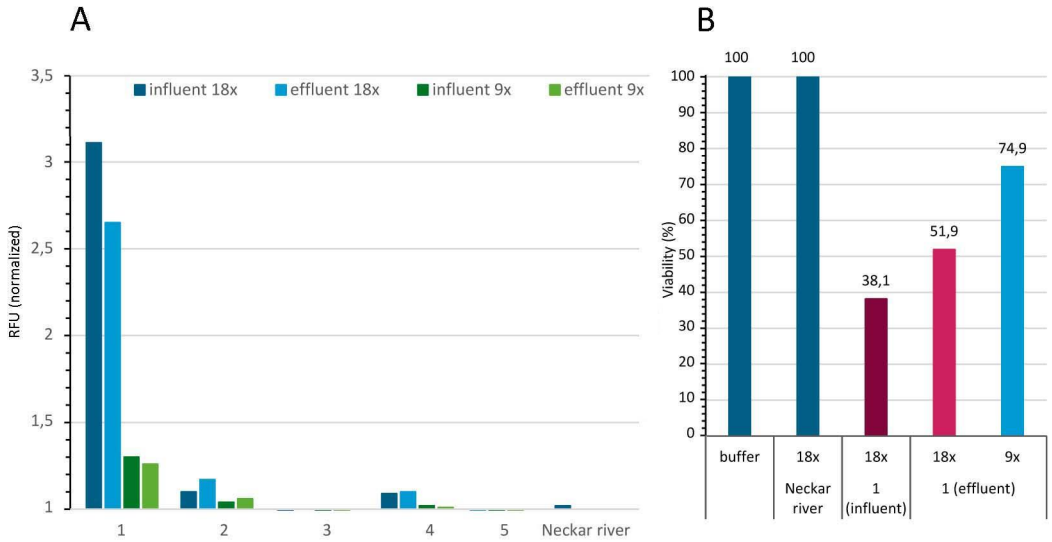
G protein-coupled receptors (GPCRs) are expressed in all living cells, and many of them are simultaneously activated on the cell membrane. These activations trigger controlled signaling cascades within locally confined regions known as signalosomes. The composition of these signalosomes varies from one cell to another.

In the breast cancer cell line A431, the membrane-bound estrogen receptor (GPER1) is expressed. We genetically modified this A431 cell line with the Ca2+ sensor protein GCaMP5 (as described in section 4.1), to investigate possible Ca^{2+} modulating activities in the influent and effluent of advanced treatment units of wastewater treatment plants.

Two minutes after adding the respective sample, we measured Ca^{2+}-dependent fluorescence, which is presented as normalized relative fluorescence units (RFU normalized). We applied influents from advanced treatment stages as both 18-fold and 9-fold concentrated samples from five wastewater treatment plants (WWTPs) (Figure 26A). In particular, the 18-fold concentrated influents and effluents from the advanced treatment unit of one WWTP (WWTP 1, using powder activated carbon as advanced purification unit) induced a significant Ca^{2+} mobilization, resembling a Ca^{2+} tsunami. This significant Ca^{2+} mobilization led to immediate cell damage, which we confirmed through the cytotoxicity MTT assay (Figure 26B).

We observed a weaker Ca^{2+} mobilization in the influents and effluents of the advanced treatment unit in WWTP 2 and 4. However, no Ca^{2+} mobilization was detected in WWTP 3 and 5, as well as in control samples (18x drinking water, 18x well water, 18x river water). The Ca^{2+} mobilization effect observed in WWTP 1 was also confirmed during a second sampling. This damage appears to persist continuously in the influent of this WWTP.

Figure 26: Ca^{2+} mobilisation induced by wastewater treatment plant samples



A: To characterize Ca^{2+} mobilization, we used the Ca^{2+} sensor cell A431-GCaMP5. Ca^{2+}-dependent cellular fluorescence was measured 2 minutes after the addition of each sample, presented in normalized relative fluorescence units (RFU normalized). Influents of advanced treatment units of WWTP 1-5 were used as 18-fold concentrated samples (in blue) and 9-fold concentrated samples (in green). 18-fold concentrated water from the Neckar was used also.

B: 18-fold and 9-fold concentrated samples were investigated for their possible cytotoxic effects using the A431 sensor cell through the MTT test. In addition to WWTP samples from WWTP 1, 18-fold concentrated Neckar river water and a buffer control were used.

