“Endocrine activity in the aquatic environment

Establishment of a bioassay battery to detect endocrine disruptive substances in various sample matrices”

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades einer Doktorin der Naturwissenschaften genehmigte Dissertation

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Tag der mündlichen Prüfung: 27.07.2015

Diese Dissertation ist auf den Internetseiten der Hochschulbibliothek online verfügbar.

Summary

Earth's accessible freshwater resources are under constant pressure. Most of the 35 million cubic kilometers freshwater are bound in glaciers (over 68 %), permanent snow und not accessible groundwater resources. Accessible freshwater is crucial for mankind as well as ecosystems functions in general. At the same time, over 50 % of all renewable and accessible freshwater flows are already used by mankind. In Europe only 21 % of the available freshwater are used and most of it is not consumed but restored. Therefore, Europe's problems related to freshwater supply do not depend on absolute quantity but on distribution, alterations of the hydromorpholgy and an increasing number of (anthropogenic) pollutants. Because of their potential to disrupt the reproduction over altering the endocrine system one important and heavy discussed group of micropollutants are the endocrine disruptive compounds (EDCs). In the last years more and more (potential) EDCs have been detected in the environment as well as organic tissues of humans and wildlife.

For the prediction of a substance's endocrine activity a first screening with QSAR tools (e.g. OECD QSAR Toolbox, VirtualToxLab) is useful. These predictions actually are good in case of receptor binding properties if the chemical structure is known. To screen samples for known EDCs, chemical analysis is a fast and sensitive approach. For example, to date it is possible to detect estrogens in the low $ng \cdot L^{-1}$ range (e.g. 0.3 $ng \cdot L^{-1}$ 17ß-Estradiol) in groundwater samples with a LC system coupled to a triple-stage quadrupole mass spectrometer with electrospray ion source (LC-ESI-MS^2). Nevertheless, to detect unknown endocrine disruptive potentials of a substance or to screen an environmental sample with unknown composition, bioassays are the methods of choice.

In the present study, the two recombinant receptor-reporter assays lyticase assisted yeast estrogen screen (LYES) and "estrogen receptor mediated chemical activated luciferase gene expression" (ER CALUX®) assay as well as the H295R steroidogenesis assay, that allows the detection of effects on the whole steroid synthesis pathway, were applied. Additionally, a variation of the *in vivo* reproduction test with the New Zealand mudsnail *Potamopyrgus antipodarum* (Pa-Repro) has been used. This bioassay battery therefore covers receptor-mediated activities as well as activities on the process of steroid genesis and impacts on whole organisms as well as populations.

To get insights into the development as well as international validation of knew test systems for the detection of EDCs the validation of the newly developed H295R was scientifically attended. It could be shown that the H295R successfully detected all suspected or known EDCs with good correlations between the different laboratories. Nevertheless, beside the number of advantages of this test system some disadvantages were also figured. Those were the high variability of steroid concentration between different passages as well as the high costs for the detection of produced steroids. To overcome the disadvantages several approaches to improve the test system were pursued. At first cells were adapted to chemical defined media which lead to a more stable production of steroids. With the development of a new ELISA method for the detection of steroids in the exposure medium both costs as well as working time could be saved. Hereafter, the selected bioassay battery was applied to various sample matrices as well as single substances. Both LYES and ER CALUX® were used to assess the estrogenic activity of heterocyclic aromatic hydrocarbons (hetero-PAHs). While none of the hetero-PAHs showed estrogenicity in the LYES, nine (indole, 1-benzothiophene, benzofuran, 2-methylbenzofuran, 2,3-dimethylbenzofuran, quinoline, 6-methylquinoline, carbazole, dibenzothiophene, dibenzofuran, acridine, and xanthene) out of twelve showed significant estrogenic activity in the ER CALUX®. With the help of liquid chromatography with diode array detection (LC-DAD) and high resolution tandem mass spectrometry (LC-$HRMS^2$), it could be demonstrated that the used T47Dluc cell line was capable for metabolizing of the parent substances. To assess the endocrine disrupting potential of the biological larvicide VectoBac®, which is extracted out of *Bacillus thuringienses var. israelensis* (*Bti*), the whole bioassay battery was used. With both receptor-mediated assays LYES and ER CALUX® the previously detected estrogenic activity of three commercial available tablet formulas could be confirmed. From the subsequently investigated active substance

VectoBac® TP as well as the formula WDG only TP showed dose-dependent estrogenic activity in both assays. Neither for TP nor WDG clear influences on the steroidogenesis could be detected in the H295R. The investigation of surface and groundwater samples which were taken after the application of both TP and WDG also showed no estrogenic activity in the receptor-mediated bioassays. To elucidate the potential effect of the suspected xenoestrogen VectoBac® TP on a complex aquatic community a microcosm study was performed. Within this microcosm, the impact on phytoplankton and zooplankton as well as the grazer *P. antipodarum* could be studied. While no effects on the zooplankton were detected, a clear shift from green to blue algae could be detected at concentrations which also showed significant estrogenic activity in the LYES. No clear effects on the reproduction of *P. antipodarum* could be observed, but it is assumed that this was a result of the limited food supply due to the community shift within the phytoplankton. Nevertheless, the concentrations used in the microcosm study were ten to 50 times higher than it would be expected in the field and therefore, a shift in the ecosystem structure of routinely treated areas seems unlikely. In the last approach the three selected *in vitro* bioassays were used to assess the performance of advanced sewage treatment processes with regard to the removal of pharmaceuticals from hospital sewage before discharge into the public sewerage. The high estrogenic activity of the raw sewage sample detected in the LYES could be significantly reduced by a full scale membrane bioreactor (MBR). After subsequent ozonation the estrogenicity was reduced to the level of the solvent controls in both receptor-mediated assays. By means of liquid chromatography-tandem mass spectrometry (LC-MS^2) it could be verified that after ozonation concentrations of estrone (E1) were significantly reduced. In contrast, there was a significant increase of 17ß-estradiol production as well as aromatase activity in H295R cells after ozonation. It is hypothesized that this is partly due to formation of active by-products during ozonation.

Overall, the selected *in vitro* bioassays were suitable for the detection of endocrine activity in various sample matrices as well as single substances. The receptor-mediated bioassay ER CALUX® was able to detect estrogenic activity in all matrices used. These included groundwater, surface water and sewage as well as single substances (hetero-PAHs). Additionally, in case of the hetero-PAHs, the estrogenic activity of single substances which require metabolic activation could be detected. With the LYES investigation of medium to highly contaminated water samples was possible, low contaminated water samples could not be investigated due to the high limit of detection (LOD) as well as the relatively high estrogenicity of the solvent controls. The H295R was applicable for medium contaminated samples as well as single substances. Performance of the bioassays markedly varied throughout the present study. The ER CALUX® provided the most stable performance (very good), followed by the H295R (good), the LYES (good to moderate) and *P. antipodarum* (moderate). Problems occurred with the consistent production of testosterone (H295R), the background of the solvent controls (LYES) as well as food supply and response to the positive control (*P. antipodarum*).

Concluding, it can be stated that the selected bioassay battery is suitable to detect (potential) endocrine disrupting substances in different water sample matrices such as groundwater, surface water and wastewater. Additionally, even single substances that require metabolic activation to exert their estrogenic activity can be investigated.

Zusammenfassung

Die verfügbaren Süßwasserressourcen der Erde sind stark begrenzt. Der Großteil der 35 Millionen Kubikmeter Süßwasser ist in Gletschern (über 68 %), permanenten Schneedecken und als nicht verwertbares Grundwasser gebunden. Dabei ist verfügbares Süßwasser nicht nur überlebenswichtig für die Menschheit sondern auch für die Aufrechterhaltung des globalen Ökosystems. Nichtsdestotrotz werden weltweit bereits über 50 % des verfügbaren Süßwassers durch den Menschen genutzt. In Europa werden lediglich 21 % genutzt, wobei ein Großteil nicht verbraucht sondern wieder aufbereitet wird. Infolgedessen, bezieht sich die Problematik in Europa nicht auf die generelle Verfügbarkeit des Wassers sondern auf dessen Verteilung, Eingriffe in die hydromorphologischen Bedingungen sowie die Verschmutzung durch anthropogene Schadstoffe. In diesem Kontext wird einer Gruppe der Mikroschadstoffe besondere Aufmerksamkeit zuteil, den endokrinen Disruptoren (EDs), welche über Veränderungen des endokrinen Systems die Reproduktion beeinträchtigen können. In den letzten Jahren wurden immer mehr (potentielle) EDs sowohl in der Umwelt als auch in Geweben von Menschen und Nutz- sowie Wildtieren detektiert.

Ein erster Schritt, um eine Substanz anhand ihrer chemischen Eigenschaften als potentiellen ED zu entlarven, ist die Anwendung von QSAR Programmen wie der OECD QSAR Toolbox. In einem weiteren Schritt können (Umwelt)Proben mittels chemischer Analytik auf bekannte Substanzen mit endokriner Aktivität untersucht werden. Unter Verwendung eines Flüssigchromatographie-Systems mit angeschlossenem Massenspektrometer (LC-ESI-MS^2) ist es bereits möglich, EDs im niedrigen ng/L-Bereich (z.B. 0,3 ng/L 17ß-Estradiol) zu detektieren. Um jedoch eine Substanz mit unbekannter oder vermuteter endokriner Wirksamkeit oder eine komplexe Umweltprobe auf ihr Wirkpotential hin zu untersuchen bedarf es der Anwendung von Biotestverfahren.

In der vorliegenden Studie wurden deshalb die zwei Reportergentestverfahren „lyticase assisted yeast estrogen Screen“ (LYES) und “estrogen receptor mediated chemical activated luciferase gene expression assay“ (ER CALUX®) für die Detektion endokriner Disruptoren in komplexen Umweltproben angewendet. Zusätzlich wurde der H295R „steroidogenesis assay“ verwendet, der es ermöglicht, Effekte im gesamten Steroidgeneseweg der Nebennierenrinde abzubilden. Darüber hinaus wurde der Testorganismus *Potamopyrgus antipodarum* (Neuseeländische Zwergdeckelschnecke) in einer Mikrokosmosstudie eingesetzt. Mit dieser Auswahl an Testverfahren ist es möglich, neben Rezeptor-vermittelten Wirkungen auch (negative) Wirkungen auf die Steroidgenese sowie auf der Ebene von Einzelorganismen und sogar ganzen Populationen zu ermitteln.

Zunächst wurde die Entwicklung sowie Validierung des erst kürzlich entwickelten H295R wissenschaftlich begleitet. Dabei konnte gezeigt werden, dass der H295R in unterschiedlichen Laboratorien alle bekannten und vermuteten EDCs zuverlässig und mit einer guten Korrelation ermitteln konnte. Es zeigten sich allerdings auch einige Nachteile des Testsystems, wie etwa die hohe Variabilität in der Steroidproduktion zwischen einzelnen Passagen sowie die hohen Kosten für die Messung der Steroidkonzentrationen im Testmedium. Aus diesem Grund wurde eine Adaption der Zelllinie an chemisch definiertes Medium durchgeführt sowie ein neues sensitives und kostengünstiges ELISA-Verfahren entwickelt. Im Anschluss wurde die ausgewählte Testbatterie eingesetzt, um verschiedene Probenmatrices sowie Einzelsubstanzen auf ihr endokrines Wirkpotential zu untersuchen. Sowohl der LYES als auch der ER CALUX® wurden verwendet, um das estrogene Wirkpotential von heterozyklischen aromatischen Kohlenwasserstoffen (hetero-PAKs) zu ermitteln. Dabei konnte für keinen der eingesetzten PAKs estrogene Aktivität im LYES detektiert werden. Im ER CALUX® hingegen konnte für neun von zwölf untersuchten PAKs (darunter Acridin, Benzofuran, Xanthen und Chinolin) signifikante estrogene Aktivität ermittelt werden. Mittels chemischer Analytik (LC-DAD und LC-$HRMS^2$) konnte gezeigt werden, dass die verwendete Zelllinie T47Dluc dazu in der Lage ist, die Ausgangssubstanzen zu metabolisieren. Um das endokrine Wirkpotential des biologischen Larvizids VectoBac®, das aus dem grampositiven *Bacillus thuringinesis var. israelensis* gewonnen wird, zu untersuchen, wurde die gesamte Biotestbatterie

eingesetzt. Die bereits bekannte estrogene Aktivität verschiedener kommerziell erhältlicher Feststoffformulierungen konnte mit dem LYES und dem ER CALUX® verifiziert werden. Der untersuchte Reinwirkstoff VectoBac® TP (TP) erwies sich ebenfalls als estrogen aktiv. Jedoch konnte keine klare Auswirkung auf die Steroidgenese im H295R nachgewiesen werden. Auch nach Applikation im Freiland konnte weder für TP noch für die Formulierung VectoBac® WDG rezeptor-vermittelte estrogene Aktivität des Oberflächen- sowie des assoziierten Grundwassers ermittelt werden. Um die Auswirkungen von TP auf eine komplexe aquatische Lebensgemeinschaft zu untersuchen wurde eine Mikrokosmosstudie mit der Indikatorspezies *Potamopyrgus antipodarum* durchgeführt. Für das Phytoplankton konnte eine signifikante Verschiebung der Arten von Grünalgen hin zu Blaualgen detektiert werden. Sowohl für das Zooplankton als auch für *P. antipodarum* konnten keine klaren Effekte ermittelt werden. Allerdings wurden die Ergebnisse wahrscheinlich durch die Zunahme des Blaualgenwachstums und damit einhergehend einer Abnahme des Futterangebots beeinflusst. Da jedoch die eingesetzten Konzentrationen *Bti* zehn bis 50-mal höher waren, als die tatsächlich im Feld ausgebrachten Mengen, erscheint ein negativer Einfluss auf aquatische Ökosysteme eher unwahrscheinlich. Im letzten Teil der vorliegenden Studie wurden die drei ausgewählten *in vitro* Testsysteme verwendet, um die Eliminationsleistung erweiterter Abwasserbehandlung am Beispiel eines mittelgroßen Krankenhauses zu untersuchen. Die hohe estrogene Aktivität des Rohabwassers konnte durch den Einsatz eines Membranbioreaktors signifikant verringert werden. Nach einer anschließenden Ozonierung des vorgereinigten Wassers konnte die verbliebene estrogene Aktivität auf ein Minimum reduziert werden. Mittels chemischer Analytik konnte nachgewiesen werden, dass die vorhandenen Konzentrationen an Estron durch die Behandlungsmethoden signifikant reduziert wurden. Allerdings wurde nach der Ozonierung eine erhöhte Produktion von Estradiol im H295R ermittelt, die mit einem Anstieg der Aromatase-Aktivität korrelierte. Dies wurde auf eine Bildung von Transformationsprodukten zurückgeführt, die allerdings nicht chemisch verifiziert werden konnten.

Insgesamt zeigte sich die in der vorliegenden Studie ausgewählte Biotestbatterie als sehr geeignet, endokrin aktive Substanzen in der aquatischen Umwelt zu detektieren. Der Rezeptor-Reportergen-Test ER CALUX® konnte in allen untersuchten Probenmatrices estrogene Aktivität detektieren. Darüber hinaus konnte die Möglichkeit der Metabolisierung von Substanzen durch die verwendete Zelllinie T47Dluc nachgewiesen werden. Mit dem LYES konnten Proben mit mittlerer und hoher Belastung untersucht werden, wobei die hohe Detektionsgrenze des Testsystems keine Untersuchung von niedrig belasteten Proben erlaubt. Mit dem H295R konnten sowohl Proben mittlerer Belastung als auch Einzelsubstanzen zuverlässig auf ihre endokrine Wirksamkeit hin untersucht werden. Die Leistungsstärke der einzelnen Testsysteme wurde wie folgt kategorisiert: (1) ER CALUX® (sehr gut), (2) H295R (gut), (3) LYES (gut bis mittelmäßig), (4) *P. antipodarum* (mittelmäßig). Hauptprobleme waren dabei die ungleichmäßige Produktion von Testosteron (H295R), die hohe Hintergrundbelastung der Negativkontrolle (LYES) sowie ein eingeschränktes Nahrungsangebot (*P. antipodarum*).

Grundsätzlich konnte in der vorliegenden Studie belegt werden, dass die ausgewählte Biotestbatterie sehr geeignet ist, um (potentielle) EDs in verschiedenen Wasserproben wie Grundwasser, Oberflächenwasser und Abwasser zu untersuchen. Außerdem konnte die Eignung für die Untersuchung von Einzelsubstanzen, auch solche mit benötigter metabolischer Aktivierung, verifiziert werden.

Danksagung

Wie bei den meisten größeren Projekten haben auch zum Gelingen meiner Doktorarbeit zahlreiche Personen beigetragen. Besonders danken möchte ich an dieser Stelle:

Prof. Dr. Henner Hollert, der es mir ermöglichte diese spannende und vielfältige Arbeit am Institut für Umweltforschung der RWTH Aachen anzufertigen. Lieber Henner, ich danke Dir für all Deine Unterstützung im fachlichen wie auch privaten Bereich. Du warst eine Vorzeige-„Doktorvater“!

Prof. Dr. Andreas Schäffer, für die bereitwillige Übernahme des Zweitgutachtens und die freundliche Aufnahme am Institut für Umweltforschung.

Prof. Dr. Dr. h.c. Dieter Maier (Technologiezentrum Wasser, Karlsruhe) für die gute Zusammenarbeit in unserem *Bti*-Projekt. In diesem Zusammenhang möchte ich mich auch bei PD Dr. Norbert Becker (Kommunale Aktionsgemeinschaft zur Bekämpfung der Stechmückenplage e.V., Speyer) für seine fachliche Beratung und die Bereitstellung des Untersuchungsmaterials bedanken.

Prof. Dr. Markus Hecker (University of Saskatchewan, Saskatoon, Kanada), der mir die Teilnahme an der OECD Ringstudie für die Validierung des H295R Steroidogenesis Assay ermöglichte. Außerdem für die Möglichkeit zweier meiner Abschlussarbeitskandidaten einen Teil ihrer Untersuchungen am Toxicology Center in Saskatoon durchzuführen.

Prof. Dr. Johannes Pinnekamp, Dr. Wilhelm Gebhardt, Dr. Silvio Beier und Frau Dr. Claudia Klümper (Institut für Siedlungswasserwirtschaft, RWTH Aachen) für die gute Zusammenarbeit im Projekt zur Eliminierung von Spurenstoffen aus Krankenhausabwässern.

Dr. Tido Strauss (RWTH-Aninstitut gaiac e.V., Aachen), der mit seinem fundierten Fachwissen und seiner Hilfsbereitschaft maßgeblich zum Gelingen der *Bti*-Mikrokosmos-Studie beigetragen hat.

Dr. Martin Krauss (Helmholtz-Zentrum für Umweltforschung (UFZ), Leipzig) für die spontane und hervorragende Ausführung der chemischen Analysen innerhalb der Studie zur estrogenen Aktivität von hetero-PAKs.

Dem Ökotoxzentrum (Dübendorf, Schweiz) und im besonderen Frau Dr. Petra Kunz für die gute Zusammenarbeit und die vielen wertvollen Tipps in Bezug auf die Optimierung des Yeast Estrogen Screen.

Herrn MSc Markus Brinkmann (Institut für Umweltforschung, RWTH Aachen) für die großartige Zusammenarbeit in der Studie zur estrogenen Aktivität von hetero-PAKs.

Dem Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV) für die Finanzierung der Studie zur Spurenstoffelimination in Krankenhausabwasser, der Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg (LUBW) für die Finanzierung des *Bti*-Projektes sowie Valent Biosciences (insbesondere Ernest Dankwa) für die Unterstützung der *Bti*-Mikrokosmos-Studie.

Ein besonderer Dank gilt auch meinen studentischen Mitarbeitern innerhalb der einzelnen Projekte. Namentlich Herrn Dipl. Biol. Tilman Floehr, der mit seiner hervorragenden Diplomarbeit mit großem Engagement und Erfolg einen bedeutenden Teil der praktischen Arbeiten im Rahmen der Studie zur Spurenstoffelimination in Krankenhausabwasser beigetragen hat. Innerhalb des *Bti*-Projektes war mir Herr Dipl. Biol. Nils Lippmann eine große Hilfe, da er im Zuge seiner ausgezeichneten Diplomarbeit die Mikrokosmos-Studie ausführte. Ebenso wichtig wenn auch etwas geringer war der Beitrag von Frau BSc Annett Müller, Frau Katharina Kubiak und Herrn MSc Stefan Schmitz. Zuletzt aber nicht letztens gilt mein ganz besonderer Dank Herrn MSc Marc Wollenweber, der sowohl mit seiner BSc- als auch mit seiner MSc-Arbeit und sogar darüber hinaus durch seinen unermüdlichen und erfolgreichen Einsatz im Labor maßgeblich zu den Ergebnissen des *Bti*-Projektes beigetragen hat.

Die Zeit in der eine solche Arbeit entsteht hat natürlich ihre Höhen und Tiefen. Diese sind umso besser zu meistern wenn man sich an seinem Arbeitsplatz wohlfühlt. Ich danke deshalb dem gesamten Institut für Umweltforschung für die freundliche Aufnahme und die zahlreichen Hilfestellungen. Im Besonderen danke ich allen Mitarbeitern des Lehr- und Forschungsgebietes Ökosystemanalyse von Prof. Henner Hollert für die tolle Arbeitsatmosphäre. Meinen Kollegen und Freunden im Doktorandenzimmer der AG Hollert gebührt dabei ein ganz besonderes Dankeschön: Herr MSc Markus Brinkmann, Herr Dipl. Biol. Sebastian Hudjetz, Herr Dipl. Biol. Tilman Floehr, Frau Dr. Sabrina Schiwy, Frau Dipl. Biol. Marit Ernst, Herr Dr. Hanno Zielke, mit euch ein Büro zu teilen war mir eine große Freude. Vielen Dank für all die Unterstützung, Aufmunterungen zur rechten Zeit, guten Gespräche im fachlichen wie auch privaten und natürlich die schönen Feiern! Ein ebenso großes Dankeschön gebührt natürlich auch Frau Dipl. Biol. Kerstin Bluhm und Herrn Dr. Thomas-Benjamin Seiler, mit denen ich in den vergangenen Jahren das Büro teilen durfte. Danke, für all die guten Gespräche und eure Unterstützung in fachlichen wie auch privaten Fragen. Und natürlich gebührt auch Frau Simone Hotz ein ganz großes Dankeschön, für all ihre Unterstützung im Labor und darüber hinaus die Urlaubsbetreuung meiner Katzen.

Hinter allem, was ich tue, steht mein Grundpfeiler im Leben, meine Familie. Ich danke euch für unendlich viele große und kleine Taten, für eure bedingungslose Liebe und Unterstützung. In den vergangenen Jahren immer an meiner Seite warst dabei Du, Beat. Ohne Deine Liebe und Unterstützung hätte ich das alles nicht geschafft.

Und immer, wenn ich mich in anstrengenden und frustrierenden Zeiten fragte, wozu das alles war die Antwort klar. Für mich, um mir selbst zu beweisen, dass ich es kann. Aber noch viel mehr für meine zwei größten Schätze im Leben, Celia und Lucia, um euch neben meiner grenzenlosen Liebe die größtmögliche Sicherheit für eine sorgenfreie Zukunft zu geben.

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Chapter 1

Introduction

1.1 Water pollution

Earth's total water resources add up to 1.4 billion cubic kilometers. The majority of this water (97 %) is salt water, while 35 million cubic kilometers (2.5 %) are classified as freshwater only. Furthermore, most of it is bound in glaciers (over 68 %), permanent snow und not accessible groundwater resources (Gleick & Palaniappan 2010). To date, from all renewable and accessible freshwater flows over 50 % are already used by mankind (Postel et al. 1996). Concurrently, accessible freshwater is not only crucial for human drinking water supply, sanitation facilities as well as industry and agriculture but also for the ecosystems functions in general. The so called provisioning functions of water are inseparably linked to healthy ecosystems. Availability of water for human needs is therefore depending on a good ecological status of the water providing ecosystems (Werner 2012).

In Europe only 21 % of the available freshwater are used. The abstracted water is used for agriculture (33 %), public water supply (18 %), industry (14 %; without cooling water) and power generation and non-defined uses (38 %) (Nixon et al. 2000). Additionally, most of the abstracted water is not consumed but restored. Apparently, Europe's problems related to freshwater supply do not depend on absolute quantity but on distribution (Nixon et al. 2000), alterations of the hydromorpholgy (Werner 2012) and pollution (Collins et al. 2011).

Since the industrial revolution, freshwater resources are exposed to an increasing number of (anthropogenic) pollutants like fertilizers, pesticides, pharmaceuticals, personal care products, metals and other industrial chemicals. They originate from industry, agriculture, mining, waste disposal and sewage treatment plants and other sources from the urban environment but also from accidental release during transport and production. In the European Union (EU) approximately 200 million tons of toxic chemicals (subject to the EU Dangerous Substances Directive) are produced every year (Collins et al. 2011). According to data reported under the Water Framework Directive (WFD), European surface waters are polluted by high levels of heavy metals (e.g. in the Czech Republic, Germany, Sweden and the United Kingdom), Polycyclic Aromatic Hydrocarbons (PAHs; e.g. in the rivers Elbe and Rhine) and diethylhexyl phthalate (DEHP; e.g. in the river Danube) (Collins et al. 2011).

Excursus: **Water Framework Directive**

The Water Framework Directive (WFD; 2000 / 60 / EC)) of the European Union (EU) was adopted in 2000 and represents a framework for water protection and management covering all types of water bodies from groundwater to coastal waters. The ultimate objective of the directive is to achieve **“good ecological and chemical status”** for all waters by 2015. In case of the **ecological status** biological factors like abundance of aquatic flora and fish fauna are monitored. Additionally, chemical and physical factors like salinity, temperature and availability of nutrients as well as pollution by chemicals are considered. Furthermore, the hydromorphological features like water flow and structures of the riverbed are covered. The classification scheme includes five categories: high, good, moderate, poor and bad. In terms of the **chemical status** environmental quality standards for 53 substances of high concern have been established (Environmental Quality Standards Directive; 2008 / 105 / EC) (European Commission 2012). In case of groundwater resources the objectives lie on a good chemical and quantitative status (Groundwater Directive; 2006 / 118 / EC).

(European Parliament 2000)

Schwarzenbach et al. (2010) stated that there are two major classes of chemical pollutants regarding their concentrations in the environment: macropollutants occur in the milligram per liter range while micropollutants occur in the range of nanogram ($ng \cdot L^{-1}$) to microgram ($\mu g \cdot L^{-1}$) per liter. The former include pollutants such as nitrogen, phosphorous and salt, the latter a vast number of synthetic and natural substances found in the aquatic environment. In most cases micropollutants do not exert adverse effects in an acute but chronic manner and therefore pose a longterm threat to humans and biota.

Because of their potential to disrupt the reproduction over altering the endocrine system one important and heavy discussed group of micropollutants are the endocrine disruptive compounds (EDCs), which include natural as well as synthetic steroids, pharmaceuticals, pesticides, organotin compounds, polychlorinated biphenyls (PCBs), phthalates, dioxins, phenol derivatives etc. (Falconer et al. 2006).

1.2 The vertebrate endocrine system

The endocrine system is one of the two fundamental communication and regulatory systems in the body. Communication occurs via hormones that are excreted in the blood or lymph by a number of ductless glands. The hormones are then transported to their targets, mostly cells with specified hormone receptors. The main functions of this internal communication system are the control of reproduction as well as growth and development and the maintenance of the metabolism. There are three types of hormones which can be distinguished by their chemical structure conformity, the protein- or peptide hormones, the steroid hormones and the amino acid derivates (Bartke & Constanti 2003, Kleine & Rossmanith 2010). This study will deal only with the steroid hormones, wherefore they solely will be described in the next paragraph.