Source: own research, SIZ Zellkulturtechnik

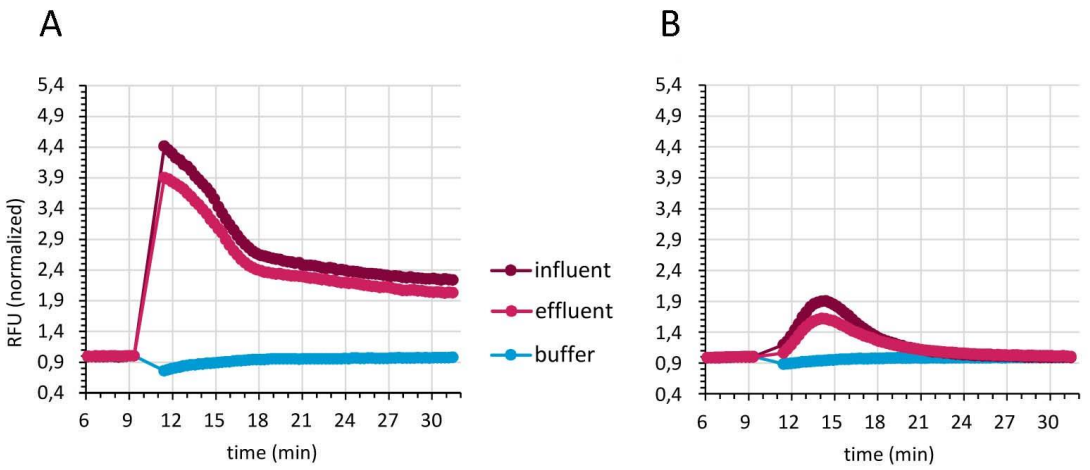
To evaluate potential endocrine-disrupting effects, we utilized a sensor cell with functional GPER1 (HSC-GCaMP3-GPER1 SK6, 4.1.1) alongside cells without functional GPER1 (HSC-GCaMP5, 4.1.1) to characterize influent and effluent of the advanced treatment unit of WWTP 1 for GPER1-dependent Ca^{2+} mobilization.

Surprisingly, both 18-fold concentrated influent and effluent of the WWTP 1 advanced treatment unit induced a robust Ca^{2+} mobilization in the GPER1-expressing sensor cells. This Ca^{2+} wave in the GPER1-expressing sensor cells exceeded the intensity of the Ca^{2+} tsunami observed in A431 sensor cells (Figure 27A). The compounds, which trigger this effect, are polar and are not efficiently eliminated from the wastewater through activated carbon treatment. In contrast, the 18-fold concentrated influent and effluent from the advanced treatment unit of WWTP 1 induced only a minor Ca^{2+} mobilization in the HSC-GCaMP5 cell line lacking functional GPER1 (Figure 27B). This suggests the involvement of the GPER1 receptor, although the Ca^{2+} mobilization seems to be influenced by mechanisms independent of GPER1.

The compounds responsible for the Ca^{2+} mobilization exhibit high oxidation stability. We conducted ozone treatment on both the influent and effluent of the activated carbon unit at WWTP 1, using an ozone dose of 0.5 mg ozone per mg of dissolved organic carbon. Subsequently, we analyzed samples before and after ozonation for their Ca^{2+} mobilization activity across three cell lines (Figure 28).

Surprisingly, ozone treatment did not result in any alteration of the Ca^{2+} mobilization activity. These compounds, which neither bind to activated carbon nor can be oxidized by ozone, demonstrate resistance to common advanced treatment techniques employed by WWTPs, indicating the need for alternative strategies for their removal.

Figure 27: Ca^{2+} mobilisation in HSC-GCaMP5-GPER1 SK6



18-fold concentrated influent and effluent samples of the WWTP 1 advanced treatment unit induce a significantly stronger Ca^{2+} mobilization in the GPER1-expressing sensor cells HSC-GCaMP5-GPER SK6 (A) compared to the non-GPER1-expressing control cell HSC-GCaMP5 (B).

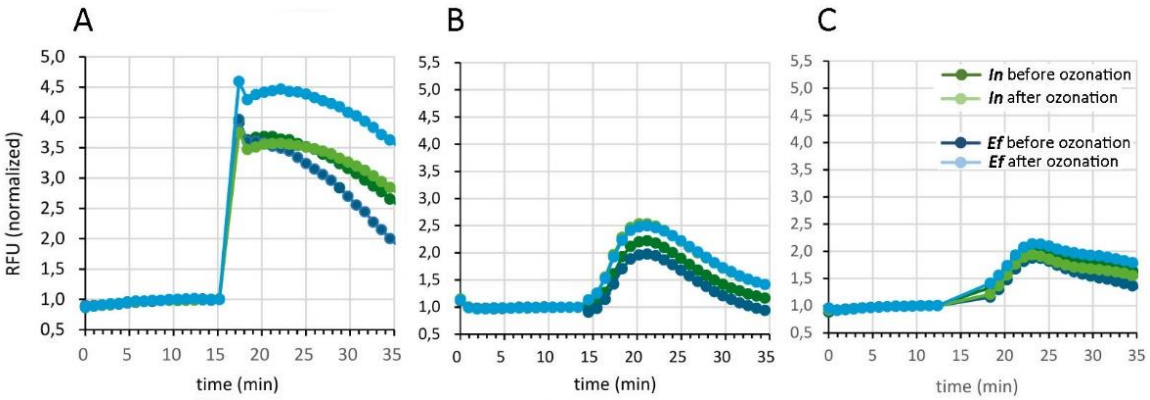
Source: own research, SIZ Zellkulturtechnik