1.2.1 Steroids

Steroid hormones are derivates of the steran skeleton differing in altered functional group composition and grade of ring condensation. Primary substance of the vertebrate steroidogenesis is cholesterol which is the precursor of pregnenolone from which over various steps the five main steroid classes, gestagens, glucocorticosteroids, mineral corticosteroids, androgens and estrogens, derive (Tab. 1.1) (Kleine & Rossmanith 2010).

Table 1.1 Steroid classes[1]

Steroid class	Example	Function	References
Gestagens	Progesterone	Nidation, maintenance of pregnancy	1
Glucocorticosteroids	Cortisol	Gluconeogenesis, inhibition of the hypothalamic-pituitary and the hypothalamic-pituitary-gonadal systems, control of inflammatory responses	1
Mineral corticosteroids	Aldosterone	Control of the sodium reabsorption and potassium secretion in the kidney	1
Androgens	Testosterone	Development of males, osteogenesis	1
Estrogens	Estradiol	Key regulator of growth, differentiation and function in e.g. the reproductive tract, mammary gland, skeletal system and cardiovascular system	1, 2

[1](Kleine & Rossmanith 2010); [2](Hall et al. 2001)

The steroidogenesis (Fig. 1.1) takes place in the cells of different organs and tissues but the first step of hormone synthesis is always the transport of the membrane lipid cholesterol into the mitochondria. This step is mediated by the steroidogenic acute regulatory protein (StAR) which has to be constitutively synthesized due to its instability.

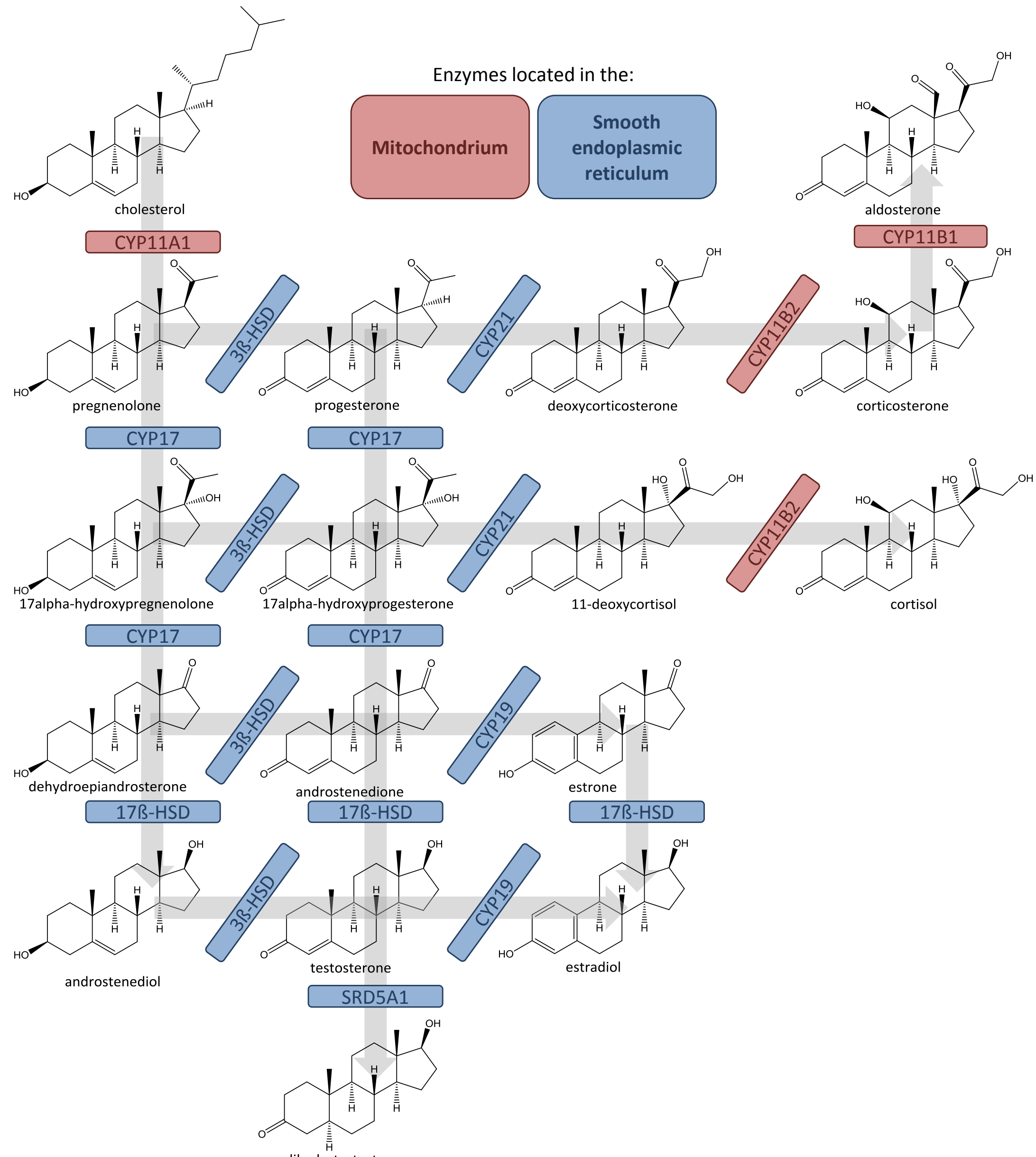

Figure 1.1 Steroidogenesis with steroids and steroidogenic enzymes (CYP11A1 = cholesterol side chain cleavage enzyme; CYP11B1 = steroid 11β-hydroxylase; CYP11B2 = aldosterone synthase; CYP17 = steroid 17α-monooxygenase; CYP19 = aromatase; CYP21 = steroid 21-hydroxylase; 3β-HSD = 3β-hydroxysteroid-dehydrogenase; 17β-HSD = 17β-hydroxysteroid-dehydrogenase; SRD5A1 = steroid 5α-reductase) (Hanukoglu 1992).

In the initial step of the steroidogenesis cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (P450scc or CYP11A1). The cytochromes P450 (CYPs) are the major class of steroidogenic enzymes. Furthermore, 3β- and 17β-hydroxysteroid-dehydrogenases as well as the 5α-reductases are involved (Arukwe 2008, Kleine & Rossmanith 2010). Cholesterol is mainly synthesized in the liver and transported via lipoproteins to the endocrine glands. The adrenal cortex is the main location for synthesis of steroids. It is divided in three zones where different classes of steroids are synthesized: *Zona glomerulosa* (mineral corticosteroids), *Zona fasciculata* (gluco corticosteroids) and *Zona reticularis* (gestagenes, androgens and estrogens). Further sites for the synthesis of estrogens are the ovary, the placenta and the testis, for gestagenes the *Corpus luteum*, the follicles and the placenta and for androgens the testis and the ovary. Central control of the steroidogenesis takes part in the hypothalamus and the pituitary. Releasing-Hormones (RH) and Release-Inhibiting-Hormones (IH) from the hypothalamus are signals for inhibition or induction of the pituitary hormones release which control the peripheral endocrine glands. Due to the presence of Pregnenolone in almost all steroids producing glands synthesis of the diverse steroids is depending on the presence of specific receptors and the involved enzymes. In the adrenal cortex 17-, 21- and 11-hydroxylases are present. Therefore, all paths of the steroidogenesis are possible in this part of the adrenal gland (Hanukoglu 1992, Payne & Hales 2004, Silbernagl 2012).

Excursus: **Vertebrate steroids in prosobranch mollusks**

Prosobranch mollusks are able to produce vertebrate-type steroids like estrogens and androgens. To date, almost all necessary enzymes for steroidogenesis have been detected in mollusks wherefore an endogenous production is suggested. Furthermore, it could be proofed that steroids are crucial for the regulation of sexual differentiation and reproduction. Recently, Stange et al. (2012) found an estrogen-receptor transcript in the mudsnail *Potamopyrgus antipodarum* and could prove that it is regulated under estrogenic and androgenic influence. Taken together, due to their ecological relevance and widespread distribution as well as the homologues to the vertebrate endocrine system prosobranch mollusks like *P. antipodarum* are promising test species for EDCs in the aquatic environment. Of course, it has to be taken in mind that the extrapolation of effects on steroid metabolic pathways from one phylum to the other is difficult.

(Janer & Porte 2007, Lafont & Mathieu 2007, Oehlmann et al. 2007)

Targets of steroids in the cells are highly specific nuclear receptors in the cytoplasm and / or the nucleus. Due to their lipophilic properties steroids enter the cell over passive membrane transport. After binding to the receptor, the receptor-ligand complex mostly forms a dimer with a second one and the resulting homo- or heterodimer binds to hormone responsive elements (HREs) on the DNA. Thus, expression of the related genes is activated (Krauss 2006). In the present study the main focus lies on estrogen receptor (ER) binding capacity of EDCs. Therefore, the next paragraph exemplary explains normal activation and possible disruption ways of the ER pathway.

1.2.2 ER signaling

To date, two intracellular isoforms of the human ER are known, ER alpha (hERα) and ER beta (hERβ). Both DNA- and ligand-binding domains of the two receptors are highly homologous, wherefore they bind to the same response elements on the DNA and show analogical binding affinities to several estrogens. Nevertheless, some substances show different binding affinities to the ERs. For example, phytoestrogens show a significantly higher affinity to the ERβ. Furthermore, some compounds can act as agonists as well as antagonists depending on the affected tissues. While E2 is a pure agonist in all effected tissues, the breast cancer drug Tamoxifen acts as antagonist in the breast but as agonist in bone, cardiovasculature and uterus. As expression of the two receptors occurs in different tissues their physiological functions within the body are thought to be quite distinct. The classical mechanism of estrogen activity is ligand-dependent. Binding of a ligand to the receptor leads to a conformational change and subsequently to the formation of homo- or heterodimers as well as the binding to estrogen response element (ERE) consensus sequences (5'-GGTCANNNTGACC-3' (Driscoll et al. 1998)) in the target genes. In some cases ligand-depending estrogen action is even possible in genes without EREs for example over binding to the transcription factor activation protein-1 (AP-1). Another way of ER-signaling is ligand-independent. In those cases the ER is activated by polypeptide growth factors (GF) like the epidermal GF (EGF) in the female reproduction tract. At last, also nongenomic transactivating ways of estrogen activity are known. In those cases, fast responses of tissues like breast and bones to estrogens are mediated through membrane-coupled ERs (Hall et al. 2001, Singleton & Khan 2003).

1.3 Endocrine disruptive compounds

Endocrine disruption has been a blazing theme in eco(toxico)logical sciences since the early 90th of the 20th century (Colborn et al. 1993). Mainly initiated by the publication "Our stolen future: are we threatening our fertility, intelligence and survival?: a scientific detective story" of Theo Colborn and colleges (Colborn et al. 1996) the topic now is also heavy discussed in public. Already in the 1940s observations over the ecological impacts of the insecticide dichlorodiphenyltrichloroethane (DDT) on bald eagles were made. But it took over 30 years of research and heavy debates until the ban of DDT in western countries in the 1970s (Grier 1982, Patisaul & Adewale 2009). Another prominent case of endocrine disruptive effects of manmade chemicals was the investigation of imposex (growth of male sex organs) in female marine snails exposed to the antifouling agent tributyl tin (TBT) (Bryan et al. 1987). Even impacts on humans were observed in conjunction with Diethylstilbestrol (DES; 4,4'-(3E)-hex-3-ene-3,4-diyldiphenol) (Giusti et al. 1995) and polychlorinated biphenyls (PCBs) (Kuratsune et al. 1972).

In the last years more and more (potential) EDCs have been detected in the environment as well as organic tissues of humans and wildlife (Amaral Mendes 2002). Beside the natural and synthetic steroids a large number of substances and metabolites have been recognized as steroid receptor binders. Subsequently, several substances have the potential to disrupt the natural steroidogenesis independent from receptor binding (Tab. 2.2). And the number of actual and potential EDCs is still rising (Hotchkiss et al. 2008).

The Worlds Health Organisation (WHO) defines an EDC as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations. In case of substances that seem to have characteristics to cause adverse effects but no proof is given the term potential EDC was created (WHO 2002). Furthermore, the Organisation for Economic Co-operation and Development (OECD) extended the definition to the term of possible EDCs. A possible EDC has been tested for endocrine disruption in *in vitro* bioassays but no proof in *in vivo* assays or the environment has been given so far (Kortenkamp et al. 2011).

Table 1.2 Outline of anthropogenic endocrine disruptive compounds

Substance	Structure[1]	ER agonist[2]	Steroidogenic active[2]
Atrazine (herbicide)	Cl, N, N, N, N H, N H	-	+
Bisphenol A (starting substance for polymer plastics)	HO, OH	+++	+
Diclofenac (nonsteroidal anti-inflammatory drug)	O, OH, Cl, H N, Cl	- (++)	-
2,4-Dichlorophenol (metabolite of pesticides)	OH, Cl, Cl	+	+
Perfluorooctane sulfonate (PFOS) (fluorosurfactant)	F F F F F F F F, O, F, S, F, OH, F, F F F F F F, O	-	+

[1]ChemBioDraw Ultra (Perkin Elmer, Waltham, USA; free trial version); [2]OECD QSAR Toolbox

1.4 Detection of EDCs

1.4.1 QSAR

To predict an endocrine activity of a substance a first screening with QSAR tools (e.g. OECD QSAR Toolbox, VirtualToxLab) is useful (Tab. 1.3).

Table 1.3 Example for OECD QSAR Toolbox data of a screening for acridine

Acridine

N *

Carcinogenicity	Structural alert for genotoxic carcinogenicity (heterocyclic polycyclic aromatic hydrocarbons)
Toxic Hazard Classification	High
Estrogen Receptor Binder	Non binder, without OH or NH_2 group
Simulated Rat Liver Metabolism (S9)	
Metabolites	8 (1 Epoxide, 4 Phenols, 3 not categorized)
Estrogen Receptor Binder	2 moderate binder, 2 strong binder, 4 non binder

*ChemBioDraw Ultra (Perkin Elmer, Waltham, USA; free trial version)

In case of receptor binding and / or as long as the chemical structure is known, predictions on potential activities are quite good. But with respect to alteration of the whole steroidogenesis and substances with unknown modes of action models are mostly overextended (Kase et al. 2009, Seifert et al. 1999). These results from the various targets for disruption in the chain of steroid genesis like inhibition or activation of enzyme kinetics, building and secretion of basic and intermediate products and feedback (Cronin & Worth 2008).

1.4.2 Extraction and sample preparation

Due to the complexity of the matrices and the often very low concentrations ($ng \cdot L^{-1}$) of the analytes, extraction as well as clean-up steps prior to chemical analysis of environmental water samples are mandatory (Sosa-Ferrera et al. 2013). In case of *in vitro* bioassays it strongly depends on the sample structure and the test system used. While the investigation of native water samples is sometimes possible, sediments have always to be extracted. Binding assays such as enzyme linked receptor assays (ELRA) are suitable for crude water samples (Seifert et al. 1999). Cell based assays are partly suitable for testing of

native water samples. For example, the Yeast estrogenic screen (YES) has shown to be suitable in several studies using native samples (Wagner & Oehlmann 2009) (see also Chapter 6). In case of assays with mammalian cell lines problems with bacterial and fungal contaminations as well as general toxicity often occur. In a recent investigation spiked crude sewage samples were successfully applied to the ERα CALUX® (data not published). Investigation of native samples in *in vivo* test systems is possible in most cases (Tuikka et al. 2011).

1.4.2.1 Water (liquid) samples

Preparation of water samples for investigation reaches from mere filtration steps to exhaustive extraction with subsequent clean up steps and fractionation. The first step is mostly a filtration of the native sample with glass fiber filters (±0.45 µm) to remove particles and microorganisms and adding of preservatives like acids (HCl, H_2SO_4) or solvents (formaldehyde, methanol) to prevent biodegradation (Li et al. 2013, Matějíček 2012, Samaras et al. 2011, Stasinakis et al. 2012, Wang et al. 2012a, Wang et al. 2012b, Zhang et al. 2011). To date, the most commonly used method for extraction of EDCs from water samples is solid phase extraction (SPE). The advantages of this technique are the relatively small amount of solvent needed as well as the broad range of available sorbents for different analytes (López de Alda & Barceló 2001, Pavlović et al. 2007). The most commonly used sorbents are the non-polar alkyl-bonded silica sorbent C-18, the polymeric sorbents Oasis HLB (Waters, Germany) and Lichrolut ENV+ (Merck Millipore, Germany) as well as the mixed polymeric and cation-exchange sorbent Oasis MCX (Waters, Germany) (Comerton et al. 2009). Additional methods for extraction of water samples are solid phase micro extraction (SPME), liquid-liquid extraction (LLE) and passive samplers (Camilleri et al. 2012, Sosa-Ferrera et al. 2013). Advantages as well as disadvantages of the different extraction methods for liquid samples are summarized in table 1.4.

Table 1.4 Extraction methods for EDCs in water samples

Method	Principle	Advantages	Disadvantages	References
Solid phase extraction (SPE)	Substances in sample (mobile phase) are adsorbed to a sorbent (e.g. C-18; stationary phase) in a cartridge	+ Low amount of solvent + Off-line or on-line	- plugging of cartridges - risk of channeling - low sample processing rates - small cross-sectional area	1, 2, 3
Solid phase micro extraction (SPME)	Substances in sample are adsorbed to a thin layer of a polymeric phase (stationary phase) e.g. on a stir bar	+ Very low amount of solvent + Small sample volume + Fast + high-enrichment factors	- limited choice of sorbent coating - reduced sorption capacity	1, 2
Liquid liquid extraction (LLE)	Substances in sample migrate into the solvent (mobile phase)		- high amount of solvent - relatively low selectivity, specificity and reproducibility	1
Passive samplers	Substances in surface water are adsorbed to a polymeric sorbent (e.g. POCIS; stationary phase)	+ time weighted average concentration of the substances + more realistic overview of environmental concentrations	- calibration is difficult - unsteady environmental conditions - biofouling	4, 5

[1](Sosa-Ferrera et al. 2013), [2](Comerton et al. 2009), [3](Sosa Ferrera et al. 2004), [4](Camilleri et al. 2012), [5](Vrana et al. 2005)

1.4.2.2 Sediment (solid) samples

Due to the hydrophobic capacities of many EDCs, for example synthetic estrogens, sediments and suspended particular matter are important sinks and thus even sources for EDCs in the aquatic environment. Therefore, partially high loads of EDCs in the aquatic ecosystems are adsorbed to particles and not direct available in the water phase (Lai et al. 2000, Westrich 1986).

Subsequent to sampling sediments are often homogenized, sieved and finally dried (air, heat or freeze drying) to prevent the substances from further biodegradation during storage. Previously, the most common extraction method for organic pollutants in solid matrices was the Soxhlet extraction (Liu et al. 2004, Seiler et al. 2008). But mainly due to its high consumption of organic solvents as well as the long duration of the extraction Soxhlet is more and more replaced by other techniques (Luque de Castro & García-Ayuso 1998). Methods replacing Soxhlet currently are ultrasonic extraction (USE), microwave-

assisted extraction (MAE) and pressurized liquid extraction (PLE) (Pavlović et al. 2007, Sosa-Ferrera et al. 2013). Advantages as well as disadvantages of the different extraction methods for solid samples are summarized in table 1.5.

Table 1.5 Extraction methods for EDCs in sediment samples

Method	Principle	Advantages	Disadvantages	References
Soxhlet extraction	Substances adsorbed to a solid matrix are transferred to a organic solvent for several cycles under high temperature	+ extraction of high amounts of sample is possible + no further cleaning steps are necessary + relatively cheap + easy to handle	- large amount of solvent - long duration - possible thermal decomposition of substances - concentration step is necessary	1
Ultrasonic extraction (USE)	Substances are transferred to a organic solvent through a low-frequency sound wave	+ relatively fast + relatively cheap + easy to handle	- poor reproducibility - further clean-up and / or enrichment necessary	2
Microwave-assisted extraction (MAE)	Substances are transferred to a organic solvent through heating and accelerated pressure	+ fast + low amount of solvents + increased sample throughput	- only for polar and thermally stable substances	2, 3
Pressurized liquid extraction (PLE)	Substances are transferred to a organic solvent under high pressure (and temperature) in a closed flow-through system	+ high reproducibility + low amount of solvents + fast + high extraction rates	- high acquisition costs	2, 3

[1](Luque de Castro & García-Ayuso 1998), [2](Pavlović et al. 2007), [3](Sosa-Ferrera et al. 2013)

1.4.3 Analytical methods

1.4.3.1 Chemical analysis

To screen samples for known EDCs, chemical analysis is a fast and sensitive approach. Nevertheless, chemical analysis of complex environmental samples requires extensive extraction as well as clean-up procedures and sometimes even derivatization to make the detection of EDCs in very low concentrations ($ng \cdot L^{-1}$) possible (Sosa-Ferrera et al. 2013, Streck 2009). Because of the diversity of substance classes detection of multiple EDCs should be performed with both gas (GC) and liquid (LC) chromatography coupled with mass spectrometry (MS). While LC-MS^2 is suitable for polar and less volatile or thermodegradable compounds such as antibiotics and betablockers, GC-MS^2 is to be preferred for non-polar and / or volatile compounds like PAHs. In general, GC-MS has

lower limits of detection (LOD) than LC-MS and is less affected by matrix effects but requires a more complicated sample preparation (Comerton et al. 2009, Sosa-Ferrera et al. 2013, Streck 2009). For example, according to Vulliet et al. (2008), to date it is possible to detect estrogens in the low $ng \cdot L^{-1}$ range (e.g. 0.3 $ng \cdot L^{-1}$ 17ß-Estradiol) in groundwater samples with a LC system coupled to a triple-stage quadrupole mass spectrometer with electrospray ion source (LC-ESI-MS^2).

1.4.3.2 Bioassays

To detect an unknown endocrine active potential of a substance or to screen an environmental sample with unknown composition, *in vitro* and where appropriate *in vivo* bioassays are the methods of choice (Hecker & Hollert 2009, Kunz et al. 2014). However, with this methods no qualitative or quantitative detection of a substance is possible (Kase et al. 2009). While the majority of the *in vitro* assays focus on receptor binding and / or activation as well as proliferation, only a small number covers the pathways of steroidogenic activity (Hecker & Giesy 2008). The applicability of several bioassays to detect estrogenic and androgenic activity in water samples was recently assessed (Kase et al. 2009, Leusch 2008, Leusch et al. 2010). Unfortunately, there are a vast number of different *in vitro* and *in vivo* test systems and therefore, investigators of EDCs are spoiled for choice. To give a short and admittedly subjective overview an outline of suitable bioassays (Bars et al. 2011, Duft et al. 2007, Hecker & Hollert 2011, Kase et al. 2009, Knacker et al. 2010, Leusch 2008) is presented in table 1.6.

Table 1.6 Outline of bioassays for the detection of EDCs

Mechanism	Name	Cell line / Test species	References
Enzyme-linked binding			
	Enzyme Linked Receptor Assay (ELRA)	None / Diverse	1
	Enzyme Linked Immunosorbent Assay (ELISA)	Non / Diverse	2
Cell Proliferation			
	E / A-Screen	MCF-7 / Human	3, 4, 5
Recombinant receptor-reporter			
	(L)Yeast Estrogen / Androgen Screen (YES / YAS)	Saccharomyces cervisiae / Yeast	6, 7
	Estrogen / Androgen Receptor mediated Chemical Activated LUciferase gene eXpression (ER / AR-CALUX®)	T47D and U2OS / Human	8, 9
	T47D-KBluc	T47D / Human	10
	MVLN; MELN	MCF-7 / Human	11, 12
	HELN	HeLA / Human	12
Steroidogenesis			
	H295R Steroidogenesis assay (H295R; OECD 456)	NCI-H295R / Human	13, 14
Reproduction			
	Fish Short Term Reproduction Assay (OECD 229)	*Pimephales promelas*	15
	P. antipodarum Reproduction Test (Pa-Repro)	*Potamopyrgus antipodarum*	16

[1](Seifert et al. 1999), [2](Nunes et al. 1998), [3](Sonnenschein & Soto 1998), [4](Soto et al. 1995), [5](Szelei et al. 1997), [6](Purvis et al. 1991), [7](Routledge & Sumpter 1996), [8](Legler et al. 1999), [9](Sonneveld et al. 2005), [10](Wilson et al. 2004) [11](Demirpence et al. 1993), [12](Balaguer et al. 2001), [13](Hecker et al. 2011), [14](OECD 2011), [15](OECD 2012c); [16](Duft et al. 2007)

In vitro

Binding assays like the enzyme-linked receptor assay (ELRA) or the enzyme linked immunosorbent assay (ELISA) are fast and relatively cheap methods to detect single substances (ELISA) and substances as well as mixtures with the ability of receptor-binding (ELRA). Compared to cell-based assays, binding assays are not affected by

cytotoxic substances in unprocessed complex environmental samples but are suitable for screening of biochemical binding. ELISAs are highly sensitive (ng·L^{-1} range) as well as simple and are available for a great number of synthetic and natural substances. Additionally, they can be produced in accordingly equipped laboratories. Disadvantages of the method are its vulnerability to cross reactions (e.g. specificity) and the limitation to one analyte. ELRAs share the advantages of the ELISA technique but are also able to detect e.g. the whole estrogenic activity of complex samples and less vulnerable to cross reactions (Caron et al. 2010, Huang & Sedlak 2001, Nunes et al. 1998, Seifert et al. 1999).

Cell proliferation assays like the E-(A-)Screen measure the estrogenic (androgenic) activity of single substances and mixtures containing substances with similar modes of action (e.g. ER-binding). They are robust, relatively cheap and easy to apply and have a low method quantification limit (0.2 ng·L^{-1}; MQL) (Leusch 2008). The induction of cell proliferation was thought to be one major characteristic of a substance to classify it as a (xeno)estrogen (Sonnenschein & Soto 1998), but to date it is proven that it can also be mediated through other pathways (OECD 2012b). Another major drawback of this method is the use of (human) breast-cancer cells (MCF-7) which express several steroid receptors. Therefore, the investigation of complex mixtures is partly limited due to antagonistic effects. Furthermore, this method is relatively slow as cell proliferation occurs only after several days (Connolly et al. 2011).

There are several recombinant receptor-reporter or reporter-gene assays (RGAs) with the main focus on mammalian (vertebrate) cell lines but also some widely used yeast systems. The Yeast Estrogenic Screen (YES) developed by Routledge & Sumpter (1996) is one of the first RGAs and is still performed in many studies regarding estrogen activity in environmental samples (Bovee & Pikkemaat 2009, OECD 2012a). The yeasts were transformed with a constitutively expressed human ERα as well as an inducible ß-Galactosidase reporter gene expression cassette and are suitable to measure the estrogenic effect of inducible substances that bind to the receptor either in an agonistic or antagonistic way. Because it is cheap and relatively fast, the YES is useful as a first screening of many samples and of highly polluted samples. Compared to some mammalian (vertebrate) cell lines the yeasts lack the ability to transform substances into (more) active metabolites. Additionally, the yeast cell wall acts as a barrier for

certain substances. As a result the MQL is relatively high (±4 $ng \cdot L^{-1}$) (Leusch 2008). One of the first developed RGAs with mammalian cells is the MVLN assay (Bovee & Pikkemaat 2009). It is based on the human MCF-7 breast-cancer cell line which includes endogenous steroid receptors (e.g. ERα) and was transfected with a plasmid containing the estrogen-responsive luciferase reporter gene pVit-tk-Luc (Demirpence et al. 1993). Another RGA based on the MCF-7 cell line is the MELN assay. In this case, cells have been stably transfected with a plasmid containing the estrogen-responsive luciferase reporter gene pERE-βGlob-Luc-SVNeo (Balaguer et al. 2001). The MELN assay has a MQL of 0.27 $ng \cdot L^{-1}$ and seems to be sensitive towards anti-estrogenic substances in complex mixtures (Leusch 2008). To overcome the potential interactions of endogenous steroid receptors in the used cell lines a further assay for the detection of estrogenic activity in mammalian cell lines was developed by Balaguer et al. (2001), the HELN. For this assay the human HeLa cervical cancer cell line was stably transfected with pERE-βGlob-Luc-SVNeo as well as an ERα expression cassette. The commercially available "estrogen receptor mediated chemical activated luciferase gene expression" assay (ER CALUX®) and the free available KBluc assay initially both based on the human T47D breast-cancer cell line. This cell line includes endogenous ERα as well as ERβ and is transfected with plasmids containing different estrogen-responsive luciferase reporter gene expression cassettes (T47Dluc: pEREtata-Luc; T47D-KBluc: pGL2.TATA.Inr.luc). Subsequent established versions of the ER and AR CALUX® work with the human U2OS osteosarcoma cancer cell line which does not express endogenous estrogen or androgen receptors. Therefore, the human ERα (ERα CALUX®) or ERβ (ERβ CALUX®) in combination with the estrogen-responsive luciferase reporter gene vector pEREtata-Luc were transfected into the cells resulting in more selective responses towards ER ligands (Sonneveld et al. 2005). Both assay systems are highly sensitive with MQL between 0.1 $ng \cdot L^{-1}$ (ER CALUX®) and 0.2 $ng \cdot L^{-1}$ (KBluc) (Leusch 2008). One of the advantages of all RGAs is the possibility to detect both agonists and antagonists of the relevant receptors. They are fast and reliable as well as sensitive systems to detect hormonal effects whereas the mammalian systems are more sensitive than the yeast based systems (Connolly et al. 2011).

While the proliferation assays and RGAs exclusively concentrate on receptor-based mechanisms there are few *in vitro* bioassays which deal with the effect of substances on

the steroidogenesis as a major part of the reproductive system. The H295R Steroidogenesis Assay is based on the human H295R-NCI adrenocortical carcinoma cell line. This cell line expresses all key enzymes of the steroidogenesis and produces all steroids of the adrenal cortex (Hecker et al. 2011, Staels et al. 1993). Therefore, effects on steroid production, enzymatic activities and gene expression are detectable (Hilscherova et al. 2004, Sanderson et al. 2000).

In vivo

With the Fish Short Term Reproduction Assay (OECD 229) it is possible to measure the impact of chemicals on the egg production (e.g. fecundity) and the biomarker endpoints vitellogenin production as well as secondary sexual characteristics. Therefore, this screening method provides information on several endocrine disrupting mechanisms. It is regularly accomplished with the fathead minnow (*Pimephales promelas*) and takes 21 days (Knacker et al. 2010, OECD 2012c). As the use of vertebrates for screening purposes should be avoided alternative methods should be preferred if possible. Therefore, great efforts for alternative methods are undertaken. In this context, the reproduction test with the mud snail *Potamopyrgus antipodarum* is a promising candidate. It sensitively detects endocrine disruption via the impact on the fecundity (e.g. produced eggs per female) of exposed individuals (Duft et al. 2007, Kase et al. 2009).

1.5 Selected test systems

In the present study, the two recombinant receptor-reporter assays lyticase assisted YES (LYES) and ER CALUX® assay as well as the H295R, that allows the detection of effects on the whole steroid synthesis pathway, were applied. Previous studies reported the applicability of these assays for the evaluation of endocrine activity in water samples (Gracia et al. 2008, Hecker & Hollert 2009, Kase et al. 2009, Leusch 2008, Leusch et al. 2010). Additionally, the *in vivo* reproduction test with the New Zealand mud snail *Potamopyrgus antipodarum* (Pa-Repro) has been used.

1.5.1 LYES

Yeasts of the strain *Saccharomyces cervisiae* have been stably transformed with an expression cassette for the human estrogen receptor α (hER α). Additionally, the cells contain an expression plasmid carrying the reporter gene lacZ, which is encoding β-galactosidase and the enhancer sequences for an estrogen responsive element (ERE) within the promoter region ensuring inducibility by hER activation.

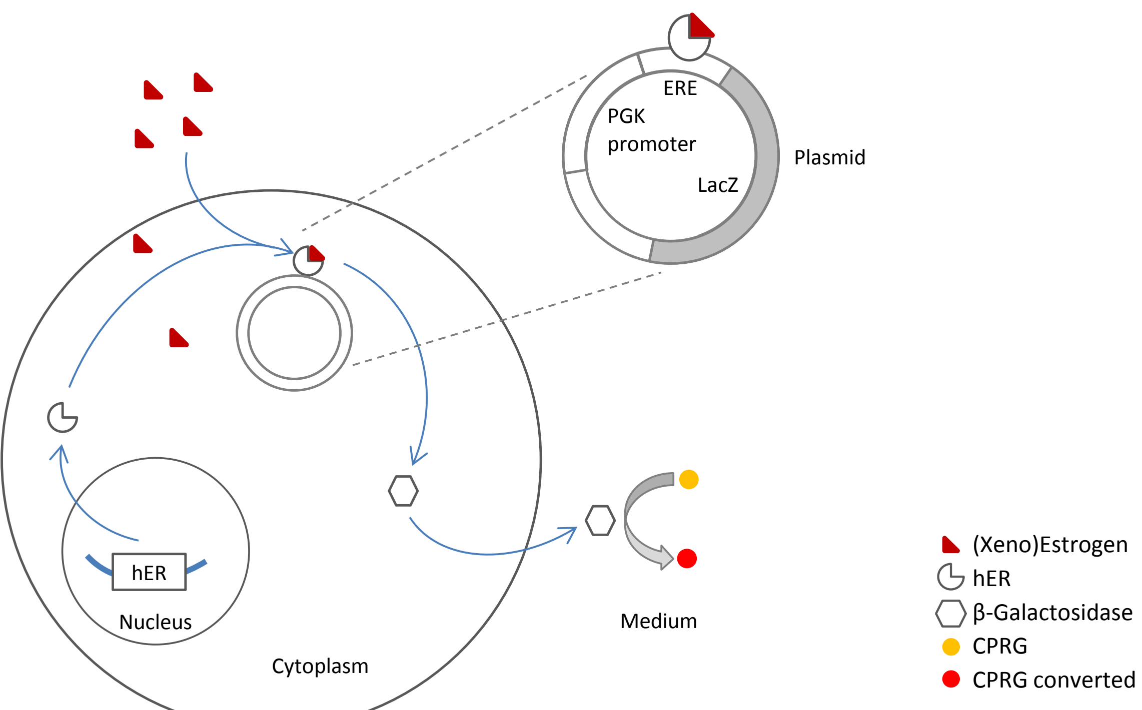

Figure 1.2 Mechanism of action in transformed yeasts of the Yeast Estrogen Screen (YES). The gene encoding the human estrogen receptor (hER) is integrated into the main genome. An expression plasmid is carrying estrogen responsive elements (ERE), the reporter gene encoding β-Galactosidase (LacZ) and the human phosphoglycerate kinase 1 (PGK) promoter. Redrawn and adapted from Routledge & Sumpter (1996).

Through binding of a ligand, expression of β-galactosidase is induced and the enzyme partly secreted into the culture medium. The ß-galactosidase converts the yellow chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) to its product chlorophenol red with a detection wavelength at 540 nm (Fig. 1.2). Conversion of CPRG is therefore directly proportional to the presence of receptor-mediated endocrine active substances (Routledge & Sumpter 1996). In the present study the lyticase assisted YES system according to Schultis & Metzger (Schultis & Metzger 2004) has been applied. It includes a lyticase assisted digestion step to shorten the test and increase the sensitivity.

1.5.2 ER CALUX®

Human T47D breast adenocarcinoma cells expressing endogenous estrogen receptors α and β have been stably transfected with a plasmid bearing the estrogen-responsive luciferase reporter gene (pEREtata-Luc).

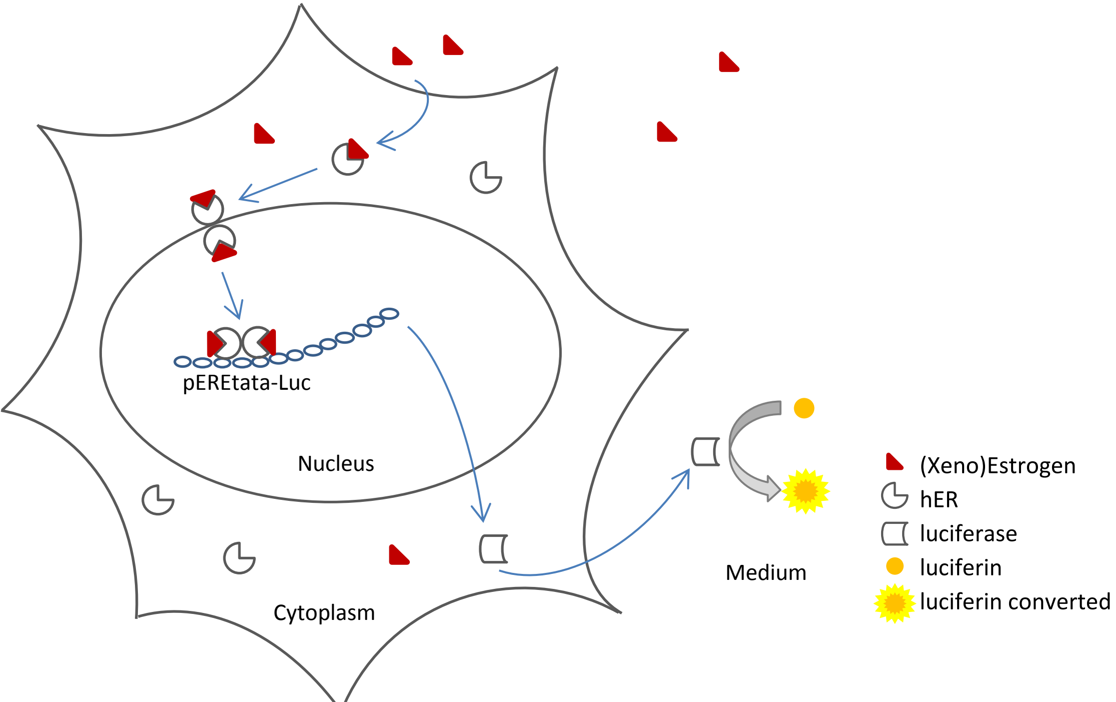

Figure 1.3 Mechanism of action in transfected T47D cells (T47Dluc) for the estrogen-responsive chemically-activated luciferase gene expression (ER CALUX®). The cells contain endogenous human estrogen receptors (hER) and the estrogen-responsive luciferase reporter gene pEREtata-Luc is stably transfected into the genome. Redrawn and adapted from Legler et al. (1999).

In case of binding of an estrogen active agent to the receptor the expression of the enzyme luciferase is induced and the enzyme is partly secreted into the culture medium. Luciferase activity can easily be measured through light emission after addition of the substrate

luciferin. This modified cell line (T47Dluc) is sensitive and highly responsible to (anti)estrogenic compounds (Legler et al. 1999).

1.5.3 H295R

This assay is a cell-based *in vitro* assay for the detection of chemicals with the potential to disrupt steroid production over various pathways (e.g. enzyme inhibition or gene expression) (Fig. 1.4). The H295R cell line expresses all the genes that encode all key proteins of steroid genesis downstream of cholesterol (Fig. 1.1). Production of steroid hormones can be measured with radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) as well as LC-MS (Hecker et al. 2011, Hecker et al. 2006).

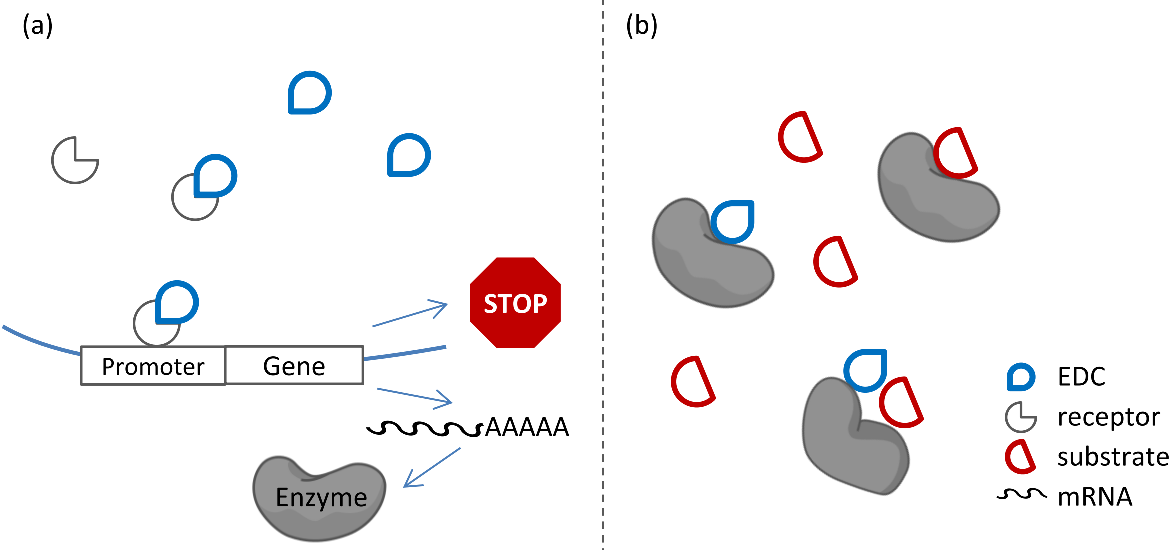

Figure 1.4 Possible mechanisms of steroidogenic disruption pathways in the H295R Steroidogenesis assay. Endocrine disruptive compounds (EDCs) can act over altering of gene expression (a) and inhibition of enzyme activities (b).

1.5.4 Pa-Repro

The reproduction test with the New Zeeland mud snail *Potamopyrgus antipodarum* is a sensitive *in vivo* test system for the detection of EDCs (Fig. 1.5). Under laboratory conditions the female adults reproduce mainly over parthenogenesis. Therefore, determination of the exact reproduction outcome for every single animal as well as for the whole population is possible. It is suitable for native samples as well as extracts and is able to express inhibition as well as promotion of reproduction (Duft et al. 2007, Fomin et al. 2009).

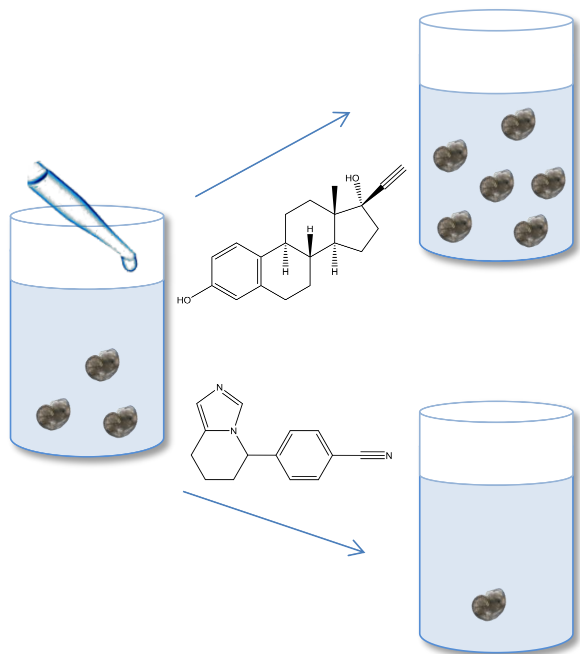

Figure 1.5 Reproduction of *Potamopyrgus antipodarum* (Pa-Repro) after exposure to endocrine active substances. An estrogen (e.g. ethinyl estradiol (EE2) causes a significant increase in embryo production; substances like aromatase inhibitors (e.g. fadrozole) cause a significant decrease in embryo production.

This bioassay arrangement allows a fast and simple screening of potential endocrine active native as well as processed samples from the aquatic environment. All test systems are applicable for extracted samples while native samples can be investigated by the LYES as well as the Pa-Repro and with some limitations (mostly regarding the possible contamination of samples with bacteria or fungi) even with the ER CALUX®. They cover the range of receptor-mediated activities as well as activities on the process of steroid genesis and impacts on whole organisms as well as populations.

1.6 Aims and objectives

This study aims to give insights into the application as well as the development and establishment of *in vitro* and *in vivo* bioassays for the detection of (possible) EDCs in the aquatic environment. The main objective was the establishment of a suitable bioassay battery to detect (possible) EDCs in different compartments of the aquatic environment as well as different sample matrices at the laboratories of the Institute for Environmental Research, RWTH Aachen University, Germany. With the application of the selected battery on different kinds of samples the suitability to detect a set of relevant endpoints should be verified. The selected endpoints and according bioassays were:

(1) Estrogen-receptor binding and activation (LYES and ER CALUX®);
(2) Alteration of the steroidogenesis (H295R);
(3) Alteration of the reproductive outcome (Pa-Repro).

For the successful establishment of the bioassay battery the present study aimed to positively respond to the following questions:

(A) Were the selected bioassays suitable to detect potential endocrine disrupting effects? Were there differences in between the investigated sample matrices?
(B) How was the performance of the selected bioassays within this study? Were the data comparable to other studies?
(C) Which kind of data analysis should be applied?
(D) How could the selected bioassays be further developed?
(E) Did the data revealed with the *in vivo* test system correlate with data revealed from the *in vitro* test systems?

In the first part of this study, the development of a new *in vitro* test system, the H295R Steroidogenesis assay (H295R), was scientifically attended. The properties of the H295R cell line to express all the necessary genes for the production of vertebrate steroids make it a unique base for the detection of substances that potentially disrupt the steroidogenesis. The H295R was developed and validated to become an OECD guideline by Hecker et al. (2011, 2007, 2006). In the present study, the outcomes of the inter-laboratory validation study are summarized. The test protocol was validated by the screening of 28 chemicals that were selected based on their known or suspected endocrine activity, or lack thereof. It could be

demonstrated that the H295R protocol successfully identified the majority of chemicals with known and unknown modes of interaction with the production of T and E2.

In the second part of the study the two recombinant receptor-reporter assays LYES and ER CALUX® were applied to the investigation of heterocyclic aromatic hydrocarbons (hetero-PAHs), a substance class that is frequently detected in groundwater wells and therefore could threaten the safety of drinking water. While there was no detectable estrogenic activity in the LYES the nine hetero-PAHS acridine, xanthene, indole, 2-methylbenzofuran, 2,3-dimethylbenzofuran, dibenzofuran, dibenzothiophene, quinoline and 6-methylquinoline showed estrogenic activity in the ER CALUX®. Estradiol equivalent factors (EEFs) between $2.85 \cdot 10^{-7}$ and $3.18 \cdot 10^{-5}$ were detected which is in the range of other known non-steroidal xenoestrogens (e.g. alkylphenols or bisphenol A) that are found in surface water. Chemical analyses revealed the metabolic capacity of the T47D cell line. Among the detected metabolites were hydroxides and their keto tautomers, sulfates, sulfoxides, and N-oxides. Because of their high concentrations measured in ground water, the metabolized hetero-PAHs could cause a potential risk for human health and should be the subject of further research. Furthermore, it could be shown that test systems without the ability for biotransformation, such as the LYES, are susceptible to underestimate the estrogenic potential of complex water samples. Consequently, when using such test systems a metabolic activation step (e.g. S9 liver fraction) should be included in the bioanalytical identification of potential EDCs.

A secondary objective of the present study was the comprehensive assessment of the endocrine disruptive effects of the biological mosquito larvicide *Bacillus thuringiensis israelensis* (*Bti*), which is regularly applied to large surface water areas in sensitive habitats of the aquatic ecosystem. The third part of this study, therefore, includes two chapters dealing with the evaluation of *Bti*. At first the potential endocrine disruptive effects were investigated with all three *in vitro* bioassays of the established battery. The already revealed estrogenic activity in the LYES was proofed for three commercially available *Bti* formulations as well as for the active substance VectoBac® TP (TP). In contrast, the investigated liquid formula as well as the formulation VectoBac® WDG were tested negative. With the ER CALUX® the estrogenic potential of TP again could be confirmed. In the H295R, significant but weak effects with no dose-response-relationship on the production of E2, and 21-hydroxyprogesterone (WDG) as well as T (TP) by H295R cells could be observed. The

investigated field samples as well as the samples from the artificial pond showed no significant increase of estrogenic activity after application of TP or WDG in the ER CALUX®. To investigate potential changes in the composition and abundance of algae and zooplankton populations after exposure to TP an aquatic microcosm study was conducted. In addition, the indicator-species *Potamopyrgus antipodarum* was integrated into the system to deliver information on a possible endocrine disruption of higher organisms. After exposure to a high concentration (20 $mg \cdot L^{-1}$) of TP a shift from green algae (Chlorophyta) to blue algae (Cyanobacteria) occurred. In contrast, the application of TP showed no impact on zooplankton communities. Clear effects on the reproduction of *P. antipodarum* could not be observed. Nevertheless, differences between the treatment-groups seemed to be associated with quantity and quality of the available food, which might be indirectly related to the application of TP (e.g. community shifts in phytoplankton and periphyton). Overall, a shift in the ecosystem structure in routinely treated areas seems unlikely, since maximum concentrations of TP utilized for mosquito control up to 50 times lower than the concentrations used in this study.

In the fourth part of the present study sewages from a medium sized country hospital with an advanced sewage treatment system (membrane bioreactor (MBR) and ozonation (O3)) were investigated with all three *in vitro* bioassays of the established battery. A first screening with the LYES revealed high estrogenic activity of the raw sewage (up to 35.8±8.7 $ng \cdot L^{-1}$ EEQ) which was significantly decreased after MBR and ozone treatment (up to 2.3±0.3 $ng \cdot L^{-1}$ EEQ). Results were confirmed by use of the CALUX® which detected activities of up to 0.2±0.11 $ng \cdot L^{-1}$ EEQs. Additionally, substance-specific analyses demonstrated efficient removal of most of the measured estrogenic compounds by ozonation. In contrast, treatment with ozone resulted in higher estradiol production and aromatase activity in H295R cells. It was hypothesized that this could partly be due to formation of by-products during ozonation. Overall, results obtained in this study demonstrated applicability of the used *in vitro* assays for monitoring of endocrine-modulating potency of treated sewage.

Chapter 2

Frequently applied methods

2.1 Sample preparation

All chemicals were purchased from Sigma Aldrich (Munich, Germany).

2.1.1 Solid phase extraction (SPE)

Parts of the liquid samples (see chapters 5 and 7) were extracted by use of solid phase extraction (SPE) with Oasis HLB cartridges (Waters) according to previously described methods by Bratkowska et al. (2010) and Cahill et al. (2004). Cartridges were conditioned with 4 ml hexane, followed by 4 ml acetone, 4 ml methanol and finally 4 ml demineralized water (pH 3). The pH of the samples was set to 3 by adding hydrochloric acid. Extraction was performed at a flow rate of 15-20 $ml \cdot min^{-1}$. For treated sewage and surface water 2000 ml, for untreated sewage a volume of 500 ml were concentrated. After drying under a gentle stream of nitrogen, cartridges were eluted with three times 4 ml acetone and 4 ml methanol. Extracts were again dried under a gentle stream of nitrogen and redissolved in ethanol or dimethylsulfoxide (DMSO) for dosing in bioassays and in methanol for chemical analyses (Pinnekamp et al. 2009). Prior to use in assays samples were stored in 1-4 ml amber glass vials with Teflon lids at ≤ -20°C.

2.1.2 Lyophilisation

Another set of liquid samples (see chapter 5) was processed through freeze drying (lyophilisation). Prior to lyophilisation turbid samples were centrifuged to remove particles from the solution. Centrifuged solutions as well as water samples were dried for approximately 96 hours in a manifold freeze-dryer (Christ, Germany) at 0.22 bar. The dried samples were reconstituted with 2x100 ml dichloromethane (DCM) in an ultrasonic bath. DCM was subsequently evaporated using a rotary evaporator until only 1-3 ml remained. The remaining solvent was removed under a low stream of nitrogen. Dry extracts were reconstituted in ethanol or DMSO and stored at ≤ -20°C in 1 ml amber glass vials with Teflon lids.

2.1.3 Liquid-liquid extraction (LLE)

Steroids as well as metabolites in assay medium were extracted by use of liquid-liquid extraction according to Hecker et al. (2006). For the H295R (see chapter 3 and 7), 450 µl of the assay medium were mixed with 550 µl nanopure water in a glass test tube. 2.5 ml

diethyl ether (chapter 7) or a 1:1 mixture of hexane and ethylacetate (chapter 3) were added and the test tube was vortexed for 1 minute (min) for the actually extraction step. Afterwards, test tubes were centrifuged for 10 min at 2,000 rounds per minute (rpm). The solvent fraction was collected in a second test tube and the process was repeated once. For the LC-DAD analysis (see chapter 4), 2 ml of the assay medium were extracted in the same manner but using 4 ml ethyl acetate instead. Extracts were dried under a gentle nitrogen stream and stored at -80°C until further analysis (H295R) or reconstituted in HPLC-grade acetonitrile (chapter 4) or methanol (chapter 3), respectively.

2.1.4 Pressurized liquid extraction (PLE)

Parts of the solid samples were extracted according to Beck et al. (2008) with acetone p.a. using a SpeedExtractor® (Büchi, Switzerland). Extraction columns were loaded with 10 g of each sample mixed with 10 g of quartz. The extraction was performed twice, at 30°C and 60°C (using separate loaded columns for each run). The device was operated under the following conditions: two extraction cycles, extraction pressure 120 bar. Each run contained an additional column loaded with 20 g of quartz sand only as the process control. Extracts were reduced in volume to about 2 ml with a rotary evaporator (Heidolph, Germany) and transferred to 4 ml amber glass vials. Subsequently, 500 µl DMSO were added as a keeper and the remaining acetone was removed under a gentle nitrogen stream. Upon removal of acetone, an additional 500 µl of DMSO were added (resulting in 1 ml DMSO).

2.2 Bioassays

2.2.1 Lyticase Yeast Estrogen Screen (LYES)

The Lyticase Yeast Estrogen Screen (LYES) was conducted as described by Routledge & Sumpter (1996) with modifications according to Schultis & Metzger (2004) and Wagner & Oehlmann (2009).

2.2.1.1 Cell culture

Recipes for media and nutrient agar plates are given in table 2.1. As long as not stated otherwise all chemicals were purchased from Sigma Aldrich (Munich, Germany).

Table 2.1 Recipes for cell culture of the yeasts

Amino acid solution (DO) with adenine	100 mg adenine, 100 mg arginine, 500 mg asparagic acid, 500 mg glutamic acid, 100 mg histidine, 150 mg isoleucine, 500 mg leucine, 150 mg lysine, 100 mg methionine, 250 mg phenylalanine, 2000 mg serine, 1000 mg threonine, 150 mg tyrosine and 750 mg valine in 500 ml ultrapure water (Millipore, Merck, Darmstadt, Germany); sterile filtered
Nutrient solution (SD)	13.4 g Yeast Nitrogen Base w / o Amino Acids (Difco, Becton Dickinson, Heidelberg, Germany) and 40 g D-glucose in 200 ml ultrapure water, sterile filtered
Growth medium	1 ml DO and 1 ml SD in 8 ml ultrapure water (sterile)
Freeze medium	Growth medium containing 40 % glycerol (sterile)
Nutrient agar plates	5 g Agar-agar in 200 ml ultrapure water; autoclaved Completion with 25 ml DO and 25 ml SD

For long time storage, yeasts were kept as cryo cultures in freeze medium in 2 ml reaction tubes (Eppendorf, Hamburg, Germany) at ≤ -70°C. Prior to testing, cryo cultures were plated on nutrient agar at 30°C for approximately 30 h to obtain single colonies with a diameter of 1 mm. Plates were stored at 2-8°C for a maximum of four weeks. For test purposes, yeasts had to be transferred to a liquid culture. Therefore, one colony was transferred to 10 ml growth medium in a 45 ml reaction tube (Falcon®, Corning, Amsterdam, The Netherlands) and incubated at 30°C and 750 rpm for 24 h. The liquid culture was stored at 2-8°C for a maximum of one week.