Per- and polyfluoroalkyl substances (PFAS) represent a vast category of synthetic chemicals. They are known to pose potential health risks and exhibit persistence both in the environment and within the human body. PFAS have been reported to alter calcium signaling and calcium homeostasis. By disturbing the Ca^{2+} modulating function of mitochondria, PFAS may cause mitochondrial Ca^{2+} overload and increase mitochondrial ROS possibly inducing oxidative stress and mitochondrial dysfunction (Ehrlich *et al.*, 2023). These substances can enter ecosystems through waste streams. In the context of global ecological concerns related to PFAS in drinking and surface waters, the focus has gradually shifted from long-chain to short-chain hydrophilic PFAS. Conventional PFAS adsorption technologies are inadequate to address the challenges posed by hydrophilic PFAS.

As a response to this issue, we analyzed the influent and effluent of WWTP 1 advanced treatment unit to determine unusually high concentrations of short-chain hydrophilic PFAS, in particular trifluoroacetic acid (TFA), Perfluoropropylamine (PFPrA), and Perfluorobutyric acid (PFBA).

For assessing the possibility of oxidative metabolization or degradation of PFAS compounds we treated influent and effluent of the WWTP 1 advanced treatment unit with ozone using an ozone dose of 0.5 mg ozone per mg dissolved organic carbon. Throughout the ozone treatment process, we observed no significant alterations in the tested PFAS levels in both the influent and the effluent of the activated carbon unit (Table 19). The TFA levels, approximately 3 µg/L, align with the typical range found in other wastewater treatment plants in Germany. This suggests that there are no notably elevated levels of short-chain PFAS compounds such as TFA, PFPrA, or PFBA, and there are no apparent precursors that could be oxidized into these compounds.

Figure 28: Ca^{2+} mobilization in three cell lines by ozone treated samples



18-fold concentrated influent and effluent samples of the WWTP 1 advanced treatment unit induce Ca^{2+} mobilization in the cell line HSC-GCaMP5-GPR SK6 (A), HSC-GCaMP5 (B) and A431 (C). ***In***: influent, ***Ef***: effluent. Ozon untreated (before ozonation) and ozone treated (after ozonation) samples were analyzed comparatively.
Source: own research, SIZ Zellkulturtechnik

Table 19: short-chain PFAS analysis in WWTP 1 effluents after additional ozonation

Advanced treatment unit	TFA (before ozonation)	TFA (after ozonation)	PFPrA (before ozonation)	PFPrA (after ozonation)	PFBA (before ozonation)	PFBA (after ozonation)
Influent WWTP 1	3.17 µg/L	3.22 µg/L	0.10 µg/L	0.11 µg/L	<0.01 µg/L	<0.01 µg/L
Effluent WWTP 1	3.16µg/L	3.18 µg/L	<0.02 µg/L	<0.02 µg/L	<0.01 µg/L	<0.01 µg/L

The cell-based cAMP and Ca^{2+} signaling assays developed in this study offer a valuable approach for characterizing the modulation of cAMP and Ca^{2+} signalosomes. Given the simultaneous expression and activity of numerous G protein-coupled receptors within a single cell, along with their intricate regulation, pinpointing effects specifically to one receptor type presents challenges. Moreover, the complex composition of compound mixtures found in WWTP effluents results in overlapping activities. To analyze GPER1-specific signaling effectively, it becomes imperative to fractionate the mixture into distinct fractions. This was not possible within the scope of this work. To achieve this, a combination of hydrophobic resin and ion exchange resin-based solid-phase extraction is recommended. Subsequently, polar and non-polar compound fractions should be employed to assess cAMP and Ca^{2+} mobilization effects using the newly developed sensor cell lines. Fractions exhibiting activity can then undergo non-target analysis. The integration of WWTP effluent fractionation, effect-based in vitro activity assays, and non-target analysis promises to be a robust method for identifying micropollutants resistant to WWTP advanced treatment methods.

6.4 Aspects for further applications in the future

Under REACH regulations, only minimal knowledge requirements are mandated for chemicals with low annual tonnage, particularly regarding potential health risks. This approach seems to stem from the assumption that low-volume chemicals necessitate assessment primarily for

short-term exposure risks. Given their limited production volumes, it's improbable that large segments of the population would be exposed to substantial quantities of these chemicals over prolonged periods. However, the costs escalate with the complexity of studies needed to evaluate long-term exposure effects, including developmental and reproductive toxicity. There's a growing shift towards adopting effect-based assessment methods, aiming to classify substances without solely relying on traditional animal studies. One notable framework is the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors (OECD, 2018), offering a structured approach to evaluate evidence on endocrine disruption and integrate new study types as they emerge. While the newly developed cAMP and Ca^{2+} signalosome assays shouldn't replace traditional animal toxicology studies, they can complement them by providing additional insights otherwise inaccessible. These assays have the potential to aid in defining hazard categories for individual substances.

7 List of references

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