2.2.1.2 Test procedure

Recipes for test medium, buffer and reaction solutions are given in table 2.2. As long as not stated otherwise all chemicals were purchased from Sigma Aldrich (Munich, Germany).

Table 2.2 Recipes for test procedure of the LYES

Copper sulfate solution (CSS; 10mM)	2.5 g $CuSO_4 \cdot 5H_2O$ in 1 liter ultrapure water; autoclaved
Assay medium (1 plate)	10 mg ampicillin, 10 mg streptomycin, 7.5 ml DO, 7.5 ml SD and 148 µl CSS
lacZ buffer	8.52 g Na_2HPO_4, 0.75 g KCl and 0.25 g $MgSO_4 \cdot 7H_2O$ in 950 ml ultrapure water; pH 7; autoclaved Completion with 1 g sodium dodecyl sulfate (SDS) and ultrapure water up to a final volume of 1 liter
lacZ solution (1 plate)	2 mg Chlorophenol Red-β-D-galactopyranoside (CPRG), 0.4 mg lyticase (from *Arthrobacter luteus*, 250 $U \cdot mg^{-1}$) and 25 µl 2-Mercaptoethanol in 10 ml lacZ buffer

Generally, each well of a 96-well microtiter plate was filled with 75 µl reference water (tap water) or 75 µl of the liquid samples. Estrogenic potency was assessed relative to a 17ß-Estradiol (E2) standard curve (7 concentrations beginning at 1 pM and ending at 100 pM) running in parallel on a separate plate. E2 standards in ethanol were diluted in test medium and 25 µl of the corresponding E2 concentration were added to the reference water. Extracted samples or single substances were solved in ethanol and diluted in test medium as well. The solvent control contained test medium with ethanol and the blank only test medium. Yeasts from the liquid culture were diluted five times in test medium and 20 µl of the yeast solution was added to all wells except for the blanks. Plates were covered with breathable sealing membranes (Breathe-Easy®, Sigma Aldrich, Munich, Germany) to allow oxygen exchange. After incubation at 30°C and 750 rpm for 24 h cell density estimation was determined by optical density at 595 nm. Exposure was terminated by addition of 100 µl lacZ solution per well. The amount of converted CPRG was measured at 540 nm. Samples were at least tested in independent duplicates.

2.2.1.3 Data analysis

Data analysis was performed with Excel (Microsoft Corporation, Redmond, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, USA). At first, absorbance of each well was corrected for the blank (Eq. 2.1) and the cell density at 595 nm (Eq. 2.2).

$$Corrected\ cell\ number = OD595 - mean\ blank_{595nm} \qquad \textbf{Equation 2.1}$$

$$Corrected\ OD540 = \frac{OD540 - mean\ blank_{540nm}}{corrected\ cell\ number} \qquad \textbf{Equation 2.2}$$

Estradiol equivalents (EEQs) were calculated as described by Wagner & Oehlmann (2009). Corrected optical densities were inserted in an inversion of the four parameter logistic function (Eq. 2.4) which was fitted to the curve parameters obtained from the standard curve (Eq. 2.3).

$$y = \frac{Bottom + (Top - Bottom)}{1 + 10^{((logEC50 - E2\ conc.) \cdot Slope))}} \qquad \textbf{Equation 2.3}$$

$$EEQ\ (M) = 10^{\left(logEC50_{E2} - \frac{\log\left(\frac{TOP_{E2} - BOTTOM_{E2}}{corrected\ OD540_{Sample} - BOTTOM_{E2}} - 1\right)}{SLOPE_{E2}}\right)} \qquad \textbf{Equation 2.4}$$

EEQs were then corrected by the mean EEQ of the negative control of each replicate to exclude activities caused by the solvent as well as the used tap water.

To determine the performance of the test system (Armbruster & Pry 2008) the limit of detection (LOD; Eq. 2.5) and the limit of quantification (LOQ; Eq. 2.6) were calculated as EEQs by implementing the traditional applied formulas (Eq. 2.7) into the standard curve.

$$LOD\ (M) = EC50 \cdot \left(\left(\frac{Max_{E2}}{(SD_{control} \cdot 3) - 1}\right)^{\frac{1}{Slope}}\right) \qquad \textbf{Equation 2.5}$$

$$LOQ\ (M) = EC50 \cdot \left(\left(\frac{Max_{E2}}{(SD_{control} \cdot 10) - 1}\right)^{\frac{1}{Slope}}\right) \qquad \textbf{Equation 2.6}$$

$$LOD\ /\ LOQ = mean_{control} + x \cdot standard\ deviation_{control}$$

As the mean of the control always is subtracted from the data it is not implemented into the adapted formulas.

2.2.2 ER CALUX® Assay

The ER CALUX® was performed according to the method of Legler et al. (1999) with minor modifications according to the standard operation procedure (SOP) of BDS (2007).

2.2.2.1 Cell culture

Recipes for media, buffer and trypsine solution are given in table 2.3. As long as not stated otherwise all chemicals were purchased from Sigma Aldrich (Munich, Germany).

Table 2.3 Recipes for cell culture of the T47D cell line

Growth medium	500 ml Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (DMEM / F12; Gibco®, Life Technologies, Darmstadt, Germany) containing phenol red and GlutaMAX™, 41 ml fetal calf serum (FCS; Biowest, Nuaillé, France), 0.68 g $NaHCO_3$, 5 ml 100x non-essential amino acids solution (MEM; Gibco®, Life Technologies, Darmstadt, Germany) and 1 ml penicillin-streptomycin solution (5,000 $U \cdot ml^{-1}$; Gibco®, Life Technologies, Darmstadt, Germany; sterile filtered
Phosphate buffered saline (PBS)	0.2 g of potassium chloride (KCl), 0.2 g of potassium phosphate (KH_2PO_4), 1.15 g of sodium phosphate (Na_2PO_4) and 8 g of sodium chlorid (NaCl) in 1 liter ultrapure water, pH 7.2, sterile filtered
Trypsine solution	0.05 % trypsine in PBS; sterile filtered
Freeze medium	7 ml growth medium, 1 ml DMSO and 2 ml FCS

For long time storage, cells were kept in freeze medium in 2 ml cryo tubes (Greiner Bio-One, Frickenhausen, Germany) at -196°C in the gas phase over liquid nitrogen. For regular cell culture, cells were transferred to 10 ml growth medium in 75 cm^2 tissue culture (TC) flasks and incubated at 37°C and 7.5 % CO_2 until they reached 85-95 % confluence. Subsequently, cells were rinsed with PBS twice and trypsinated. Detached cells were transferred to new TC flasks and diluted 1:3 with fresh growth medium. T47Dluc cells can be subcultured a maximum of 40 passages.

2.2.2.2 Test procedure

The recipe for assay medium is given in table 2.4. As long as not stated otherwise, all chemicals were purchased from Sigma Aldrich (Munich, Germany).

Table 2.4 Recipes for test procedure of the ER CALUX®

Assay medium	480 ml DMEM / F12 (Gibco®, Life Technologies, Darmstadt, Germany) without phenol red, 25 ml charcoal stripped FCS, 0.63 g NaHCO3 and 5 ml MEM; sterile filtered

T47Dluc cells were diluted in assay medium until a density of $1 \cdot 10^5$ cells was achieved. The outer wells of a 96-well microtiter plate were filled with 200 µl PBS. 100 µl cell solution was seeded into the remaining inner wells. After 24 h incubation at 37°C and 7.5 % CO_2 medium was renewed. After another 24 h medium again was removed and cells were exposed to a dilution series of the single substances or extracted samples (solved in DMSO) in assay medium for 24 h. In addition, an E2 standard dilution series (7 concentrations beginning at 0.3 pM and ending at 30 pM) was included on each plate. Exposure medium was removed and luciferase activity was measured after adding 50 µl of PBS and 50 µl of SteadyLite® (Perkin Elmer, USA) with a luminescence counter (Infinite® M 200, Tecan, Switzerland). Samples were at least tested in independent duplicates.

2.2.2.3 Data analysis

Data analysis was performed with Excel (Microsoft Corporation, Redmond, USA). At first, mean values (given as relative luminescence units (RLU)) of the standard and samples were subtracted by the mean value of the DMSO control. Concentrations of EEQs were calculated as described by Legler et al. (1999) and BDS (2007). The corrected E2 concentrations were used for the construction of a calibration curve (sigmoidal fit, four parameter logistic function) (Eq. 2.8).

$$y = \frac{Max_{E2}}{1+\left(\frac{E2\ conc.}{EC50}\right)^{Slope}} \qquad \textbf{Equation 2.8}$$

Corrected luciferase responses of the samples were then interpolated in the linear range of the E2 standard curve (Eq. 2.9).

$$EEQ\ (pM) = EC50_{E2}\left(\left(\left(\frac{Max_{E2}}{corrected\ RLU_{Sample}}\right) - 1\right)^{\frac{1}{Slope_{E2}}}\right) \qquad \textbf{Equation 2.9}$$

LOD (Eq. 2.10) and LOQ (Eq. 2.11) again were calculated as EEQs (see chapter 2.2.1.3).

$$LOD\ (pM) = EC50 \cdot \left(\left(\frac{Max_{E2}}{(SD_{control} \cdot 3) - 1}\right)^{\frac{1}{Slope}}\right) \qquad \textbf{Equation 2.10}$$

$$LOQ\ (pM) = EC50 \cdot \left(\left(\frac{Max_{E2}}{(SD_{control} \cdot 10) - 1}\right)^{\frac{1}{Slope}}\right) \qquad \textbf{Equation 2.11}$$

2.2.3 H295R Steroidogenesis Assay

The H295R Steroidogenesis assay was conducted according to the methods of Hecker et al. (2011, 2007).

2.2.3.1 Cell culture

Recipes for media, buffer and trypsine solution are given in table 2.5. As long as not stated otherwise all chemicals were purchased from Sigma Aldrich (Munich, Germany).

Table 2.5 Recipes for cell culture of the H295R cell line

Stock medium	15.6 g Dulbecco's Modified Eagle's Medium / Nutrient Mixture F-12 Ham containing L-glutamine and 15mM Hepes and 1.2 g sodium bicarbonate(Na_2CO_3) in 1 liter ultrapure water; sterile filtered
Supplemented medium	12.5 ml Nu-Serum™ (Corning Life Sciences, Amsterdam, The Netherlands) and 5 ml ITS + Premix (Corning Life Sciences, Amsterdam, The Netherlands) in 482.5 ml stock medium, sterile filtered
Phosphate buffered saline (PBS)	0.2 g KCl, 0.2 g KH_2PO_4, 1.15 g Na_2PO_4 and 8 g NaCl in 1 liter ultrapure water, pH 7.4, sterile filtered
Trypsine solution	0.05 % trypsine in PBS; sterile filtered
Freeze medium	7.5 ml Nu-Serum and 5.26 ml DMSO in 92.5 ml stock medium

H295R cells were purchased from ATCC (LGC Standards, Wesel, Germany). For long time storage cells were kept in freeze medium in 2 ml cryo tubes (Greiner Bio-One, Frickenhausen, Germany) at -196°C in the gas phase over liquid nitrogen. For regular cell culture, cells were transferred to 10 ml supplemented medium, resuspended and centrifuged at 125·g for 5 min. Medium was discarded and the pellet was resuspended in 10 ml fresh supplemented medium in 75 cm^2 tissue culture (TC) flasks and incubated at 37°C and 5 % CO_2 until they reached 85-95 % confluence. Subsequently, cells were rinsed with PBS twice and trypsinated. Detached cells were transferred to new TC flasks and diluted 1:3 with fresh supplemented medium. H295R cells can be subcultured a maximum of 15 passages (derived from ATCC stock).

2.2.3.2 Test procedure

After a minimum of four passages H295R cells were seeded in a 24-well microtiter plate at a density of $3x10^5$ cells per ml. After 24 h the medium was discarded and 1 ml fresh supplemeted medium was added to each well. Cells were then exposed with 1 µl of a

dilution series (in DMSO) of the extracted samples or single substances as well as the two reference substances forskolin and prochloraz (quality control plate; QC) for 48 h. Subsequently, the medium was removed and extracted using liquid-liquid extraction with diethyl ether or a 1:1 mixture of hexane and ethylacetate (see 2.1.3). The amount of steroids was either determined in an enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' recommendations (Cayman Chemicals Europe, Tallinn, Estonia) as well as Grund et al. (2011) and / or by use of liquid chromatography tandem mass spectrometry (LC-MS^2). The LC-MS^2 consisted of an Agilent 1200 series HPLC system (Santa Clara, CA, USA) connected to an API 3000 triplequadrupole MS^2 system (PE Sciex, Concord, ON, Canada). Both LC and MS were controlled by AB Sciex Analysts 1.4.1 software (Applied Bioscience, Foster City, CA, USA). Chromatographic separation was done on a Betasil C18 column (Thermo Scientific, Waltham, MA, USA) using a mixture of ACN and 0.1 % formic acid in water as the mobile phase. The device was operated according to Chang et al. (2010). Furthermore, aromatase enzyme activity was measured (chapter 7) using a tritiated water release assay (^{3}H Release Aromatase Assay) as described by Lephart & Simpson (1991) with minor modifications according to Sanderson et al. (2001a). After removal of the assay medium (see above) cells were washed with PBS twice and exposed to a solution of 54 nM ^{3}H-androstenedione in stock medium at 37°C under a 5 % CO_2 in air atmosphere for 1.5 h. The reaction was stopped reaction by putting the microtiter plates on ice for 5 min. Subsequently 200 µl of the solution from each well were transferred to 1.5 ml reaction vials containing 500 µl chloroform. After short vortexing the vials were centrifuged and 100 µl of the supernatant were transferred to reaction vials containing dextran coated charcoal. After short vortexing and a lag phase of 5 min the vials were centrifuged and 125 µl of the supernatant were transferred to scintillation vials (Research Products International Corp., Mount Prospect, USA) containing 4 ml scintillation cocktail Bio-Safe II (Research Products International Corp., Mount Prospect, USA). The release of tritiated water, e.g. aromatase activity, was counted for 10 min in a scintillation counter (LS6500 Multi-Purpose Scintillation Counter, Beckman Coulter, Brea, USA).

2.2.3.3 Data analysis

For determining the steroid concentrations in the ELISA measured absorption was first corrected for the mean of the non-specific binding (NSB) through the tracer. Concentrations were then calculated by inserting the corrected values in a polynomial cubic function based on the estradiol standard (Eq. 2.7).

$$y = ax^3 + bx^2 + cx + d \qquad \textbf{Equation 2.7}$$

Concentrations of the steroids measured by means of LC-MS were calculated by comparison to the deuterium-labeled internal standards 21-HPT-d_8, 17-HPT-d_8, ASD-d_7, progestrone-d_9, testosterone-d_5 (100 µg·L^{-1}) and cortosol-d_4 (1 mg·L^{-1}) (C / D / N Isotopes Inc., Pointe-Claire, Canada).

For all data fold inductions (changes) compared to the SC were calculated whereas the SC was set to one.

2.2.4 MTT Assay

The MTT assay was conducted according to Sanderson et al. (2001b) with some modifications according to Blaha et al. (2004).

2.2.4.1 Test procedure

In case of the H295R assay the MTT was applied during the main test procedure directly after removal of the medium. For the ER CALUX® the MTT was performed separately prior to the main test. In both cases cells were treated in the same way as for the main assays (see chapter 2.2.2.2 and 2.2.3.2). After exposure cells were rinsed with PBS and subsequently exposed to a 0.5 mg·ml^{-1} solution of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) in unsupplemented assay medium for 30 min at 37°C and 5 % (H295R) or 7.5 % (ER CALUX®) CO_2 in air atmosphere. MTT solution was removed and lysis of the cells was implemented through addition of DMSO. After homogenization on an orbital shaker for 15 min absorbance at 492 nm was measured.

2.2.4.2 Data analysis

Viability of the cells was expressed as relative changes to the NC in percent, whereas the NC was set to 100 %.

Chapter 3

Development and inter-laboratory validation study of the H295R steroidogenesis assay

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Large parts of this chapter have been published in *Environmental Science and Pollution Research*

3.1 Abstract

Due to increasing concerns regarding the potential of chemicals to interact with the endocrine system of humans and wildlife, various national and international programs have been initiated with the aim to develop new guidelines for the screening and testing of these chemicals in vertebrates. In the current study, the validation of the H295R steroidogenesis assay is documented. With this test system, it is possible to detect inhibition or induction of sex steroid hormone production as well as alteration of enzyme activity or gene regulation. The aim of the inter-laboratory study was the development of an Organization for Economic Cooperation and Development (OECD) test guideline.

A previously optimized and pre-validated protocol was used to assess the potential of 28 chemicals of diverse structures and properties to validate the H295R steroidogenesis assay. These chemicals are comprised of known endocrine-active chemicals and chemicals that were not expected to have effects on the targeted endpoints, as well as a number of chemicals with unknown modes of action at the level of the steroidogenic pathway. A total of seven laboratories from seven countries participated in this effort. In addition to effects on hormone production, confounding factors, such as cell viability and possible direct interference of test substances with antibody-based hormone detection assays, were assessed. Each participating laboratory had to demonstrate that they were able to conduct the assay within the margin of predefined performance criteria.

With a few exceptions, all laboratories met the key quality performance parameters, while only 2 % (testosterone) and 7 % (17ß-estradiol) of all experiments were excluded due to non-compliance of these parameters. Of the 28 chemicals analyzed, 13 and 14 affected production of testosterone (T) and 17ß-estradiol (E2), respectively, while 11 and 8 did not result in significant effects on T and E2 production, respectively. Four and six chemicals produced ambiguous results for effects on T and E2 production, respectively. Significant interference of test chemicals with some of the antibody-based hormone detection systems occurred for four chemicals. Only one of these chemicals, however, significantly affected the ability of the detection system to categorize the chemical as affecting E2 or T production. With one exception, the H295R steroidogenesis assay protocol successfully identified the

majority of chemicals with known and unknown modes of interaction as inducers or inhibitors of T and E2 production. Thus it can be considered a reliable screen for chemicals that can alter the production of sex steroid hormones. Based on the results obtained during this validation study and the accordingly revised test protocols an OECD draft test guideline was developed (OECD 456).

Additionally, in the present study further improvements on the usability of the test system were developed. These included an adaption of the H295R cell line to suspension culture as well as the development of a highly sensitive but cost reducing enzyme linked immunosorbent assay (ELISA) for the detection of steroids in the exposure medium directly.

Keywords: endocrine disruption, estradiol, H295R, hormone production, transferability, sex steroid, steroidogenesis, testosterone, validation, OECD

3.2 Introduction

Due to increasing concerns regarding the potential of chemicals to interact with the endocrine system of humans and wildlife, various national and international programs have been initiated with the aim of developing new guidelines for screening and testing of these chemicals in vertebrates (Endocrine Disruptor Screening and Testing Advisory Committee 1998, OECD 1998, 2012). One of the leading and first nationally, legally binding programs was the endocrine disruptor screening program (EDSP) of the US Environmental Protection Agency (EPA), which employs a battery of *in vitro* and *in vivo* screening assays to assess the endocrine disrupting potential of chemicals. Specifically, the US Congress included a provision in the Food Quality Protection Act of 1996 that requires EPA to "… develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen…" (21 U.S. Code §346a (p)). Subsequent to passage of the act, EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a committee of scientists and stakeholders that was charged with the duty to provide EPA with recommendations on how to implement its EDSP. Upon recommendations from EDSTAC, the EDSP was expanded to include the androgen and thyroid hormone systems and wildlife effects as well as the originally mandated effects relating to estrogen. In addition to the US EPA activities, the Organisation for Economic Cooperation and Development (OECD) initiated a high-priority activity in 1998 to revise existing, and to develop new, test guidelines for the screening and testing of potential endocrine disrupting compounds (EDCs). The OECD conceptual framework for testing and assessment of potential EDCs comprises five levels, each level corresponding to a different level of biological complexity (OECD 2012).

The objective of the steroidogenic screening assay is to detect substances that would disrupt estradiol and testosterone production. It is intended to identify substances that interact with the biochemical pathway of steroidogenesis beginning with the sequence of reactions occurring after the gonadotropin-releasing hormone receptors (GnRHR) up through the production of the terminal sex steroid hormones testosterone and estradiol / estrone. The

steroidogenic assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamus or pituitary gland or on storage or release of sex steroid hormones.

One of the assays recommended by EDSTAC as a Tier 1 screen was an *in vitro* rodent minced testis assay to detect chemicals with the potential to disrupt steroid hormone production (Endocrine Disruptor Screening and Testing Advisory Committee 1998). Despite its long history of use, the rodent minced testis assay had not been optimized at the time that it was recommended by EDSTAC. EPA conducted a series of studies to optimize the assay and evaluate its suitability to serve a function in the EDSP testing battery. Preliminary inter-laboratory studies exhibited large variability within and among laboratories (Johnson & Feder 2005). However, the seemingly insurmountable problem of assessing cytotoxicity specific to Leydig cells led EPA's advisory committee to recommend that EPA abandon further work on the minced testis assay. As a consequence, there was a need for a less variable and more reliable *in vitro* test system as an alternative to the minced testis assay. One assay that offered promise with regard to the characterization of inducers and / or inhibitors of sex steroid production was the H295R steroidogenesis assay (Hecker & Giesy 2008, Hecker et al. 2006).

Development and standardization of the H295R steroidogenesis assay as a screen for the evaluation of the effects of chemicals on the synthesis of sex steroids has been conducted in a multistep process. The results of the assay optimization process and the pre-validation efforts undertaken to date have been reported previously (Hecker et al. 2007, Hecker et al. 2006). After initial development of the assay, US EPA presented a progress report on the development of the H295R assay to an OECD committee and invited member countries to join the USA in its further standardization and validation. This invitation was accepted by laboratories in Japan, Denmark, Germany, Hong Kong, and Korea.

In the present study, the results of an inter-laboratory study that was part of the final validation of a H295R protocol in accordance with the OECD guidelines for validation (OECD 1996) are presented. Using three model chemicals tested by five independent laboratories, an inter-laboratory pre-validation study was conducted to develop the H295R

steroidogenesis assay protocol (Hecker et al. 2007). These studies indicated that the H295R test protocol was capable of characterizing the effect of chemicals on the production of testosterone (T) and 17ß-estradiol (E2). The goal of the present project was to further validate the H295R steroidogenesis assay by assessing the transferability, flexibility, and applicability of an improved and revised protocol across several laboratories using an extended test set of 28 chemicals selected and approved by the OECD Validation and Management Group for Non-Animal Testing (VMG NA).

Subsequently to validation, further developments to enhance cell performance as well as to cheapen the test system were conducted. The adherent H295R cell line was adapted to chemical defined medium (CDM5) and brought into suspension culture which led to a prolonged growth period and therefore, fewer passages were needed. Additionally, a new ELISA protocol was developed which significantly lowered costs as well as duration of the H295R steroidogenesis assay.

3.3 Material & Methods

3.3.1 Study protocol

Based on the results obtained during the initial pre-validation studies (Hecker et al. 2006), a standardized H295R steroidogenesis assay protocol was developed (OECD 2011) (see chapter 2.2.4). To ensure sufficient basal E2 production cells were to be cultured under standard cell culture conditions for a minimum of four to five passages (cell age was not to exceed ten passages). For the inter-laboratory study cells were exposed to seven concentrations between 0.0001 and 100 µM of the test chemical in triplicate. At the end of the exposure period hormones were extracted using ethyl ether (see chapter 2.1.3). One laboratory (Lab 3) did not conduct extraction because the medium was directly used in a radio immunoassay (RIA). Cell viability in each well was analyzed immediately after removal of medium by means of the MTT assay (see chapter 2.2.1) (Mosmann 1983). All concentrations, where cell viability was less than or equal to 80 %, were excluded from the data analysis. Concentrations of hormones in medium were measured using commercially available hormone detection kits, namely enzyme linked immunoassays (ELISA; Lab 1, 2, 4 and 6) and radio immunoassays (RIA; Lab 3). Responses measured by means of antibody-based assays in the QC plate experiments were confirmed by instrumental techniques (liquid chromatography mass spectroscopy (LC-MS)) at Lab 1 following the method described by Chang et al. (2010) (data not shown). Each experiment was repeated three times with exception of Labs 1 and 3, where one and two replicate experiments were conducted per chemical, respectively.

Laboratories were required to demonstrate competence in performing all of the procedures that are part of the H295R steroidogenesis assay prior to testing chemicals (Tab. 3.1). The QC also served as a benchmark for determining laboratory competence prior to the initiation of chemical testing.

Table 3.1 Performance criteria to be met by each laboratory during experiments

System	Parameter	Comparison to / Between	T	E2
Hormone detection system	Sensitivity	Detectable fold decrease relative to SC	≥2-fold	≥2-fold
	Precision	CV among replicate measures (absolute concentrations) of the same well for SCs	≤25 %	≤25 %
Cell assay	Basal hormone production (SCs)	Fold greater than LOQ of hormone detection system	≥5-fold	≥2.5-fold
	Precision (SCs)	CV among absolute concentrations of replicate wells	≤30 %	≤30 %
	Sensitivity (induction at 10 µM forskolin)	Fold greater than SC	≥2-fold	≥7.5-fold
	Sensitivity (inhibition at 3 µM prochloraz)	Fold less than SC	≥0.5-fold	≥0.5-fold

Induction and inhibition refer to the relative change in hormone production after exposure to 10 µM forskolin or 3 µM prochloraz, respectively, in the QC plates
CV = Coefficient of variation (%), LOQ = limit of quantification, SC = solvent control

Prior to initiation of the actual exposure experiments, each chemical was tested for potential interference with the hormone detection system used. This was of particular relevance for antibody-based assays such as ELISAs and RIAs because it has been previously shown that some chemicals can interfere with these tests (Puddefoot et al. 2002, Shapiro & Page 1976).

3.3.2 Participating laboratories

Seven laboratories from the USA, Denmark, Germany, Japan, Hong Kong, and Canada, each with different levels of experience in conducting the H295R steroidogenesis assay, were invited to participate in this validation study. Inclusion of laboratories with different levels of proficiency in conducting the assay was essential to evaluate the completeness of the test protocols and their transferability. Each laboratory was assigned a random code number (1–7) as part of the study. However, part way through the study, two of the seven laboratories decided to cease their participation in the validation studies. Thus, with the exception of the QC exposure data, only the data for the remaining five laboratories that completed the validation studies is presented (Labs 1, 2, 3, 4, and 6).

3.3.3 Selection and testing of chemicals

A total of 28 chemicals were selected in this study to validate the H295R steroidogenesis assay as a screen for potential effects of endocrine-disrupting chemicals on the production of T and E2. These chemicals were selected based on their known or suspected endocrine activity, or lack thereof, and included inhibitors and inducers of different potencies as well as positive and negative controls. Where possible, the test set of chemicals was harmonized with those used in other steroidogenesis assays currently under development or in validation (e.g., the Registration, Evaluation, Authorization and Restriction of Chemical substances (REACH) program).

Prior to the start of the validation studies, all chemicals were pre-analyzed by the lead laboratory (Lab 1). To reduce the workload for individual laboratories, each of the other groups tested only 17 to 18 chemicals. Each chemical set consisted of a “core group” of 12 chemicals that were tested in parallel by all laboratories. In addition, three laboratories plus the lead laboratory conducted assays on a different set of five or six chemicals selected from the 16 chemicals that did not comprise the core chemicals. The 16 non-core chemicals were divided into three subgroups of five to six chemicals, and each chemical subgroup was tested by one of the three laboratories as well as the lead laboratory.

3.3.4 Further improvement of the H295R

For effective adaption of the H295R cell line to suspension culture different approaches were applied including epigenetic resetting by DNA demethylation. Successively, H295R cells were cultured within three different kinds of media: (1) DMEM F12 / Ham's (Sigma Aldrich, Munich, Germany) including standard H295R supplements and containing 5 µM 5-Aza-2’-deoxycytidine (5-Aza-dc), (2) ProVero™1 (Lonza, Cologne, Germany) and (3) “CDM5” (working title). 5-Aza-dc is a demethylating agent that inhibits DNA methyltransferase activity which presumably reactivates silenced genes (Ferguson et al. 1997). The serum-free medium ProVero™1 is a non-animal origin, protein-free medium for the culture of MDCK and Vero cell lines containing low levels of human recombinant insulin. The chemical

defined medium "CDM5" (working title) is designed for biopharmaceutical production and contains neither proteins nor steroids.

To replace the cost extensive, error prone as well as time consuming steroid extraction as well as the detection step with commercially available ELISA kits (Cayman Chemicals Europe, Tallinn, Estonia) a new ELISA method was developed. Therefore, 96-well-plates with high sorption capacities were coated with monoclonal antibodies for estradiol (E2), testosterone (T) or progesterone (Pro) (Tab. 3.2) at 100 µl per well. Plates were incubated for 12 hours (up to one week) at 2-8°C. Afterwards remaining plate surface was covered with skimmed milk powder (in PBS) at 200 µl per well for 1 hour at 37°C. Plates were washed three times with PBS-T and one time with PBS only. In a low-binding plate (TPP, Trasadingen, Switzerland) 60 µl per well of samples, standards and controls in cell culture medium were mixed with 60 µl of tracer in PBS (Tab. 3.2).

Table 3.2 Reagents and solutions for the alternative ELISA

Solution	Formula	Dilution
Antibodies	Monoclonal antibodies (α-estradiol-6 mAB, α-testosterone-3 mAB, α-progesterone-3 mAB; Antibodies-online, Aachen, Germany) in PBS (Sigma-Aldrich, Munich, Germany)	E2 1:4000 T 1:3000 P 1:1000
Blocking	5 % skim milk powder (Sigma-Aldrich, Munich, Germany) in PBS	
Washing	0.5 % Tween-20 (Roth, Karlsruhe, Germany) in PBS	
Tracer	17ß-estradiol(6)-short linker-HRP conjugate (Randox Laboratories, Wülfrath, Germany) in PBS 17ß-testosterone-3-HRP conjugate (Randox Laboratories, Wülfrath, Germany) in PBS Progesterone-3-HRP conjugate (Antibodies-online, Aachen, Germany) in PBS	1:2000
Substrate	Mix 10 ml 0.1 M KPO_4 / citrate (pH 6.0; Sigma-Aldrich, Munich, Germany) with 500 µl MeOH containing 5 µl of 5 mM 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich, Munich, Germany) in DMSO. Prior to incubation add 5 µl H_2O_2 (30 %)	

100 µl per well of this mixture were transferred to the ELISA plates and incubated at room temperature for one to two hours. Plates were washed three times with PBS-T and one time with PBS only. Per well 100 µl of the substrate solution were added and plates were

incubated for 20 to 45 minutes until Signal / Blank ratio at A_{640nm} reached >2.0. Reaction was stopped by adding of 3 M HCl (50 µl per well) and the absorption at 455 nm was measured immediately.

3.3.5 Statistical analysis

All data were expressed as mean±standard error of the mean (SEM). To examine the relative changes in hormone production, results were normalized to the mean solvent control (SC) value for each assay, and results were expressed as percent change relative to the SC. Prior to conducting statistical analyses, the assumptions of data normality and variance of homogeneity were evaluated. Normality was evaluated using standard probability plots or the Shapiro-Wilk's test. If the data were normally distributed or approximated a normal distribution, differences between chemical treatments and SCs were analyzed using one-way analysis of variance (ANOVA) followed by a two-sided Dunnett's test. If data were not normally distributed, the Kruskal-Wallis test followed by the Mann-Whitney U test was used. Data analysis was conducted using pooled replicate experiments. All statistical analyses were conducted using SYSTAT 11 (SYSTAT Software, Point Richmond, CA). Differences were considered significant at $p<0.05$.

3.4 Results & Discussion

3.4.1 Laboratory performance assessment

With a few exceptions, all of the laboratories met the key quality performance parameters for conducting the H295R assay protocol (Tab. 3.1; Fig. 3.1).

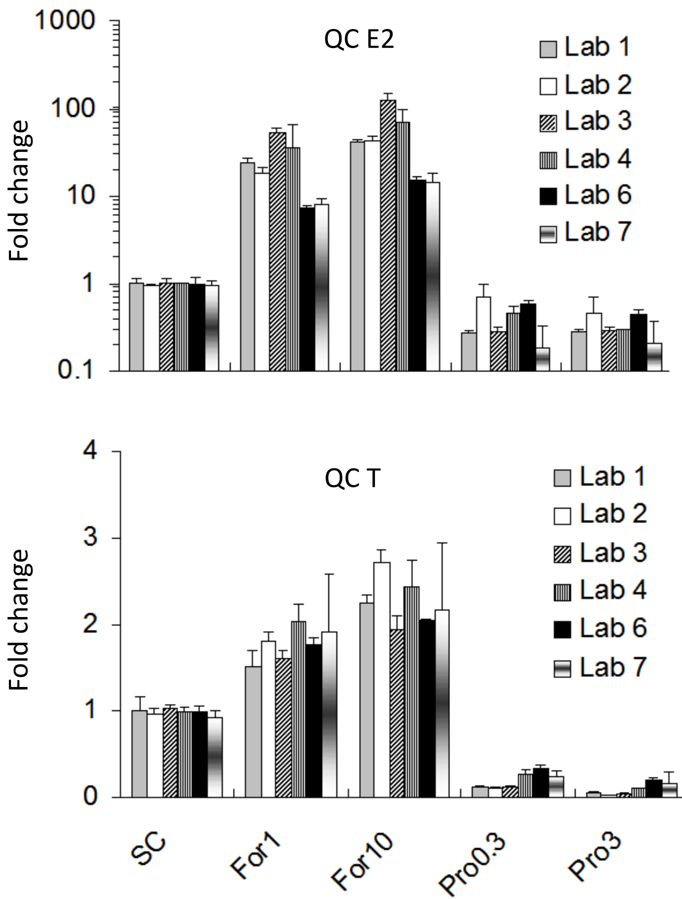

Figure 3.1 Comparison of changes in the concentrations of testosterone (T) and 17ß-estradiol (E2) relative to the solvent controls (SC = 1) in the quality plates (QC) among laboratories (Lab). For1 = 1 µM Forskolin; For10 = 10 µM Forskolin; Pro0.3 = 0.3 µM Prochloraz; Pro3 = 3 µM Prochloraz. Error bars = 1×standard deviation. Bars represent means of four independent experiments. (Lab 5: only T data from two experiments.)

However, at Lab 2, there was a greater increase (forskolin) and a lesser decrease (prochloraz) in T concentrations when compared to the other laboratories. Furthermore, at one laboratory, there were instances when decreases in E2 or T production could not be measured due to low basal hormone production (Tab. 3.3). In addition, in rare occasions,

there was an increase in variation among replicate wells such that the data could not be used. However, this only occurred at one laboratory during a single experiment (Lab 4; chemicals: letrozole, paraben, molinate, ethylene dimethanesulfonate (EDS); experiment 1), where the average coefficient of variation (CV) of the SCs was 48 %, which is almost 20 % greater than the QC criterion of 30 % for this parameter. None of the results obtained during these experiments was used for the data evaluation. However, it should be emphasized that these were rare events that did not impact the overall validity and utility of data produced during these studies. Overall, only 2 % or 7 % of all experiments for T and E2, respectively, were excluded due to exceedance of permitted variation. Relative changes in the production of T and E2 after exposure to forskolin and prochloraz in the QC plates were comparable both within and among laboratories (Fig. 3.1), indicating that the H295R steroidogenesis assay functioned similarly at all laboratories. Coefficients of variation for relative changes measured after exposure to forskolin and prochloraz were between 12 % and 13 % and between 44 % and 77 %, respectively, for T, and between 62 % and 73 % and 31 % and 55 %, respectively, for E2. There were no significant decreases in cell viability between any of the different treatment groups (results not shown).

Table 3.3 Lowest observed effect concentrations (LOECs) and strength and direction of change for testosterone (T) and estradiol (E2) after exposure to the 12 core chemicals.

	Lab 1[a]		Lab 2		Lab 3		Lab 4		Lab 6	
	LOEC (µM)	Max change	LOEC (µM)	Max change	LOEC (µM)	Max change	LOEC (µM)	Max change	LOEC (µM)	Max change
					Testosterone					
Prochloraz	0.0001	⇩⇩⇩⇩	0.1	⇩⇩⇩⇩	0.0001	⇩⇩⇩⇩	0.01	⇩⇩⇩⇩	0.01	⇩⇩⇩
Aminoglutethimid	100[b]	⇩⇩⇩	100[b]	⇩⇩	10	⇩⇩⇩	100[b]	⇩⇩	100[b]	⇩⇩⇩
Letrozole	100[b]	⇩⇩	100[b]	⇩⇩	100[a, b]	⇩⇩	100[b]	⇩⇩	100[b]	⇩⇩
Nonoxynol-9	10[b, c]	⇩	10[b, c]	⇩	n.d.[d]		10[b, c]	⇩	10[b, c]	⇩⇩
Molinate	n.d.		n.d.		100	⇩	n.d.		n.d.	
Benomyl	n.d.		n.d.		n.d.		n.d.[mu]		n.d.	
EDS	n.d.		n.d.		n.d.		n.d.		n.d.	
HCG	n.d.		n.d.		n.d.		n.d.		n.d.	
Paraben	10	⇧	n.d.		1	⇧	n.d.		n.d.	
Atrazine	100[b]	⇧	1	⇧	100[b]	⇧	n.d.		n.d.	
Forskolin	10	⇧⇧	1	⇧⇧	1	⇧	1	⇧⇧	1	⇧⇧
Trilostane*	0.1[mu]	⇧⇧⇧	0.01[mu]	⇧⇧⇧	1[mu]	⇧⇧⇧⇧	1[mu]	⇧⇧⇧⇧	0.01[mu]	⇧⇧⇧⇧
					17ß-Estradiol					
Letrozole	0.001	⇩⇩	0.001	⇩⇩	0.0001[mu]	⇩⇩⇩	0.01	⇩⇩⇩	0.01	⇩⇩
Prochloraz	0.1	⇩⇩	1	⇩	0.1	⇩⇩	1	⇩⇩	0.1	⇩⇩
Aminoglutethimid	100[b]	⇩⇩	10[mu]	⇩⇩	10	⇩⇩	100[b, e]	⇩⇩	100[b]	⇩⇩
Benomyl	n.d.		n.d.		n.d.[a]		n.d.[a]		n.d.	
EDS	n.d.		n.d.		n.d.		n.d.		n.d.	
Nonoxynol-9	n.d.		n.d.		n.d.		n.d.		n.d.	
HCG	n.d.		n.d.	⇧	n.d.[a]		n.d.[a]		n.d.	
Paraben	n.d.[mu]	⇧⇧⇧	10	⇧	10[mu]	⇧⇧	n.d.	⇧⇧	n.d.	
Molinate	100[b]	⇧⇧[mu]	100[b]	⇧	100[mu b]	⇧⇧	100[mu b]	⇧⇧	100[b]	⇧⇧
Atrazine	10	⇧⇧⇧	1[mu]	⇧⇧⇧	1[mu]	⇧⇧⇧⇧	10[mu]	⇧⇧⇧	0.1	⇧
Forskolin	0.01[mu]	⇧⇧⇧⇧	0.1[mu]	⇧⇧⇧⇧	0.1[mu]	⇧⇧⇧⇧	0.1[mu]	⇧⇧⇧⇧	0.01[mu]	⇧⇧
Trilostane*	1[mu]	⇧⇧⇧⇧	100	⇧	0.1[mu]	⇧⇧⇧⇧	1[mu]	⇧⇧⇧⇧	1[mu]	⇧⇧⇧

LOECs measured by Dunnett's or Mann–Whitney U test ([mu]); ranges refer to maximum values measured in repeated experiments

⇩ = >0.5-fold; ⇩⇩ = 0.5-fold to >0.25-fold; ⇩⇩⇩ = 0.25-fold to >0.1-fold; ⇩⇩⇩⇩ = ≤0.1-fold;

⇧ = <2-fold; ⇧⇧ = 2-fold to <4-fold; ⇧⇧⇧ = 4-fold to <20-fold; ⇧⇧⇧⇧ = ≥20-fold

n.d. = not detectable, * = uncertainty due to cross-reactivity; [a]Only one experiment was conducted or considered for data evaluation; [b]Effects occurred at greatest non-cytotoxic concentration; no dose–response; [c]Greatest concentration cytotoxic; [d]Cytotoxicity observed at concentration at which effects occurred at other laboratories = 10; [e]Not statistically significant; p=0.051

3.4.2 Core chemical exposure experiments

There were chemical-specific differences in the response of prochloraz T production after exposure of H295R cells to the 12 core chemicals (Tab. 3.3). With a few exceptions, the observed chemical-specific responses of T production were comparable among laboratories and could be grouped into three different types of effects: inducers, inhibitors, and negative reference chemicals. Among the inducers, exposure to trilostane resulted in the greatest fold changes (>10-fold induction) in T concentration when compared to SCs. The least fold changes were observed for the atrazine exposures where induction of T production was less than 1.5-fold with the exception of Lab 2, at which maximum induction was 2.4-fold. No effect on T production was observed after exposure to atrazine at Lab 6. Exposure to prochloraz resulted in a greater than 15-fold reduction of T production at the greatest concentration tested (100 µM) at all laboratories with the exception of Lab 4 where an up to 4.5-fold reduction was observed. The greater LOEC reported for Lab 2 is likely a function of the relatively great variation among replicate experiments at 0.01 M (CV=35 %). It is unclear why T production by cells was more sensitive to exposure with prochloraz at Labs 1 and 3. However, a concentration-dependent response was observed starting at 0.01 M, which is similar to the response patterns at the other labs. Therefore, it cannot be excluded that the significant reduction at 0.0001 and 0.001 M represents an artifact. Exposure to the other inhibitors resulted in less than 4-fold changes in T production. When chemicals exhibited a less than 1.5-fold change in T production, they were categorized as negatives. This threshold was defined based on the average variation observed across all laboratories among replicate experiments. Some of these negative chemicals could have been categorized as inhibitors in individual cases (molinate: Lab 4; benomyl: Lab 1). However, even in situations where inhibition was observed at an individual laboratory, changes were always less than 2-fold and typically were not concentration-dependent. For instance, exposure to nonoxynol-9 resulted in a decrease in T concentrations at non-cytotoxic concentrations at two of five laboratories for which data was available. Relative to the SCs, inhibition of T production at Lab 1 was 29 % (1 µM), while at Lab2, it was 47 % (10 µM). However, it should be noted that exposure to 10 µM

nonoxynol-9 resulted in an average increase in cell viability at Lab 2 (138 % viable cells relative to the SCs), and thus the observed reduction in T production may be an artifact due to the correction for cell viability, especially as no such increase was observed by any of the other groups. The greatest letrozole concentration resulted in a significant decrease in T at all laboratories.

Significant differences in E2 production were observed for H295R cells exposed to the 12 core chemicals. The direction of the effect for each chemical was comparable among laboratories (Tab. 3.3). Three chemicals inhibited E2 concentrations (letrozole, prochloraz, and aminoglutethimide), while human chorionic gonadotrophin (HCG), EDS, benomyl, and nonoxynol-9 did not elicit any clear (>1.5-fold) effects at non-cytotoxic concentrations. For inducers of E2 production, the magnitude of the response ranged between 20-fold or greater (forskolin) to <3-fold (paraben) than SCs. The most potent inducer of E2 production was forskolin. Exposure to forskolin resulted in increases in E2 production at concentrations greater or equal to 0.1 µM, while exposure to other inducers typically did not reveal effects at concentrations less than 1 µM. While responses for E2 after exposure to atrazine appeared to be greater or equal to two orders of magnitude more sensitive than at the majority of the other labs no concentration-dependent response pattern occurred up to 1 µM. In fact, increases in E2 concentrations did not follow a concentration response at lesser concentrations and were very small (1.16-fold greater than SCs). The most potent inhibitors were letrozole and prochloraz, exposure to which resulted in marked reductions of E2 at concentrations greater 0.001 and 0.1 µM, respectively. The exception to this pattern was exposure to letrozole at Lab 6, for which significant reductions occurred at concentrations greater than 0.01 µM. In contrast, exposure to aminoglutethimide only caused a clear reduction in E2 concentrations at the greatest concentration tested. Variation between laboratories did not exceed 2-fold for a given concentration with the exception of trilostane.

3.4.3 Supplemental chemical exposure experiments

For the additional 16 chemicals, the H295R steroidogenesis assay was able to categorize inducers and inhibitors of T and E2 (Tab. 3.4). One exception was dinitrophenol, which was identified as a significant inhibitor of T at all concentrations tested at the 1st lab. However, changes in T were not concentration-dependent and the magnitude of the effect was weak (inhibition did not exceed 0.67-fold relative to the SC at any given exposure concentration). Therefore, it is possible that this response represents an artifact. Some of the chemicals identified as inhibitors of T showed a biphasic response where slight increases in hormone production were observed at concentrations of up to 1 µM. However, with the exception of genistein, none of these changes exceeded 1.5-fold. Compared to the 12 core chemicals, there was greater variation among the responses observed at different laboratories for the 16 supplemental chemicals.

Table 3.4 Lowest observed effect concentrations (LOECs; detected by Dunnett's test) and strength and direction of change observed for the 16 test chemicals

	Testosterone				17ß-Estradiol			
	LOEC [µM]		Max change		LOEC [µM]		Max change	
	1st Lab[a]	2nd Lab[b]	1st Lab	2nd Lab	1st Lab[a]	2nd Lab[b]	1st Lab	2nd Lab
Ketoconalzole	1	1	⇩⇩⇩	⇩⇩⇩	10	10	⇩⇩	⇩⇩
Genistein	10	10	⇩⇩	⇩⇩⇩	10	10	⇧⇧⇧⇧	⇧⇧⇧⇧
Finasteride	10	100[c]	⇩⇩	⇩⇩	n.d.	100[c]	n.d.	⇩
Bisphenol A	10	10	⇩⇩	⇩	10	1	⇧⇧	⇧⇧
Dinitrophenol	0.0001	100[c]	⇩	⇩⇩	n.d.	n.d.	n.d.	n.d.
Piperonyl butoxide	10	10	⇩	⇩	n.d.	n.d.	n.d.	n.d.
Spironolactone	1	1	⇩⇩⇩	⇩⇩	n.d.	n.d.	n.d.	n.d.
Fenarimol	n.d.	10	n.d.	⇩⇩	n.d.	1	n.d.	⇩⇩
Danazol	n.d.	n.d.	n.d.	n.d.	1	10	⇩⇩⇩	⇩⇩
DEHP	n.d.	n.d.	n.d.	n.d.	1[d]	1	⇧⇧	⇧⇧
Dimethoate	n.d.	n.d.	n.d.	n.d.	10	n.d.	⇧⇧	n.d.
Flutamide	n.d.	n.d.	n.d.	n.d.	10	n.d.	⇧⇧	n.d.
Glyphosate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Prometon	n.d.	n.d.	n.d.	n.d.	100[c]	100[c]	⇧⇧⇧⇧	⇧⇧
Tricrecyl phosphate	10	n.d.	⇧	n.d.	10	n.d.	⇧⇧⇧	n.d.
Mifepristone	0.1	n.d.	⇧	n.d.	0.1	1	⇧⇧	⇧⇧

Chemicals tested at the second laboratories (2nd Lab) were as follows: Lab 2: Piperonyl butoxide, ketoconazole, prometon, DEHP, flutamide, and danazol; Lab 3: Bisphenol A, fenarimol, genistein, finasteride, and dinitrophenol; and Lab 4: Spironolactone, mifepristone, tricrecyl phosphate, dimethoate, and glyphosate.

⇩ = >0.5-fold; ⇩⇩ = 0.5-fold to >0.25-fold; ⇩⇩⇩ = 0.25-fold to >0.1-fold; ⇧ = <2-fold; ⇧⇧ = 2-fold to<fold; ⇧⇧⇧ = 4-fold to <20-fold; ⇧⇧⇧⇧ = ≥20-fold

n.d. = not detectable; [a]Lead laboratory (Lab 1); [b]Participating laboratory (Labs 2, 3, and 4); [c]Effects occurred at greatest non-cytotoxic concentration; no dose–response; [d]Considered because there was a clear concentration–response at all but the greatest concentration

Approximately 19 % and 31 % of the chemicals showed a significant response for T and E2, respectively, at only one of the two laboratories where they were tested (Tab. 3.4). These were fenarimol, finasteride, dimethoate, flutamide, and tricrescyl phosphate for E2, and fenarimol, mifepristone, and tricrescyl phosphate for T. It is unclear what the bases for these differences are, but it should be noted that in four out of the eight cases where such incongruencies were observed (E2: dimethoate, tricrescyl phosphate; T: mifepristone, tricrescyl phosphate), they were associated with Lab 4. In all four cases, these chemicals were identified as inducers by Lab 1, while no statistically significant effects were reported by the other testing group. Also, at the same laboratory, some of the cell viability data revealed no effects where significant decreases were observed at Lab 1 (tricrescyl phosphate and spironolactone). These results indicate that there might have been some issues related to dosing or supply of the chemicals. Finally, basal E2 production measured by Lab 4 was approximately three-to-four times greater than that measured by Lab 1 (~200 vs. ~50 pg·ml^{-1}), indicating that cells were at a later passage when used for the experiment. This supports the need for stringent conditions regarding the age of the cells as well as the need for further optimizing of the cell line (see also chapter 3.4.6). Nevertheless, QC criteria were met by Lab 4 in all experiments conducted.

3.4.4 Confounding factors—interference with hormone detection assays

The analysis of cross-reactivity of each chemical with the antibodies of the immunoassays used at most of the laboratories revealed interaction with a few chemicals at the greatest concentrations tested. A large interaction of the E2 immunoassay with trilostane (up to 100 % of the overall response measured at the greatest test concentration) was observed at all laboratories with the exception of Lab 2. Similarly, a less pronounced cross-reactivity of trilostane was also reported for the T antibodies (up to 60 % of overall response at the greatest concentration tested). However, since at most of the laboratories only the greatest chemical concentration was evaluated, an adjustment of the concentration–response curves could not be performed. However, an attempt to correct for the interaction with the antibodies at this greatest concentration (greatest three concentrations for Lab 1) indicated that while the induction of E2 after exposure to trilostane is likely to be solely due to this cross-reactivity, the induction of T could not be explained alone by this factor (Fig. 3.2).

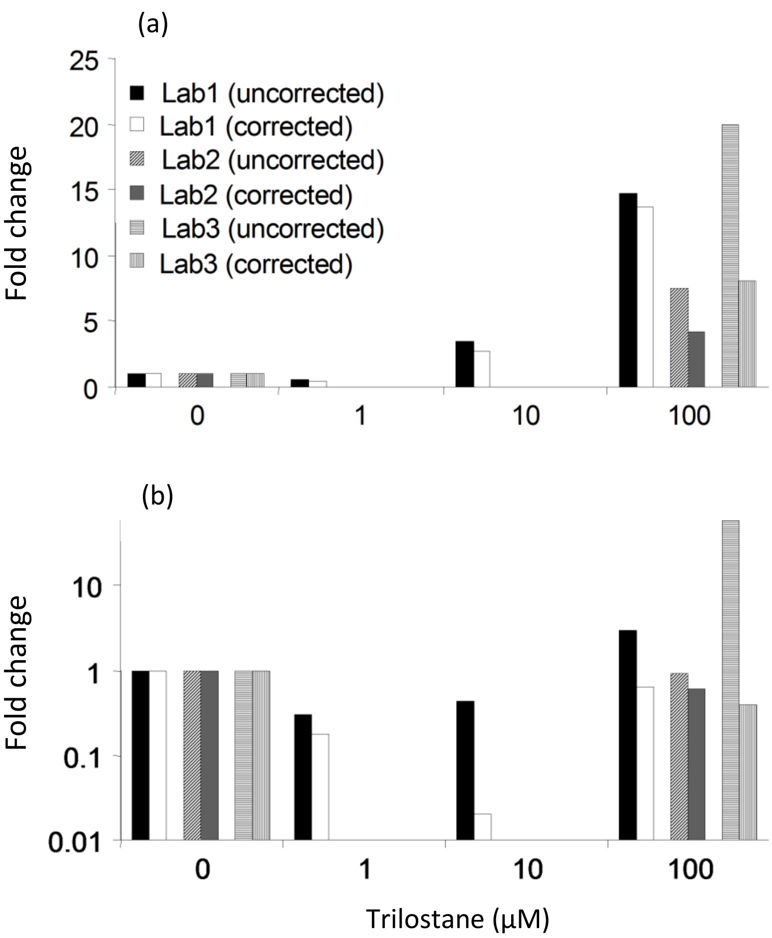

Figure 3.2 Changes in the concentrations of testosterone (T) and estradiol (E2) relative to the solvent controls (SC=1) after exposure to trilostane with (a) and without (b) adjustment of final hormone concentration for interference with the antibody-based hormone detection system. Bars represent average responses of one (Lab 1), two (Lab 3), and three (Lab 2) independent experiments.

Similar interactions of trilostane with hormone detection systems have been also observed by other authors (Puddefoot et al. 2002, Shapiro & Page 1976). In addition, nonoxynol-9, paraben, and prochloraz also interacted with the E2 immunoassays. However, since the cross-reactivity of prochloraz, paraben, and nonoxynol-9 at the greatest concentrations tested were either low or these concentrations were excluded due to marked cytotoxicity, this factor had no effect on the interpretation of the results. Significant interactions of the chemicals with the hormone detection assays that occurred at non-cytotoxic concentrations were only observed for T after exposure to spironolactone, finasteride, and danazol at Lab 1 and for E2 after exposure to genistein at Labs 1 and 3. When uncorrected

data (Fig. 3.2 b) for spironolactone, finasteride, and danazol were compared to the data corrected (Fig. 3.2 a) for this interference, significant impacts on the overall trend / response were not observed (data not shown). Similarly, while genistein interference with the E2 ELISA antibodies reduced the magnitude of the response by approximately 30 %, it did not change the overall trend of the response. However, further analyses are required to address possible uncertainties resulting from the interference of a test chemical with the hormone detection system utilized.

3.4.5 Predictive power and accuracy of H295R steroidogenesis assay

In addition to the ability of an assay to produce reliable and transferable results, the potential of data obtained with an *in vitro* test to be predictive of effects at higher organizational levels is one of the key parameters relevant to its use as a screening tool. Comparisons of the *in vivo* and *in vitro* effects of prochloraz, ketoconazole, fenarimol, prometon, and aminoglutethimide have been made previously (Hecker et al. 2006, Villeneuve et al. 2007), and the findings reported in this study were similar to those reported by these authors. Comparing with results from *in vivo* studies the potential of a cell line to metabolize xenobiotics is crucial. In principle, to a certain extent, H295R cells are capable of xenobiotic biotransformation. For example, both CYP1A1 and CYP1B1 are expressed in this cell line (Sanderson et al. 2001). Through oxygenation CYP1A1 catalyzes the initial step of the conversion of several chemicals to more polar metabolites and subsequently increased excretion. On the other hand, it catalyzes the metabolic activation of e.g. polycyclic aromatic hydrocarbons (PAHs) to their carcinogenic metabolites. Therefore, it plays a critical role in chemical carcinogenesis and toxicity (Ma & Lu 2007).

A comparison of the effects of E2 inducers observed in the H295R validation studies and the findings of *in vivo* studies showed that the results were comparable for six out of ten chemicals tested: atrazine (Spanò et al. 2004, Wetzel et al. 1994), mifepristone (Fassett et al. 2008, Wang et al. 1994), danazol (Peters et al. 1980), tricresyl phosphate (Latendresse et al. 1995), flutamide (Andrews et al. 2001), and genistein (Harrison et al. 1999) (Tab. 3.5). The results obtained with H295R cells for inhibitors of E2 production corresponded to the findings of *in vivo* studies for five out of six chemicals studied: letrozole (Kumru et al. 2007), aminoglutethimide (Berman & Laskey 1993, Rocha Monteiro et al. 2000), prochloraz (Brande-Lavridsen et al. 2008, Vinggaard et al. 2005), ketoconazole (Rocha Monteiro et al.

2000), and fenarimol (Ankley et al. 2005). In only three cases opposite trends among results for E2 production obtained with the H295R steroidogenesis assay and *in vivo* tests were observed. Exposure to DEHP, prometon, and bisphenol A (BPA) *in vivo* resulted in an inhibition (Davis et al. 1994) and no effect (Villeneuve et al. 2006, Yamasaki et al. 2002) on E2 concentrations, respectively, while all three chemicals caused a significant increase in E2 in the present study. However, the increase of E2 concentrations observed with the H295R cells for prometon may have been an indicator for the decrease in the expression of secondary sex characteristics observed in male fish (Villeneuve et al. 2007). In case of BPA, the lack of response in the *in vivo* studies is likely due to the administration route, which was via gavage. Previous studies have reported that orally administered BPA has very low bioavailability and is rapidly excreted (Pottenger et al. 2000).

Table 3.5 Comparison of data obtained with the H295R steroidogenesis assay (this study) with *in vivo* data

Chemical	Testosterone		Estradiol		Species	Reference
	H295R	*in vivo*	H295R	*in vivo*		
Aminoglutethimide	⇩	⇧	⇩	⇩	Fish; Rat	Bergman and Laskey 1993; Monteiro et al. 2000
Atrazine	⇧ / -	-	⇧	⇧	Fish; Rat	Spano et al. 2004; Wetzel et al. 1994
Benomyl	-	-	-	-	Rat	Carter and Laskey 1982; Spencer et al. 1998
Bisphenol A	⇧	n.d.	⇩	n.d.	-	-
Butyl paraben	-	-	⇧	n.d.	Rat	Taxvig et al. 2008
Danazol	-	n.d.	⇧	⇧	Human	Murakami et al. 1993; Peters et al. 1980
DEHP	-	-	⇧	⇩	Rat	Davis et al. 1994; Noriega et al. 2009
Dimethoate	-	n.d.	-	-	Sheep	Rawlings et al. 1998
Dinitrophenol	-	n.d.	-	n.d.	-	-
EDS	-	n.d.	-	n.d.	-	-
Fenarimol	⇩	⇧	⇩	⇩	Fish	Ankley et al. 2005
Finasteride	-	-	-	n.d.	Amphibian	Canosa and Ceballos 2001
Flutamide	-	-	⇧	n.d.	Rat	Adamson et al. 2008; Mikkilä et al. 2006
Forskolin	⇧	n.d.	⇧	n.d.	-	-
Genistein	⇩	⇩	⇧	⇧	Monkey; Rat	Harrison et al. 1999; Ohno et al. 2003
Glyophosate	-	-	-	-	Fish	Soso et al. 2006
HCG	-	-[a]	-	-[a]	-[a]	-[a]
Ketoconazole	⇩	⇩	⇩	⇩	Fish; Rat	Monteiro et al. 2000; O'Connor et al. 2002
Letrozole	⇩	⇧	⇩	⇩	Rat	Kumru et al. 2007
Mifepristone	⇧	⇧	⇧	⇧	Human	Fassett et al. 2008; Wang et al. 1994
Molinate	⇧	n.d.[b]	-	-	Rat	Ellis et al. 1998
Nonoxynol-9	-	n.d.	-	n.d.	-	-
Piperonyl butoxide	⇩	n.d.	-	n.d.	-	-
Prochloraz	⇩	⇩	⇩	⇩	Amphibian; Rat	Vinggaard et al. 2005; Brande-Lavritsen et al. 2008
Prometon	-	-	⇧	-[c]	Fish	Villeneuve et al. 2006
Spironolactone	⇩	⇩	-	n.d.[c]	Amphibian; Rat	Canosa and Ceballos 2001; Yamasaki et al. 2004
Tricrescyl phosphate	⇧ / -	n.d.	⇧	⇧	Rat	Latendresse et al. 1995
Trilostane	⇧[d]	⇩ / -	-[d]	-	Fish; Human; Rat	Jungmann et al. 1983; Villeneuve et al. 2006

⇧ = Increase; ⇩ = Decrease; - = No effect; n.d. = no data / study available; [a]H295R Assay only captures effects downstream of LH / FSH; [b]Data not considered because of inconclusive results; [c]Identified as a reproductive toxicant *in vivo*; [d]Corrected for cross-reactivity

Three chemicals that tested negative for E2 effects in the H295R, namely, benomyl, dimethoate, and glyphosate, also did not cause any changes in serum E2 concentrations *in vivo* (Soso et al. 2007, Spencer et al. 1996, Waldbillig et al. 1998). No studies describing *in vivo* effects on the production of E2 were found for the other chemicals tested. However, given the general toxic properties of chemicals, such as nonoxynol-9 (spermaticide), EDS (cytotoxicant to Leydig cells (Cooper & Jackson 1970, Kerr et al. 1985)), and dinitrophenol (metabolic poison uncoupling oxidative phosphorylation), no specific interactions with the steroidogenic pathway at non-cytotoxic concentrations would be expected.

In general, effects on T production were less consistent when the results obtained with H295R cells were compared to those of *in vivo* studies (Tab. 3.5). Out of the five chemicals found to be inducers of T production in the cells only mifepristone showed a similar trend *in vivo* (Wang et al. 1994), while three of the seven inhibitors (prochloraz: Vinggaard et al. (2005), Brande-Lavridsen et al. (2008); ketoconazole: O'Connor et al. (2002), Monteiro et al. (2000); genistein: Ohno et al. (2003)) revealed comparable trends between the results of the validation studies and previously reported *in vivo* data. However, five of the test chemicals demonstrated conflicting trends between the results obtained with the H295R cells and those from *in vivo* studies: the inducers atrazine (Spanò et al. 2004, Wetzel et al. 1994) and trilostane (Jungmann et al. 1982), the inhibitors letrozole (Kumru et al. 2007) and aminoglutethimide (Berman & Laskey 1993, Rocha Monteiro et al. 2000), and the negative chemical BPA (Yamasaki et al. 2002). As previously discussed for E2, the lack of response reported for BPA *in vivo* was likely a function of low bioavailability and rapid excretion due to the form of administration (oral; Pottenger et al. (2000)). With the exception of flutamide, 5 of the 11 chemicals that tested negative for changes in T production in the H295R steroidogenesis assay were also reported as causing no significant alterations in T concentrations *in vivo*: flutamide (Mikkilä et al. 2006), glyphosate (Soso et al. 2007), DEHP (Noriega et al. 2009), benomyl (Carter & Laskey 1982), and molinate (Ellis et al. 1998). For flutamide, a significant induction in T production was reported in rats *in vivo* (Andrews et al. 2001). Information on the effects of the other chemicals on production of T *in vivo* could not be found. The reason for the increased number of chemicals showing discrepancies between *in vivo* studies and the current work in the production of T as opposed to that of E2 is likely due to the intermediate role of T in the steroidogenesis

pathway, which makes it possible that changes in T can be better compensated by the cells than those in E2.

Overall, no chemical was falsely characterized as having no effect by the H295R steroidogenesis assay based on its known mechanism of action with the exception of T production after exposure to flutamide. However, this chemical would have been flagged due to a comparable *in vivo* / *in vitro* effect on E2. There were no studies describing the effects of the model inducer forskolin on hormone homeostasis *in vivo*. However, considering the rapid metabolism of forskolin by an organism no marked effects would be expected. Overall, these results indicate that, while not necessarily always directly predictive of a specific type of response in an organism, the H295R assay system always flagged a chemical as a potential disruptor of steroidogenic processes. Furthermore, there were only two chemicals for which both *in vivo* and *in vitro* data were available that would have been wrongly characterized as either inducers or inhibitors of each hormone tested by the H295R steroidogenesis assay (atrazine (Wetzel et al. 1994) and BPA (Yamasaki et al. 2002) for T induction and prometon (Villeneuve et al. 2006) and BPA (Yamasaki et al. 2002) for E2 inhibition). In case of BPA recent studies of Zhang et al. (2011) detected leakage of the substance from the standard laboratory material used for the H295R steroidogenesis assay into the test medium. The measured background contamination (0.38 $ng \cdot ml^{-1}$) after 24 h was not in a range near the LOEC of BPA (10 µm) in the current study but it has to be kept in mind that laboratory material can have an effect on the actual concentration in *in vitro* test systems. Furthermore, the authors detected metabolization of BPA in H295R cells. After approximately 48 h over 50 % of the substance was metabolized. Overall, investigation of BPA, as well as most other substances, should be accompanied by chemical analysis to verify the actual concentrations tested.

3.4.6 Further improvements of the H295R

Exposure to 5-Aza-dc lead to a suspension cell rate of 20 % but after a few passages insufficient proliferation and finally complete cell death were discovered. Therefore, this approach was skipped. The observed growth arrest may be caused by the reactivation of tumor suppressor genes like CDKN2A and SCGB3A1 (Fonseca et al. 2012). In contrast, replacement of the standard cell culture medium (DMEM F12 / Ham's) containing fetal bovine serum (FBS) by the serum-free medium ProVero™1 (Lonza, Cologne, Germany)

showed sufficient cell growth as well as a suspension rate of about 20 %. Unfortunately, there were issues regarding the quality between different batches, most likely due to the use of plant derived protein hydrolysates, wherefore an alternative medium was needed. In contrast, the replacement of the standard medium by a chemical defined medium for biopharmaceutical production without proteins and steroids (working title: CDM5) was overall successful. The H295R cells grew in suspension (H295R-S) with a stable cell viability of ≥95 % over 12 days without passage. Cell density increased in a linear manner from $0.5 \cdot 10^6$ cells·ml^{-1} to $2.5 \cdot 10^6$ cells·ml^{-1}, a reaction that was already described by Logie et al. (1999). Hence, the total cell density was higher than achieved in adherent cultures in tissue culture flasks. With this modification it is now possible to study the effect of a substance (or a mixture as well as environmental sample) on the steroidogenesis over a time period of up to 10 days. Within this period, medium samples can be taken and analyzed at a daily rate without changing the exposure medium. Additionally, the basal testosterone (T) production was higher compared to the adherent cells (Logie et al. 1999) but basal production of E2 was much lower (approximately 30 ng·L^{-1} after 19 passages from ATCC stock; data not shown). The quality criteria of the OECD guideline (OECD 456), however, were met by the suspension cells. The high level of T and at the same time low level of E2 could be explained by the lack of growth factors such as the epidermal growth factor (EGF). In the presence of the EGF, expression of aromatase is upregulated in H295R cells (Watanabe et al. 2006) which leads to an increased transformation of T to E2. In addition, the fast increase of steroid production over time could be decelerated (data not shown).

Another advantage of chemical defined media is the total absence of FBS. There are several drawbacks related to the usage of FBS in cell culture. First, there is the issue of animal welfare and the claim to save the lives of laboratory animals by use of alternative *in vitro* methods. FBS is a common supplement of cell culture media and includes several essential components for growth, homeostasis and survival of the cells (Barnes & Sato 1980, Even et al. 2006). The amounts of FBS that are used for *in vitro* methods are increasing mostly due to large scale manufacturing of cell therapy processes and biotechnology (Brindley et al. 2012) and to a certain extent even to the increasing number of *in vitro* test systems that are used for toxicity testing (e.g. for REACH purposes) (Hecker & Hollert 2011). While *in vitro* methods are a sufficient way to reduce suffering of laboratory animals the collection of FBS from fetal calves after slaughtering of the pregnant cow may cause severe suffering

of the fetuses. This seems to be contradictory to the 3R (replace, reduce, refine) principle (Russell et al. 1959) and is more than ethical questionable. Beside the ethical problems regarding to the use of FBS, there are also scientifically issues. On the one hand, FBS is a mixture of unknown composition which could lead to reduced reproducibility of different experimental approaches. For bioassays on endocrine disruption, for example, strongly varying steroid concentrations in the used FBS could influence the steroidogenesis within the cells. Therefore, in most cases stripping of FBS prior to use in the exposure medium is necessary. On the other hand, there is the risk of disease transmission because FBS could be contaminated with fungi, viruses, bacteria, mycoplasmas and prions. To overcome the problems related to the use of FBS as well as for the manufacture of biological products for human use, several serum free and chemical defined media have already been developed. Nevertheless, in contrast to media supplemented with FBS the alternative media are more cell type specific. Therefore, a special medium has to be used for most cell types (Even et al. 2006, Van der Valk et al. 2004). Additionally, culture medium without FBS leads to suspension cell cultures in most cases (Barnes & Sato 1980), though this was a desired side effect in the present study.

Performance of the newly developed ELISA method using monoclonal antibodies and tetramethyl benzidine / H_2O_2 as substrate (Nara et al. 2008) was good. For both the E2 and T standard clear dose-respond curves could be shown (Fig. 3.3).

According to the booklet (https://www.caymanchem.com/pdfs/582251.pdf) of the so far used ELISA kit the LOD of the assay is around 19 $pg \cdot ml^{-1}$ for E2. Estimated by the same calculation method (80 % Bound / Maximum bound) the LOD of the here established method is lower than 8 $pg \cdot ml^{-1}$ and the test system, therefore, more sensitive. The detected LOD is also in the same range as other recently developed sensitive ELISA methods (10 $pg \cdot ml^{-1}$) (Cha et al. 2010). For the Testosterone (T) ELISA the LOD of the former used method is 6 $pg \cdot ml^{-1}$ according to the booklet (https://www.caymanchem.com/pdfs/582701.pdf). In this case, the LOD of the new established ELISA method is higher (up to 30 $pg \cdot ml^{-1}$, in 100 % culture medium). But due to the high testosterone production in H295R-S cell cultures ($\mu g \cdot L^{-1}$ range), the obtained LOD is suitable for the detection of testosterone in the H295R-S culture medium. The detected LOD is also in the same range as other recently developed ELISA methods (50 $pg \cdot ml^{-1}$) (Uraipong et al. 2013).

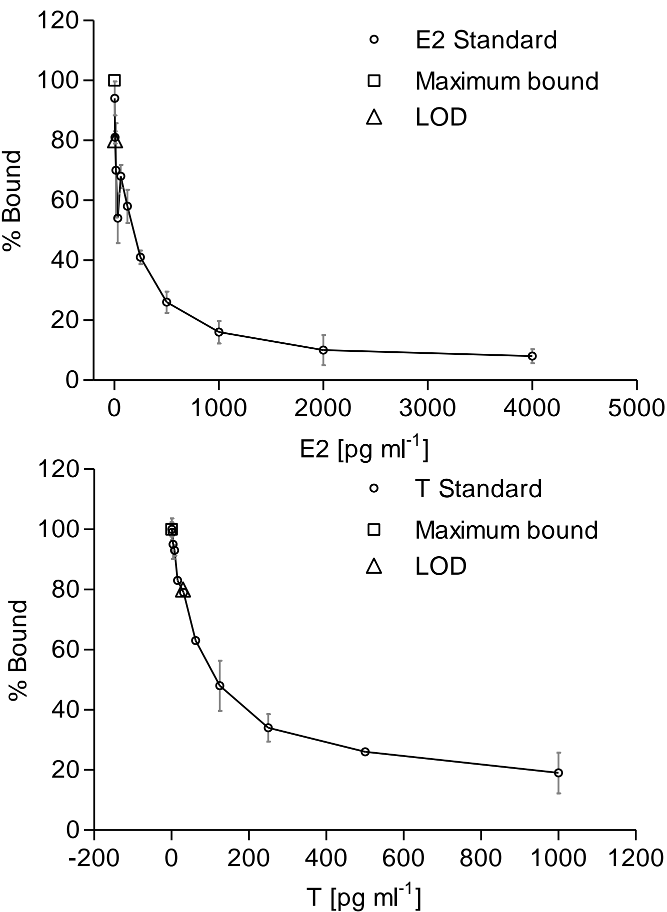

Figure 3.3 17ß-Estradiol (E2) and Testosterone (T) standards for the new developed ELISA protocols for the H295R Steroidogenesis Assay. LOD = limit of detection.

Overall, it could be shown that the developed ELISA method is highly sensitive and reliable for measurement of steroids in different cell culture mediums.

3.5 Conclusion

It was demonstrated that, with one exception, the H295R steroidogenesis assay protocol successfully identified the majority of chemicals with known and unknown modes of interaction with the production of T and E2. The results obtained in the current study confirm the findings reported for H295R cells by Hecker et al. (2006) as well as effects described in other *in vitro* and *in vivo* studies (discussed in Hecker et al. (2006) and this manuscript) for a broad range of chemicals. Major remaining limitations associated with the H295R steroidogenesis assay protocol are the relatively low basal production of E2 and its effect on quantifying the decreased production of this hormone with regard to the identification of weak inhibitors and at the same time the fast increase of basal hormone production within the passages which strongly reduces their useful life. To address this uncertainties, there should be further efforts aimed at increasing basal E2 production, e.g., by altering the cells or test protocols without affecting the potential of the cells to detect inducers of E2 production. In addition, several approaches to increase the useful life of the cell line as well as enhance the applicability of the whole test system already have been made. Furthermore, most of the variation observed among laboratories was likely due to changes in test practices during the course of this validation study. To address similar issues in the future, a number of additional performance criteria were included into the test protocols. These include the addition of a proficiency test that is required of each laboratory that plans to start using the assay or that has undergone changes in personnel, and the flexible protocols for refinement of the spacing of test chemical concentrations to enable the description of more precise concentration-response relationships. An initial comparison of H295R data from this study to *in vivo* studies from the literature demonstrated the potential of the H295R steroidogenesis assay to identify chemicals affecting hormone homeostasis in whole organisms. Particularly promising was the lack of any false negatives during the validation. Furthermore, the very low number of chemicals giving false positives represents an important aspect of this bioassay since it confirms the specificity of the test and will help avoid unnecessary additional testing. Future studies, including a larger number of chemicals with different structures and properties as well as comparison to parallel studies with whole organisms, should be conducted to confirm the predictive power of the H295R steroidogenesis assay for *in vivo* scenarios.

Acknowledgement

We thank the OECD VMG NA as well as the OECD secretariat for their support, encouragement, and guidance. Furthermore, we acknowledge many helpful discussions and manuscript review by Dr. Jerome Goldman, Endocrinology Branch, NHEERL, US EPA, Research Triangle Park, NC. Funding for this project was provided by US EPA, ORD Service Center / NHEERL (contract # GS-10F-0041L), a grant from the Western Economic Diversification Canada (project #s 6971 and 6807), and the Nordic Chemical Group (project # 2008-06-05). Conclusions drawn in this manuscript neither constitute nor necessarily reflect US EPA policy regarding the test chemicals and methods.

Chapter 4

Estrogenic activity of heterocyclic aromatic hydrocarbons

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This chapter has been published in *Environmental Science & Technology*

4.1 Abstract

High concentrations of heterocyclic aromatic hydrocarbons (hetero-PAHs) are increasingly detected in groundwater plumes at former industrial facilities where coal tar-oil was handled, e.g. wood treatment plants. Previous studies have shown that fractions of ground water with high estrogenic activity contained hetero-PAHs and their hydroxylated metabolites. To evaluate this preliminary evidence, twelve selected hetero-PAHs were screened for their estrogenic activity in the LYES and the ER CALUX® assay. All tested substances were inactive in the LYES assay. In contrast, nine hetero-PAHs (acridine, xanthene, indole, 2-methylbenzofuran, 2,3-dimethylbenzofuran, dibenzofuran, dibenzothiophene, quinoline and 6-methylquinoline) showed estrogenic activity in the ER CALUX®, with estradiol equivalence factors (EEFs) from 2.85 10^{-7} to 3.18 10^{-5}. The EEF values of the investigated substances were comparable to those of other non-steroidal xenoestrogens (e.g. alkylphenols, bisphenol A or phthalates) that are found in surface water. Chemical analyses revealed that T47Dluc cells were able to metabolize most of the substances. Among the metabolites (tentatively) identified were hydroxides and their keto tautomers, sulfates, sulfoxides, and N-oxides. Because of their high concentrations measured in ground water, we conclude that the detected estrogenicity of metabolized hetero-PAHs causes a potential risk for human health and should be the subject of further research.

Keywords: ER CALUX®, Heterocyclic aromatic compounds, hetero-PAH, NSO-HET, LYES

4.2 Introduction

A large number of synthetic heterocyclic aromatic compounds are produced by chemical industry to manufacture for example solvents, dyes or pesticides, respectively. In addition, they occur as contaminations in waste material from the mining industry, coal tar-oil processing operations, wood preservation facilities and the chemical industry (Kaiser et al. 1996). Therefore, heterocyclic aromatic hydrocarbons (hetero-PAHs) containing nitrogen, sulphur or oxygen heteroatoms represent pollutants increasingly studied at contaminated (former) industrial sites. During technical processes large amounts have been emitted into the environment since the 19th century (Alinsafi et al. 2006, Becker et al. 2001, Dyreborg et al. 1997, Thomas et al. 1995). Due to their heterocyclic structure (Fig. 4.1) hetero-PAHs are easily soluble in water and therefore transported to the ground water. As a consequence, long contaminated ground water plumes have been observed – a fact that also implies an elevated risk for human health since ground water is an important drinking water resource (Reineke et al. 2007, Tiehm et al. 2008). At former industrial sites even the soil can still contain high concentrations of heterocyclic PAHs (Meyer & Steinhart 2000, 2001).

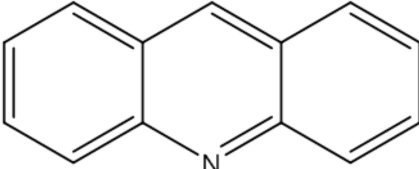

Figure 4.1 Chemical structure (ChemBioDraw Ultra (Perkin Elmer, Waltham, USA; free trial version) of the heterocyclic aromatic hydrocarbon acridine.

While extensive knowledge exists concerning environmental fate, as well as toxicological and ecotoxicological effects of PAHs (Douben 2003), such knowledge is still limited for their heterocyclic analogs. Just recently, the scientific community began to comparatively investigate a range of heterocyclic PAHs in different *in vitro* bioassays: e.g. acute toxicity to *Daphnia* and growth inhibition of green algae (Eisentraeger et al. 2008), mutagenicity (Ames assay) and embryotoxicity in the zebrafish (Peddinghaus et al. 2012) or aryl hydrocarbon (Hinger et al. 2011, Sovadinová et al. 2006) as well as retinoid receptor (Benisek et al. 2011) mediated effects. In most assays, hetero-PAHs caused similar or even higher toxicity compared to their homocyclic analogs. However, there are still data gaps, especially with regard to potential endocrine disrupting effects of hetero-PAHs.

For homocyclic PAHs, it was already demonstrated that vertebrates can transform the parent molecules into hydroxylated metabolites (e.g. Varanasi et al. 1989). In the organisms, this reaction of phase I biotransformation has the purpose to increase the water solubility of the molecule and to make it more susceptible to phase II biotransformation reactions – both processes ultimately facilitating the excretion of a compound. In permanent cell lines, which have been developed for bioanalytical determination of chemicals with mechanism-specific, e.g. dioxin-like or estrogenic effects, these biotransformation reactions could also lead to formation of active metabolites. Consequently, not only direct effects of the parent compounds but also indirect effects of metabolites have to be considered. For PAHs, e.g. benzo[*a*]pyrene and chrysene, it has been shown that indirect effects can occur in bioanalytical assays for detection of estrogenic substances (Santodonato 1997, van Lipzig et al. 2005). Furthermore, previous bioanalytical studies on the contamination of ground water with estrogenic substances have shown that fractions with high estrogenic activity contained, among others, hetero-PAHs and their hydroxylated metabolites (Kuch et al. 2010). In another study on endocrine activity of river sediments, a correlation between endocrine activity and fractions containing PAHs could be identified (Higley et al. 2012). To investigate whether heterocyclic PAHs also have the potential to act as indirect estrogenic compounds, we report on a study on the effects of selected substances in the lyticase assisted yeast estrogen screen (LYES) as well as the “estrogen receptor mediated chemical activated luciferase gene expression” assay (ER CALUX®). Most compounds tested here were suggested earlier as being representative for tar-oil contaminated sites by the project framework BMBF KORA (retention and degradation processes to reduce contaminations in groundwater and soil, cf. Blotevogel et al. 2008, Eisentraeger et al. 2008) and comprised the individual compounds indole, 1-benzothiophene, benzofuran, 2-methylbenzofuran, 2,3-dimethylbenzofuran, quinoline, 6-methylquinoline, carbazole, dibenzothiophene, dibenzofuran, acridine, and xanthene. To assess the loss of parent substance and the formation of metabolites during incubation with T47Dluc cells, supernatants were analyzed by means of liquid chromatography with diode array detection (LC-DAD) and high resolution tandem mass spectrometry (LC-HRMS^2).

4.3 Material & Methods

4.3.1 Chemicals

Indole (> 99 %), quinoline (> 98 %), carbazole (approx. 95 %), 6-methylquinoline (> 98 %), benzothiophene (> 98 %), dibenzothiophene (> 98 %) were obtained from abcr (Karlsruhe, Germany). Acridine (> 98 %) was supplied by Merck (Darmstadt, Germany). Xanthene (99 %), benzofuran (> 99 %), 2-methylbenzofuran (≥ 96 %), 2,3-dimethylbenzofuran (≥ 97 %) and dibenzofuran (approx. 98 %) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Stock solutions of the investigated hetero-PAHs were prepared in dimethyl sulfoxide (DMSO). Generalized structures of the investigated compounds are given in Figure 3.

4.3.2 LYES

For principle and general implementation of LYES see also chapter 1.4.3.2 and 1.5.1 as well as chapter 2.2.2. In the present investigation, yeast cells were exposed to a 1:2 dilution series of the heterocyclic PAHs in quadruplicate wells for 24 h. The maximum concentration of the tested chemicals was chosen according to concentrations used in the work of Hinger & Brinkmann et al. (2011).

4.3.3 ER CALUX®

For principle and general implementation of the ER CALUX® see also chapter 1.4.3.2 and 1.5.2 as well as chapter 2.2.3. In the present investigation, the cells were exposed to a 1:2 dilution series of each of the heterocyclic PAHs in triplicates. The maximum concentration of the tested chemicals was chosen according to concentrations used in the work of Hinger & Brinkmann et al. (2011).

4.3.4 MTT assay

An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay (see chapter 2.2.1) was conducted to assure that the test items did not cause non-specific cytotoxic effects in the T47Dluc cell line.

4.3.5 Data analysis

Data analysis of both ER CALUX® and LYES were conducted following the recommendations of Villeneuve at al. (2000). Mean luminescence and absorbance values, respectively, of test items and E2 standards were corrected for the response of the solvent controls. Resulting values were then divided by the maximum induction of the E2 standard (E2-max.) to scale all values from 0 (solvent control) to 1 (E2-max.). Scaled values from triplicate experiments were plotted using the software GraphPad Prism 5 (GraphPad, San Diego, USA) and fitted using four-parameter logistic regression with variable slope where top and bottom of the curve were set to 0 and 1, respectively (Figure 2 and 3). $EEF_{20\text{-}80}$ ranges, i.e. multiple estradiol equivalence estimates (EEFs, Equation 4.1) along the concentration-response curves based on a ratio of EC_{20}s, EC_{50}s and EC_{80}s, were calculated in order to both test the assumptions of parallelism and equal efficacy and give a measure of uncertainty for mass-balance analyses.

$$EEF_X = \frac{ECX_{E2}}{ECX_{sample}}$$ **Equation 4.1**

4.3.6 Prediction of estrogen receptor binding affinities

Estrogen receptor (ER) binding affinities were simulated using the (Q)SAR Toolbox 2.3 provided by the Organization for Economic Cooperation and Development (OECD). Simulations were performed for parent compounds, as well as for measured metabolites and metabolites predicted by the liver metabolism simulator of the toolbox. Results of the model were qualitatively compared with experimental data from both bioassays.

4.3.7 Determination of total metabolism by means of LC-DAD

T47Dluc cells were seeded in 6-well microtiter plates and incubated in duplicates with the highest concentration and under the same conditions as in the ER CALUX®. A control without cells was incubated under the same conditions as the cells to determine substance losses due to, e.g., sorption, precipitation or volatilization. After liquid-liquid extraction (see chapter 1.4.2.1 as well as 2.1.3) heterocyclic PAHs and respective metabolites were analyzed according to Mundt & Hollender (2005), with slight modifications. Liquid chromatography was performed using a 1200 Series LC chromatograph (Agilent,

Waldbronn, Germany) equipped with an UV-diode array detector at 1.0 ml·min^{-1} flow. Briefly, 40 µl of the reconstituted extracts were separated on a Nucleosil C18 pre-packed column (250 mm × 4 mm, 5 µm particle size; Macherey-Nagel, Düren, Germany) by gradient elution with acetonitrile (solvent A) and 5 mM potassium phosphate buffer at pH 7 (solvent B) using the following program: held 10 / 90 % (v / v) for 2 min, in 2 min up to 50 / 50 % (v / v), up to 60 / 40 % (v / v) in 8 min, up to 75 / 25 % (v / v) in 10 min, to 100 / 0 % (v / v) in 5 min, back to 10 / 90 % (v / v) in 12 min and held 6 min for equilibration. The diode array detector signals at 210 and 254 nm, as well as the spectra from 190 to 400 nm were recorded. Acridine was quantified at 254 nm, all other hetero-PAHs at 210 nm. Substance losses during incubation with and without cells were compared.

4.3.8 Identification of metabolites by LC-high resolution tandem mass spectrometry (LC-HRMS^2)

To assess the formation of potentially estrogenic transformation products, T47Dluc cells were exposed to 10 mg·L^{-1} of each heterocyclic PAH as for the determination of total metabolism. Instead of using 6-well microtiter plates, cells were seeded in 75 cm^2 tissue culture flasks to obtain a higher volume of cell culture supernatant (15 ml). To assure full compatibility with the LC-HRMS^2 method, stock solutions (10 mg·ml^{-1}) of the substances were prepared in ethyl acetate instead of DMSO. The final concentration of ethyl acetate in the exposure medium was 0.1 %. A solvent control was treated as the dilutions without addition of substance. Following 24 h incubation at 37°C, cells were detached with a cell scraper and homogenized with the cell culture supernatant using an automatic disperser. The suspension was then centrifuged at 25°C (4000 × g) and the supernatant stored at -80°C until analysis.

The samples were analyzed by LC-high resolution mass spectrometry using an Agilent 1200 LC system coupled to a LTQ Orbitrap XL (Thermo Scientific). The LC method is described in (Hug et al. 2014). Full scan chromatograms (m / z 100-1000) were acquired after electrospray ionization in positive and negative ion mode at a nominal resolving power of 100,000 referenced to m / z 400. HRMS product ion spectra were recorded in data-dependent mode using collision-induced fragmentation (CID) and higher-energy collisional dissociation (HCD) at different collision energies. Full scan spectra were manually searched for the masses of human and mammalian metabolites of the studied compounds reported

in the literature as well as possible phase I metabolites based on common pathways (e.g., the ion mass of an M+O molecule, corresponding to N-oxides, sulfoxides, hydroxylated metabolites or their corresponding keto tautomers, M+O-2H molecule corresponding to an epoxide, etc.). For detection of unknown and unexpected metabolites the software MZmine 2.10 (Pluskal et al. 2010) was used. Processing of full scan chromatograms was carried out using the settings described in (Hug et al. 2014). The obtained peak lists were searched for peaks newly formed solely by the incubation of each heterocyclic PAH that were absent in all other samples. For these peaks, molecular formulas were determined based on accurate masses and isotope patterns using the Xcalibur software (ThermoScientific) and MS^2 spectra were visually evaluated. Tentatively identified metabolites were confirmed by reference standards if these were commercially available.

4.4 Results & Discussion

4.4.1 LYES

None of the tested compounds was active in the LYES, i.e. reached 20 % induction of the E2 standard (Fig. 4.2).

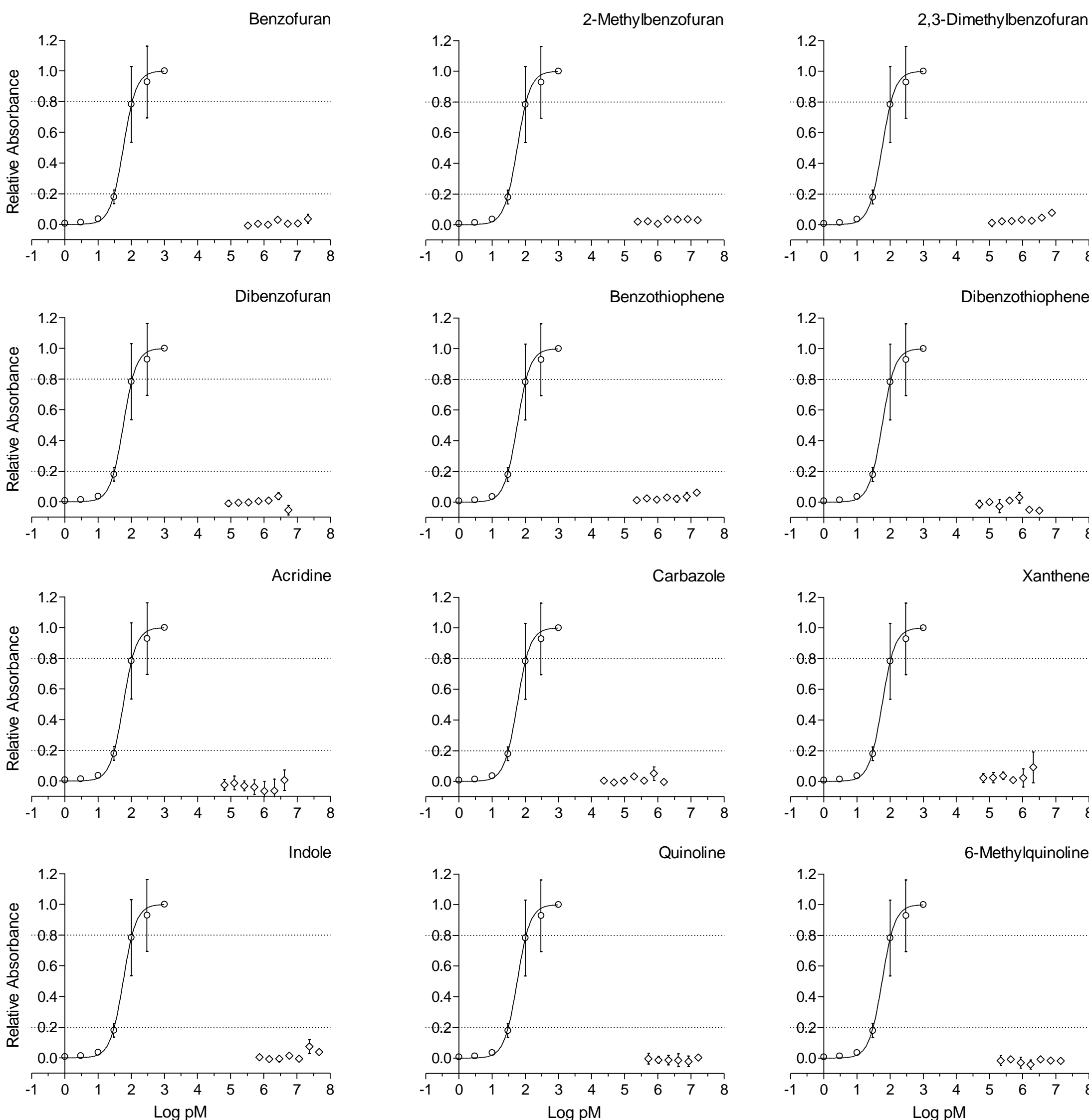

Figure 4.2 Dose-response curves and corresponding 95 % confidence bands in the LYES for all investigated heterocyclic compounds (closed circles) and the E2 standard curves (open circles) were measured in *n*=3 independent assay repetitions. Concentration values on the x-axis refer to nominal medium concentrations of the substances. Dots represent mean values from technical replicates of one respective experiment. All substances were inactive in the LYES assay, i.e. did not reach 20 % induction of the E2 standard.

The EC_{50} of the E2 standard was 57.7 ± 0.7 pM (mean ± standard error, *n*=3) throughout the whole study. Other publications including studies from our own lab report lower values in the range of 30 – 50 pM (Maletz et al. 2013, Schultis & Metzger 2004). These studies commonly used ethanol as solvent for dosing of the test items. In the present study, however, it was necessary to use another solvent, i.e. DMSO, since the substances did not readily dissolve in ethanol. Furthermore, data calculation was different. In the present study data were normalized to values between zero and one and the scaled data was used to create the dose-response curve. Bottom and top of the curve were therefore set to zero and one, respectively, which caused a shift of the EC_{50} in comparison to the data analysis in the other publications mentioned above.

4.4.2 ER CALUX®

The EC_{50} of the E2 standard was 8.1 ± 0.9 pM (mean ± standard error, *n*=12) throughout the whole study, which is within the range reported in other studies (Legler et al. 2002b, Legler et al. 1999, Maletz et al. 2013).

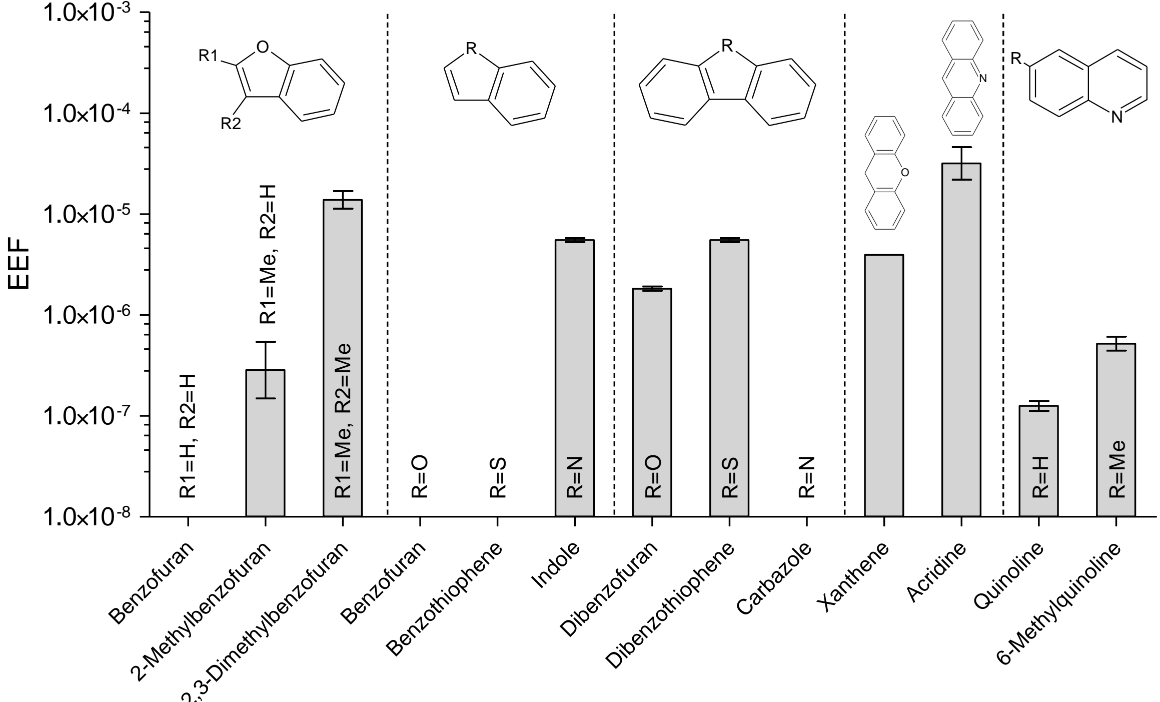

Figure 4.3 Estradiol equivalence factor (EEF) ranges of the investigated heterocyclic compounds relative to E2 as measured in the ER CALUX®. Bars refer to EEF_{50}s (i.e. the EEFs based on 50 % E2-max.), error bars represent the $EEF_{20\text{-}80}$ ranges. Benzofuran, benzothiophene and carbazole were inactive in the ER CALUX®, i.e. did not reach 20 % induction of the E2 standard.

Benzofuran, benzothiophene and carbazole did not reach 20 % induction of the E2 standard of the ER CALUX®. The substances 2-methylbenzofuran, quinoline and 6-methylquinoline, did not reach 80 % induction of the E2 standard, making slight extrapolation beyond the measured range of response necessary to establish the $EEF_{20\text{-}80}$ ranges as described by Villeneuve at al. (2000). Because acridine and 2,3-dimethylbenzofuran exhibited higher maximum induction compared to the standard, data was normalized to the maximum induction of these substances (Fig. 4.3 and 4.4).

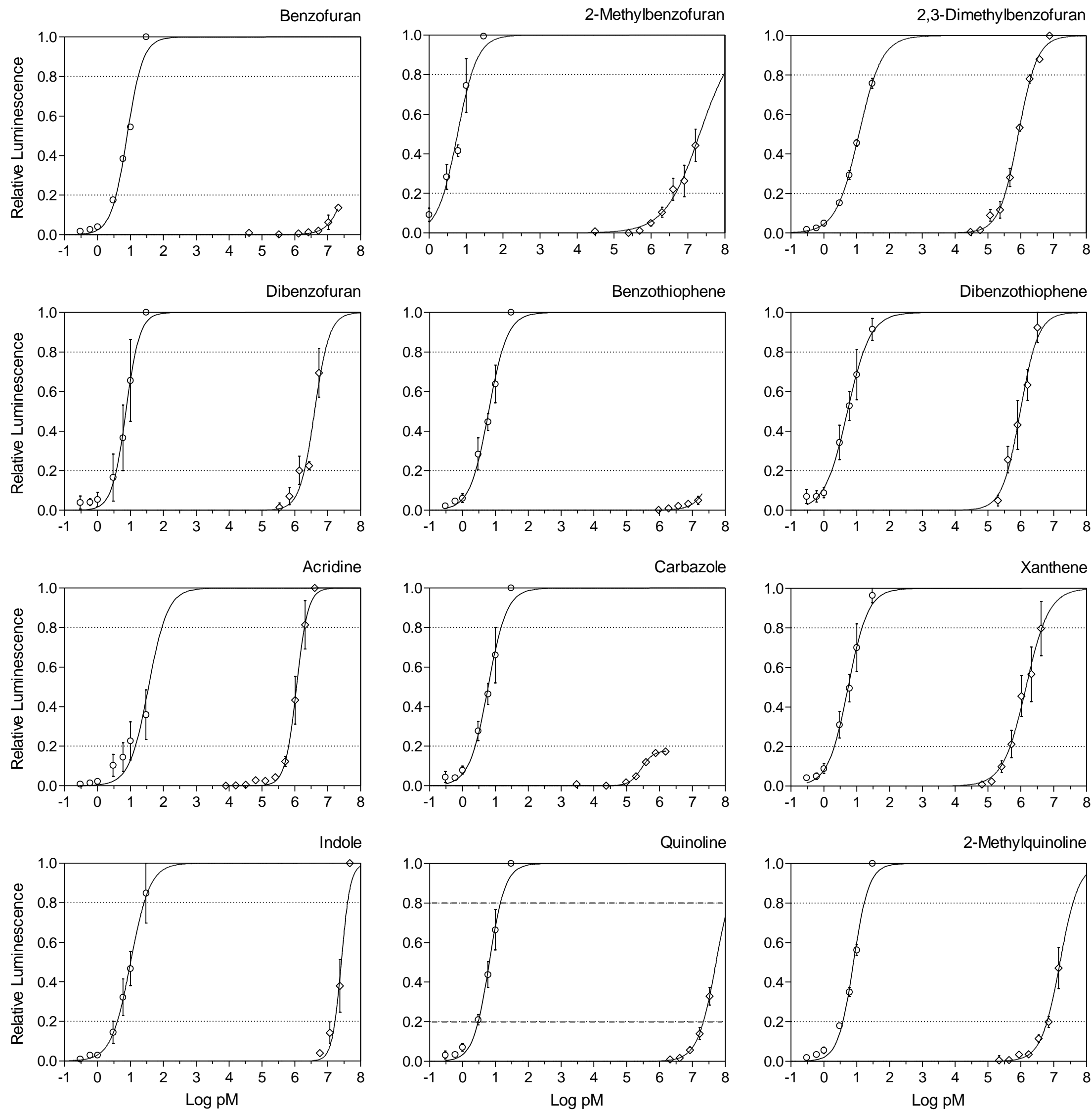

Figure 4.4 Dose-response curves and corresponding 95 % confidence bands in the ER CALUX® for all investigated heterocyclic compounds (closed circles) and the E2 standard curves (open circles) were measured in *n*=3 independent assay repetitions. Concentration values on the x-axis refer to nominal medium concentrations of the substances. Dots represent mean values from triplicate measurements in one respective experiment.

In contrast to the LYES that utilizes yeast cells, Piao et al. (1997), Kuil et al. (Kuil et al. 1998) and Spink et al. (1998) have shown that the T47Dluc cells express a number of Cytochrome P450 (CYP) isoforms as well as 17β-HSD and thus are readily capable of biotransformation and conversion of steroids. It has been demonstrated by van Lipzig et al. (2005) that the homocyclic PAHs benzo[*a*]pyrene (BaP) and chrysene were transformed into several hydroxylated metabolites during incubation with *β*-naphtoflavone-induced rat-liver S9. Furthermore, BaP itself caused a significant induction of luciferase in the ER CALUX® that was completely inhibited by the CYP inhibitor 3'-,4'-dimethoxyflavone. Similar results have been shown previously in a transfected human breast cancer cell-line, MCF-7 (Charles et al. 2000, Fertuck et al. 2001). By use of a gastrointestinal simulator, Van de Wiele et al. (2005) have demonstrated that ingested PAHs can be transformed into estrogenic metabolites by colon microbiota and may thus pose a higher risk to human health after absorption via food or drinking water than would be expected from the parent substances. We hypothesize that heterocyclic PAHs are, comparable with their homocyclic analogues, a class of indirect estrogenic compounds that are transformed into the active form (hydroxylated and other) by (mammalian) biotransformation enzymes. The observed EEFs were relatively high and within the range of other non-steroidal EDCs of high concern commonly found in surface water, e.g. alkylphenols (Preuss et al. 2006), bisphenol A, phthalates or pesticides (Houtman et al. 2006, Legler et al. 2002a).

4.4.3 Identification of metabolites

No estrogen receptor (ER) binding affinity was predicted from the structural properties of the parent substances by using the OECD (Q)SAR Toolbox (Tab. 4.1).

Table 4.1 Comparison of measured data with predicted ER binding for parent compounds and simulated metabolites (OECD QSAR toolbox liver metabolism simulator).

Substance	Maximum concentration[1]	Measured effects in the ER CALUX®		Predicted ER binding of parent substance[2]	Metabolism (LC-DAD)	Predicted ER binding of simulated metabolites[2]
	mg L^{-1}	EEF_{50} (EEF_{20-80})			%	
Benzofuran	120.9	-	-	-	n.d.	-
2-Methylbenzofuran	210.0	2.85 (1.49-5.45) 10^{-7}	+	-	n.d.	+
2,3-Dimethylbenzofuran	110.0	1.38 (1.12-1.69) 10^{-5}	++	-	n.d.	+
Dibenzofuran	56.9	1.82 (1.74-1.92) 10^{-6}	+	-	3.3	++
Benzothiophene	136.1	-	-	-	46.1	+
Dibenzothiophene	40.8	5.53 (5.27-5.80) 10^{-6}	+	-	86.7	++
Acridine	40.9	3.18 (2.20-4.61) 10^{-5}	++	-	17.0	++
Xanthene	75.5	3.94 (3.94-3.94) 10^{-6}	+	-	71.9	++
Carbazole	25.6	-	-	-	24.7	++
Indole	162.7	5.53 (5.27-5.80) 10^{-6}	+	-	90.0	-
Quinoline	226.0	1.25 (1.12-1.40) 10^{-7}	+	-	18.5	-
6-Methylquinoline	528.0	5.21 (4.45-6.09) 10^{-7}	+	-	49.3	+

EEF_{50} and EEF_{20-80} ranges are given for use in mass-balance calculations. n.d. – not determinable due to complete loss during incubation without cells; [1]according to the concentration range earlier used by Hinger et al. (2011); [2]predicted ER binding (OECD QSAR toolbox)

This is, most likely, due to the absence of hydroxy-substituted aromatic rings, which is a prerequisite for binding to the ER (Blair et al. 2000). For *Saccharomyces cerevisiae,* which is used for the LYES assay, it is generally assumed that it lacks capability of biotransformation (Connolly et al. 2011), and thus, no hydroxylation of the heterocyclic PAHs by the yeast would be expected. Thus, results from the present study confirmed the expectation that no direct estrogenic effects are caused by heterocyclic PAHs and that the used yeast strain is not capable of biotransformation.

To test the hypothesis that heterocyclic PAHs are transformed into more reactive metabolites, we first investigated the losses of parent substances from the incubation medium by means of LC-DAD. It was shown that T47Dluc cells were able to metabolize most of the substances to a significant extent (Tab. 4.1). In a next step, metabolites were detected and (tentatively) identified using LC-$HRMS^2$. We were able to detect one to four individual metabolites for each of the investigated parent substances (Tab. 4.2). For six of these compounds the identity could be confirmed by authentic reference standards (acridone, 2-hydroxyquinoline, quinoline N-oxide, oxindole, xanthone, and 2-hydroxyphenylacetic acid formed from both, benzofuran and benzothiophen). In four cases a tentative structure assignment was possible based on MS^2 fragmentation behavior and analogy to reference standards of other compounds (Tab. 4.2). For the other detected metabolites an assignment based on MS^2 spectra, retention times and ionization behavior was considered too speculative. Among the identified metabolites, only for 2-hydroxyphenylacetic acid an estrogenic activity was predicted by the OECD QSAR toolbox. However, MS^2 spectra showing neutral losses of H_2O in many cases suggest that a range of hydroxylated and thus phenolic compounds are formed, of which at least some isomers show an estrogenic activity as predicted by the QSAR.

Table 4.2 Metabolites of heterocyclic PAHs detected and (tentatively) identified in the incubation medium by high resolution tandem mass spectrometry (LC-HRMS^2).

Parent compound (RT in min)	Metabolite (bold: identified)	RT (min)	Molec. Formula	Confirmed by ref. std.	Predicted ER binding[1]	Ref.
Acridine (RT 15.8)	**Acridone**	21.7	$C_{13}H_9NO$	yes	-	
Acridine	Acridine +O	16.9	$C_{13}H_9NO$	no	+[2]	
Acridine	Acridine +2O	20.3	$C_{13}H_9NO_2$	no	++[2]	
Acridine	Acridine +2OH	9.4	$C_{13}H_{11}NO_2$	no	++[2]	
Quinoline (RT 5.9)	**2-Hydroxyquinoline**	18.2	C_9H_7NO	yes	-	(Thiebaud et al. 2013)
Quinoline	Quinoline +O	18.9	C_9H_7NO	no		
Quinoline	**Quinoline N-oxide**	14.8	C_9H_7NO	yes	-	(LaVoie et al. 1983)
Quinoline	Quinoline +OH_2	16.9	C_9H_9NO	no	-	
6-Methylquinoline (RT 11.9)	6-Methylquinoline +O	16.0	$C_{10}H_9NO$	no	+[2]	
6-Methylquinoline	6-Methylquinoline N-oxide	18.2	$C_{10}H_9NO$	no	-	(Scharping et al. 1993)
6-Methylquinoline	6-Methylquinoline +OH_2	18.0	$C_{10}H_{11}NO$	no	-	(Scharping et al. 1993)
Indole (RT 20.4)	**2-Oxindole**	17.4	C_8H_7NO	yes	-	(Skordos et al. 1998)
Indole	Indole +OH2	14.0	C_8H_9NO	no	+[2]	
Indole	Indole +OH2	19.1	C_8H_9NO	no	+[2]	
Indole	Indole -2H+O	16.6	C_8H_5NO	no	-	
Carbazole (RT 24.5)	Carbazole-sulfate	21.9	$C_{12}H_9SO_4$	no	-	

Xanthene (n.d.)	**Xanthone**	25.3	$C_{13}H_8O_2$	yes	-	
Benzofuran (n.d)	**2-Hydroxyphenylacetic acid**	16.4	$C_8H_8O_3$	yes	+	(Connelly et al. 2002)
Benzofuran	Benzofuran +2OH	17.7	$C_8H_8O_3$	no		
Benzofuran	Benzofuran $+CH_4O_2$	17.8	$C_9H_{10}O_3$	no	(+)	
Benzofuran	Benzofuran $+CH_4O_2$	19.8	$C_9H_{10}O_3$	no	(+)	
2-Methylbenzofuran (n.d.)	2-methylbenzofuran +2OH	17.7	$C_9H_{10}O_3$	no	+[2]	
2-Methylbenzofuran	2-methylbenzofuran + 2OH	18.5	$C_9H_{10}O_3$	no	+[2]	
2-Methylbenzofuran	2-methylbenzofuran -2H+O	19.1	$C_9H_6O_2$	no	-	
2,3-Dimethyl benzofuran (n.d.)	2,3-dimethylbenzofuran +O	16.3	$C_{10}H_{10}O_2$	no	+[2]	
2,3-Dimethyl benzofuran	2,3-dimethylbenzofuran -2H+O	20.2	$C_{10}H_8O_2$	no	-	
2,3-Dimethyl benzofuran	2,3-dimethylbenzofuran +2OH	16.4	$C_{10}H_{12}O_3$	no	++[2]	
Dibenzofuran (n.d.)	Dibenzofuran sulfate	23.1	$C_{12}H_8SO_5$	no	-	
Benzothiophen (n.d.)	Benzothiophen +O	15.5	C_8H_6OS	no	-	
Benzothiophen	Benzothiophen $-S+CH_4O_3$	18.1	$C_9H_{10}O_3$	no		
Benzothiophen	Benzothiophen $-S+CH_4O_3$	18.5	$C_9H_{10}O_3$	no		
Benzothiophen	**2-Hydroxyphenyl acetic acid**	16.4	$C_8H_8O_3$	yes	+	
Dibenzothiophen (n.d.)	Dibenzothiophen sulfoxide	20.9	$C_{12}H_8OS$	no	-	(Vignier et al. 1985)

Identified metabolites are given by name, those not identified by their modification as compared to the parent molecule. The retention times (RT) of the parent compounds are given for reference. n.d. – not detected due to poor ionization. [1]Predicted ER binding (OECD QSAR toolbox); [2]Different isomers possible of which some show estrogenic activity as predicted by QSAR.

4.4.4 Possible contribution to estrogenic activity in water resources

The main sources for estrogenic substances in the aquatic environment are municipal sewage and agriculture. The overall activity, however, is a result of complex substance mixtures from many different sources (Hecker & Hollert 2011, Moltmann et al. 2007). These include high amounts of pharmaceuticals in hospital sewage (Verlicchi et al. 2010) and diverse point sources such as the here investigated tar-oil contaminated sites. Most attempts to explain the overall estrogenic activity of water samples by mass-balance studies were not conclusive and detection of target analytes often underestimated the response in the bioassays (Di Dea Bergamasco et al. 2011, Leusch 2008). A number of studies confirmed that hydroxylated PAHs contributed to the estrogenicity of different complex samples (e.g., Kamiya et al. 2005, Machala et al. 2001, Muthumbi et al. 2003, Wenger et al. 2009). Previous research on contamination of ground water with estrogenic compounds identified polycyclic aromatic hydrocarbons, including hydroxylated and heterocyclic analogues, in those fractions with high estrogenic activity when applying effect-directed analyses using the E-Screen assay (Kuch et al. 2010). Further studies (Blum et al. 2011, Schlanges et al. 2008) found several heterocyclic PAHs, including quinoline, carbazole, acridine, benzofuran and benzothiophene, in concentrations up to 4 $mg \cdot L^{-1}$ in ground water samples from tar-oil contaminated sites in Germany. Acridine, which showed a higher induction in the ER CALUX® than 17β-estradiol, was found in concentrations up to 3 $\mu g \cdot L^{-1}$. Recently, acridine, quinolone and carbazole were also detected in several surface water samples from German rivers in concentrations up to 20 $ng \cdot L^{-1}$ (Siemers et al. 2015). Regarding its high potential to activate the ER in mammalian cells, acridine should possibly be included in the screening battery of chemical analyses of estrogenically active water samples in the catchment area of former tar-oil sites. Additionally, acridine and some of its transformation products are among the major photodegradation as well as biotransformation products of the antiepileptic pharmaceutical carbamazepine (Calisto et al. 2011, Kaiser et al. 2014, Kosjek et al. 2009) which is regularly found in surface, as well as drinking water in concentrations up to 1 $\mu g \cdot L^{-1}$ (surface water) or 0.258 $\mu g \cdot L^{-1}$ (drinking water), respectively (Benotti et al. 2008, Heberer 2002, Stackelberg et al. 2004). In summary, our study demonstrates that some heterocyclic PAHs have comparable estrogenicity as some non-steroidal EDCs of concern and are consequently of highest relevance in respect to the estrogenicity of water resources as well as human health.

Overall, this substance class has to be taken into account regarding the estimation of overall estrogenic activity in surface and ground water. Since ground water is an important drinking water resource the investigated hetero-PAHs could also imply an increased risk for human health (Brack et al. 2012, Grummt et al. 2013).

4.5 Conclusion

In the present investigation, it was demonstrated that certain hetero-PAHs show estrogenic activity after transformation in mammalian cells. For some hetero-PAHs, the detected maximum induction of luciferase was even higher than that of 17β-estradiol in the concentrations tested. The observed EEFs were within the range of other non-steroidal EDCs of high concern commonly detected in surface water (e.g. alkylphenols, bisphenol A, phthalates or pesticides). Regarding its high potential to activate the ER in mammalian cells, at least acridine should be added to the chemical screening battery of estrogenically active water samples. Overall, this substance class has to be taken into account regarding the estimation of estrogenic activity in surface and ground water. Since ground water is an important drinking water resource the observed hetero-PAHs could imply an increased risk for human health.

Furthermore, we could show that test systems without the ability for biotransformation, such as the LYES, are susceptible to underestimate the estrogenic potential of complex water samples. Consequently, when using such test systems a metabolic activation step should be included in the bioanalytical identification of potential EDCs.

Acknowledgement

The authors acknowledge financial support by a Seed funds project of RWTH Aachen University supported by the German Excellence Initiative and the project framework Tox-Box supported by the German Federal Ministry of Education and Research (BMBF) (funding number 02WRS1282I). Tox-Box is a constitutive part of the BMBF action plan “Sustainable water management (NaWaM)”. It is part of the funding scheme “Risk Management of Emerging Compounds and Pathogens in the Water Cycle (RiSKWa)”. M.B. received a personal stipend by the German National Academic Foundation (Studienstiftung des deutschen Volkes). The study sponsors had no influence on study design, collection, analysis or interpretation of data, or in writing of or the decision to submit the manuscript for publication. We want to express our gratitude to Mrs. Simone Hotz, for technical assistance during the experiments. The authors would like to thank Prof. J.P. Sumpter of Brunel University, United Kingdom, for supplying the yeast cells for the LYES, Martin Wagner (University of Frankfurt) for introduction into the modified LYES protocol, and Claus Bornemann (Eurofins) for supplying some metabolite reference standards.

Chapter 5

Investigation of potential endocrine disrupting effects of mosquito larvicidal *Bacillus thuringiensis israelensis* (*Bti*) formulations – *in vitro* test systems

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This chapter has been published in *Science of the Total Environment*

5.1 Abstract

Bacillus thuringiensis var. *israelensis* (*Bti*) is successfully used as a biological control agent for mosquito control. It has proven to be ecological friendly, and thus, is used in ecologically sensitive habitats. Recent investigations of the toxicological potentials of groundwater in Germany have detected estrogenic activity in five consecutive groundwater wells in a region in the Upper Rhine area (Germany) where *Bti* is applied. Together with initial confirmation of estrogenic properties of *Bti* in a yeast based estrogen screen, it was suspected that this compound can act as an environmental xenoestrogen.

In the present study, five *Bti* formulations as well as the active ingredient of *Bti*, VectoBac® TP (TP), were investigated regarding their estrogenic activity using the LYES and ER CALUX® assays. Furthermore, their potential steroidogenesis disruption properties were studied using the H295R Steroidogenesis Assay. Additionally, field samples from a Bti application area as well as samples from an artificial pond were examined.

Three of the *Bti* formulations and the active ingredient TP showed significant estrogenic activity in the LYES (up to 52 $ng \cdot L^{-1}$ estradiol equivalents (EEQ) in the 18-fold concentration) and / or the ER CALUX® (up to 1 $ng \cdot L^{-1}$ EEQ in the 18-fold concentration). In the H295R significant but weak effects with no dose-response-relationship on the production of estradiol, and 21-hydroxyprogesterone (WDG) as well as testosterone (TP) by H295R cells could be observed. The field samples as well as the samples from the artificial pond showed no significant increase of estrogenic activity after application of TP or WDG in the ER CALUX®. With the exception of the controlled laboratory experiments with direct application of *Bti* to the utilized *in vitro* test systems the present study did not reveal any significant effects of *Bti* on endocrine functions that would indicate that the application of *Bti* could cause adverse endocrine effects to organisms in aquatic ecosystems. Instead, our results support previous studies that the use of *Bti* products against mosquitos would be safe even for sensitive habitats such as conservation areas.

Keywords: *Bacillus thuringiensis israelensis*, H295R, ER CALUX®, YES, endocrine disruption

5.2 Introduction

As a biological alternative to synthetic insecticides, such as the organophosphates Fenitrothion (O,O-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate) and Temefos (*O*,*O*,*O′*,*O′*-Tetramethyl *O*,*O*′-sulfanediylbis(1,4-phenylene) diphosphorothioate), *Bacillus thuringiensis israelensis* (*Bti*) toxins are increasingly used for control of mosquitoes since the 1980s (Becker 1997, Boisvert et al. 2007). The larvicidal activity is due to the δ-endotoxins, which are synthesized during sporulation. Based on a large number of toxicity studies with non-target organisms the environmental safety of *Bti* has been suggested (Boisvert & Boisvert 2000). It was therefore recommended for use in aquatic environments and drinking water reservoirs by the World Health Organization (WHO 1999). Nevertheless, screening for endocrine activity was not included in the previous studies (El-Bendary 2006, Russell & Kay 2008, Tilquin et al. 2008).

During a study that investigated the toxic properties of groundwater in the Upper Rhine area (Germany) estrogenic activities were detected for five consecutive groundwater wells in areas with intensive *Bti* application (data not published). Because no suspected or confirmed estrogen receptor agonists were detected in these samples, it was hypothesized that *Bti* could have contributed to these activities. Subsequently, a spiked water sample, containing a commercial available *Bti* formulation, was tested in the Yeast Estrogen Screen (YES) (Routledge & Sumpter 1996). The measured estrogenic activity of the spiked sample motivated the present study. Here, we investigated the estrogenic activity of the active substance of *Bti*, five commercial available formulations and the ingredients of one of the formulations.

Attempts to explain the overall estrogenic activity of water samples by mass-balance studies are not conclusive in many cases and detection of known or suspected estrogenic active substances underestimated the response in the bioassays. Therefore, it is important to explore the potential contribution of other, so far unknown, compounds to the measured biological effects (Schulte-Oehlmann et al. 2006). In addition to natural and synthetic hormones, EDs include a range of other classes of chemicals such as phthalates, organotin compounds and hydroxylated polychlorinated biphenyls (PCBs) as well as several pharmaceuticals and personal care products. Due to their different physicochemical properties initial identification of hormonally active substances is not straightforward (Heberer 2002, Van der Linden et al. 2008). The concentration of most potent EDs in the

environment is near or below the detection limit of current chemical analysis, i.e. the lower ng·L-1 range. This becomes an issue as some of these compounds – particularly hormones – exert their effects in this range. Additionally, chemical interactions of EDs have to be taken into account to estimate the potential impacts of EDs in aquatic ecosystems.

To examine estrogenic activity of *Bti* two receptor mediated assays, the Lyticase Yeast Estrogen Screen (LYES) (Routledge & Sumpter 1996, Wagner & Oehlmann 2009) and the Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER CALUX®) assay (Legler et al. 1999), were applied. Furthermore, the H295R Steroidogenesis assay (H295R) (Hecker et al. 2011) that enables detection of effects on the steroid synthesis pathway was used to identify potential disruptors of sex steroid production. Previous studies reported the applicability of a combination of receptor-mediated and non-receptor-mediated assays for a holistic evaluation of potential endocrine activity in complex (waste)water and sediment samples (Grund et al. 2011, Hecker & Hollert 2009, Kase et al. 2009, Leusch 2008, Leusch et al. 2010, Maletz et al. 2013). As previously reviewed by Hecker & Hollert (2011) the combined use of receptor-mediated and non-receptor-mediated-methods is necessary to enable objective assessment of endocrine disrupting potentials of complex samples.

The aim of the present study was to investigate the potential estrogenic activity as well as possible impacts on steroidogenesis of *Bti* as well as its formulations usually applied in aquatic ecosystems and private water bodies. Furthermore, it should be examined if the detected activity was caused by the active ingredient or the adjuvants or the fermentation slurry, respectively.

5.3 Material & Methods

5.3.1 Samples

5.3.1.1 *Bti* formulations for the public market

In an initial experiment, four different commercially available *Bti* formulations for the public market were tested regarding their estrogenic activity in the LYES: Three solid products (tablets) and one liquid suspension. The tablets as well as the liquid formulation were obtained from local stores, online stores as well as a registered association to cover the whole range of purchase possibilities of the public market. The tablets were homogenized using a porcelain mortar and weighed. A 30-fold concentration in mg·L^{-1} was calculated depending on the dosage recommendations of the manufacturers (Tab. 5.1). This stock concentration resulted in a maximum concentration of 18-fold within the LYES.

Table 5.1 Concentration of the investigated formulas

	Solid products	A	B	C	Liquid product	D
One fold concentration	[mg·L^{-1}]	7.7	6.8	23.27	[µl·L^{-1}]	1.5
30-fold concentration	[mg·L^{-1}]	231	204	698.1	[µl·L^{-1}]	45

The homogenized tablets as well as the liquid formulation were diluted in 250 ml tap water and samples were stirred overnight. Samples were stored at 4°C in amber glass bottles with polytetrafluoroethylene (PTFE) lids.

5.3.1.2 Ingredients of product B

As one manufacturer kindly provided the composition of the tablet, screening of the individual substances contained in this product in the LYES was possible. The tablets contain the active substance as well as six adjuvants. According to the highest tested concentration of the investigated formulas, the main adjuvants were diluted in tap water and tested in an 18-fold concentration. As the composition of the screened product is proprietary single substances are not disclosed in this study. For further investigations the percentage of the active substance was set to 50 % of product B (personal communication with the manufacturer).

5.3.1.3 VectoBac® TP and WDG

The active substance VectoBac® technical powder" (TP) and the formulation VectoBac® water dispersible granule" (WDG) were produced by Valent Biosciences and have been provided by the German Mosquito Control Association (GMCA; Speyer, Germany). The manufacturing process is protected by corporate secret but as stated by Valent Biosciences the bacteria are fermented in tanks containing slurries based on either fish or soy meal. In case of TP fermentation the slurry is dried and remaining spores are inactivated through radiation sterilization. WDG is processed in the same manner but then further treated (e.g. blended with adjuvants) to enhance water solubility and granulation. 51 mg of the powder / granule were diluted in 250 ml tap water and stirred overnight. Samples were stored at 4°C in brown glass bottles with PTFE lids. To examine the development of the strogenic activity over time, one solution of VectoBac® TP was stored over a period of two month and investigated at the beginning (June) as well as the end (August) of this period.

5.3.1.4 Field samples

Water samples (Tab. 5.2) were provided by the Heinrich-Sontheimer-Laboratory (HSL) of the Water Technology Centre (TZW), Karlsruhe, Germany. Each sample consisted of two liters and was stored in two one liter polyethylene (PE) bottles.

Table 5.2 Field samples

Sample	Description	Product	Time [after application]
WS1	Surface water	none	0
WS2	Surface water	VectoBac® TP	30 min
WS3	Surface water	none	0
WS4	Surface water	VectoBac® WDG	30 min
WS5	Ground water	none	0
WS6	Artificial pond	VectoBac® TP	2 h 40 min
WS7	Ground water	VectoBac® TP	3 h 40 min
WS8	Ground water	VectoBac® TP	6 h
WS9	Ground water	VectoBac® TP	9 h 35 min
WS10	Ground water	VectoBac® TP	13 h 25 min

VectoBac® TP = Technical Powder, WDG = Water Dispersible Granule

Two surface water samples (WS1 and WS2) were taken directly before and approximately 30 min after application of a TP-sand-oil-mixture at a water depth of approximately 10 cm. Another two samples (WS3 and WS4) were taken after application of WDG ice granules (out of WDG and tap water) in the same manner. The samples were stored at 4°C and send to the RWTH Aachen University, Aachen, Germany, in 1 L PE bottles.

A third set of samples was taken after application of VectoBac® TP (WS5 to WS10) to an artificial pond (W: 2.0 m, H: 2.6 m, T: 0.4 m) that was build 12 m from a ground water well contrary to the ground water flow direction to investigate the possible distribution of VectoBac® TP within the water body (Fig. 5.1). The pond was filled with ground water and 2 Kg of a VectoBac® TP-Sand-Rapeseed Oil mixture as well as a tracer were added to the surface. Samples were taken as stated in table 5.2.

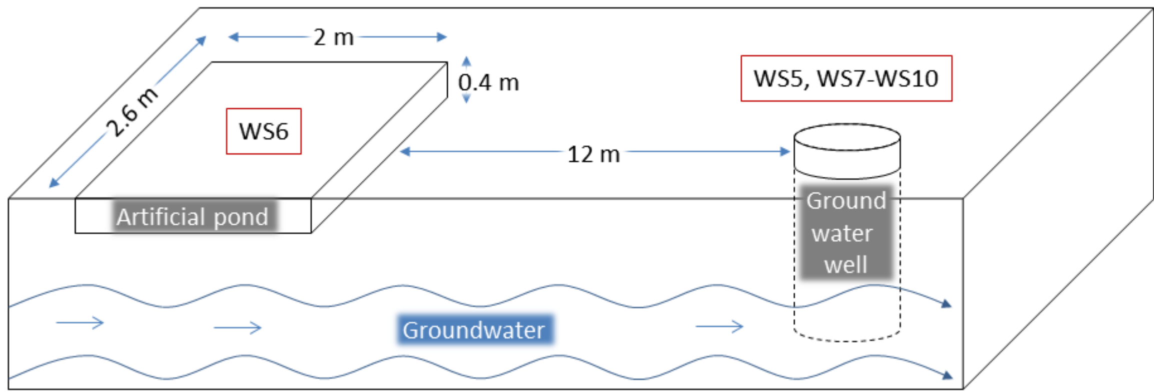


Figure 5.1 Scheme of the artificial application setup. The artificial pond was build 12 m from a groundwater well contrary to the groundwater flow direction. WS = water sample.

5.3.2 Sample Preparation

5.3.2.1 Solid phase extraction (SPE)

In the present investigation, WS1 and WS2 were extracted by use of SPE. Prior to extraction the water samples were filtered through 1.6 µm glass fiber filters (GE Healthcare Life Sciences, Solingen, Germany). For principle and general implementation of the SPE see chapter 1.4.2.1 as well as chapter 2.1.1.

5.3.2.2 Pressurized liquid extraction (PLE)

For testing of the samples in the H295R two different extraction methods were applied. A first set of TP and WDG samples was tested after extraction with a PLE device at two

different temperatures. For principle and general implementation of the PLE see chapter 1.4.2.2 as well as chapter 2.1.4.

5.3.2.3 Lyophilisation

The majority of the water samples (WS3 to WS10) as well as VectoBac® TP and WDG were processed by freeze drying (lyophilisation). For measurement in the ER CALUX® all samples were initiallys centrifuged and freeze dried. To monitor possible losses of activity during the lyophilisation an additional sample with 30 ng 17ß-Estradiol (E2) in one liter tap water was processed (PC E2). Additionally, one liter tap water was processed as a process negative control (PC H_2O). For principle and general implementation of the lyophilisation see chapter 2.1.2.

5.3.3 LYES

For principle and general implementation of the lyticase assisted yeast estrogen screen (LYES) see chapter 1.4.3.2 and 1.5.1 as well as chapter 2.2.2. In the present investigation, cells were exposed to a dilution series (18x, 15x, 12x, 9x, 6x, 3x, 1x and 0.5x the recommended application concentration) of the *Bti* formulations as well as the active substances.

5.3.4 Viability Assay

An MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay (see chapter 2.2.1) was conducted to assure that the test items did not cause non-specific cytotoxic effects in mammalian cell lines.

In the present investigation, cells were seeded into 96-well (ER CALUX®) or 24-well (H295R) microtiter plates at a density of $1 \cdot 10^5$ cells·ml^{-1} (ER CALUX®) or $3 \cdot 10^5$ cells·ml^{-1} (H295R). After 48 h (ER CALUX®) or 24 h (H295R) the cells were exposed to a dilution series of the samples for 24 h (ER CALUX®) or 48 h (H295R), respectively.

5.3.5 ER CALUX®

For principle and general implementation of the ER CALUX® see chapter 1.4.3.2 and 1.5.2 as well as chapter 2.2.3. In the present investigation, the cells were exposed to a dilution

series (18x, 15x, 12x, 9x, 6x, 3x, 1x and 0.5x concentration the recommended concentration) of the processed samples.

5.3.6 H295R

For principle and general implementation of the H295R see chapter 1.4.3.2 as well as chapter 2.2.4. In the present investigation, the amount of 17ß-estradiol (E2) was determined with an enzyme-linked immunosorbent assay (ELISA). The amount of testosterone (T), corticosterone (COR), androstenedione (ASD), progesterone (PRO), 17-hydroxyprogesterone (17-OHP) and 21-hydroxyprogesterone (21-OHP) was determined by use of liquid chromatography tandem mass spectrometry (LC-MS^2). Due to the small amount of some samples at least one measurement with three internal replicates was conducted.

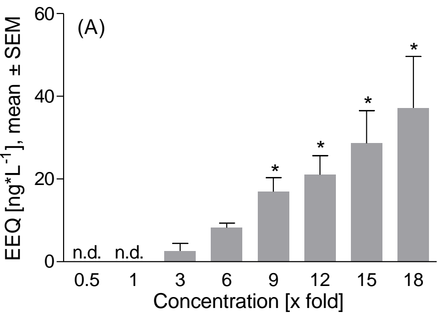
5.3.7 Statistical analysis

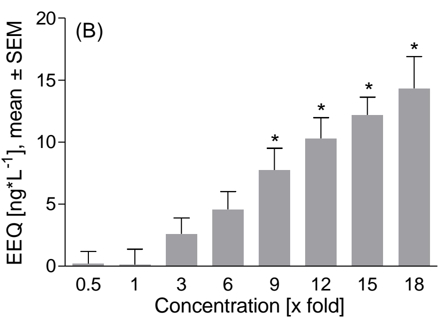
Data were statistically analysed with SigmaStat 3.5 (Systat Software, San José, USA) and presented as mean ± standard deviation (SD) or standard error of the mean (SEM) if possible. All data were tested for normality using the Shapiro-Wilk test as well as for homogeneity of variance ($p<0.05$). For data fulfilling the parametric assumptions and comparison of more than two samples a one way analysis of variance (ANOVA) followed by Dunnett's post hoc test (against control) or Bonferroni t-test (pairwise) was used to determine the treatments which differ significantly from the SC. When data did not fit the parametric assumptions, a non-parametric Kruskall-Wallis test followed by Dunn's post hoc (against control or pairwise) was used. For comparison of two samples an unpaired t-test (parametric data) or a Mann-Whitney Rank Sum test (nonparametric) was conducted. Differences were considered significant at $p<0.05$.

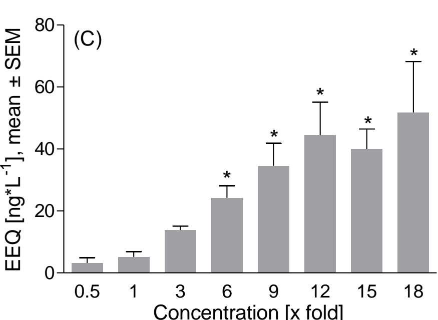
5.4 Results

5.4.1 *Bti* formulations for the public market

The three examined tablet formulations showed a dose-dependent estrogenic activity in the LYES (Fig. 5.2 A-C). No estrogenicity could be detected for the liquid formulation (Fig. 5.2 D).







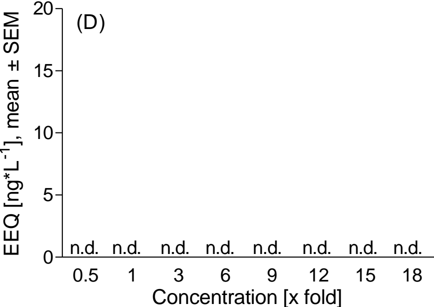


Figure 5.2 Estrogenic activity of four commercially available Bti formulations displayed as estradiol equivalents (EEQ) in the LYES. (A) – (C)=solid formulations; (D)=liquid formulation. [x fold] = x fold the recommended concentration. Bars represent mean values of three independent experiments with standard error (SEM). Data were statistically analyzed with one way analysis of variance (ANOVA) followed by Dunnett's test or non parametric ANOVA followed by Dunn's test (*=p<0.05).

The lowest activity was detected in sample B ranging between 0.2±1.0 and 14.3±2.6 ng·L^{-1} EEQs, whereas sample C showed greatest estrogenicity between 3.2±1.6 and 51.8±16.4 ng·L^{-1} EEQs. The lowest observed effect concentrations (LOECs) were determined at the six fold concentration (C) and the nine fold concentration (A and B), respectively.

5.4.2 Ingredients of product B

None of the examined ingredients of product B showed any estrogenic activity in the LYES. Therefore, they were excluded from additional investigations in the ER CALUX® or H295R, respectively.

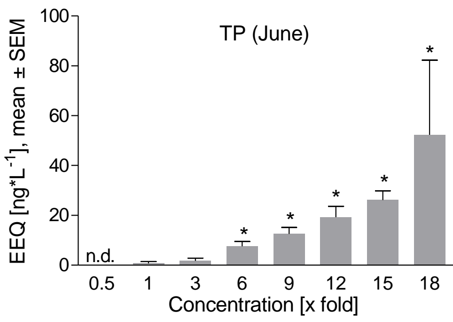
5.4.3 VectoBac® TP and WDG

5.4.3.1 LYES

VectoBac® TP showed a dose-dependent estrogenic activity in the LYES (Fig. 5.3). In contrast, the investigated batch of VectoBac® WDG showed no estrogenic activity.

Effect of storage time on estrogenic activity of TP

When exposing cells to samples immediately after their preparation (June) the LOEC was represented by the six-fold concentration (Fig. 5.3).



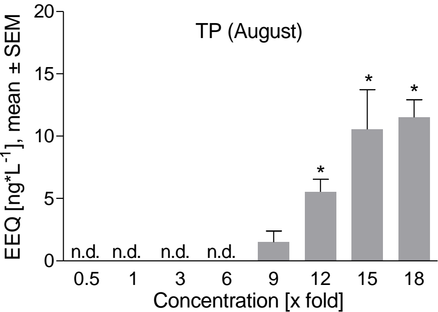


Figure 5.3 Estrogenic activity of VectoBac® TP displayed as estradiol equivalents (EEQ) directly after preparation (June) and after two months at 4°C in dark (August) in the LYES. [x fold] = x fold the recommended concentration. Bars represent mean values of two independent experiments with standard error (SEM). Data were statistically analyzed with one way analysis of variance (ANOVA) followed by Dunnett's test or non parametric ANOVA followed by Dunn's test (*=$p<0.05$). TP=Technical Powder.

Estrogenicity ranged between 0.7±0.8 ng·L^{-1} EEQ (one-fold concentration) and 52.3±30.0 ng·L^{-1} EEQ (18-fold concentration). After storage time of two months (August) at ±4°C under exclusion of light no estrogenic activity was detectable up to the nine-fold concentration, wherefore the LOEC was represented by the 12-fold concentration. Detectable estrogenic activity ranged between 1.5±0.9 ng·L^{-1} EEQ (nine-fold) and 11.5±1.4 ng·L^{-1} EEQ (18-fold). In the highest concentration, estrogenic activity therefore decreased to 22 % of the initial activity.

Effect of processing on samples for the ER-CALUX®

Processed samples were tested in the LYES to show a theoretical loss of estrogenic activity through the processing steps. Processing of the original VectoBac® TP sample led to a significant decrease of estrogenic activity as measured in the LYES (Fig. 5.4).

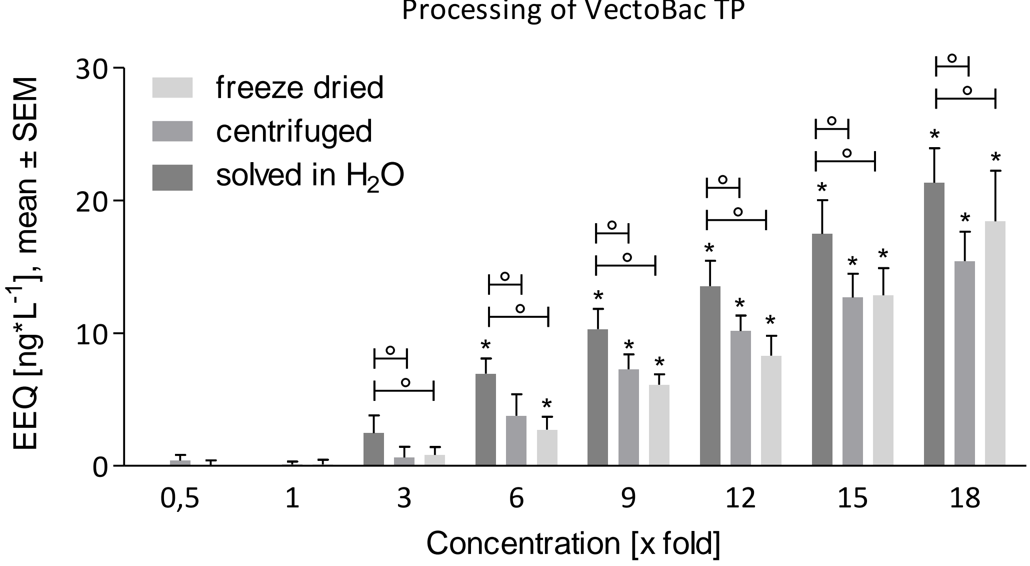
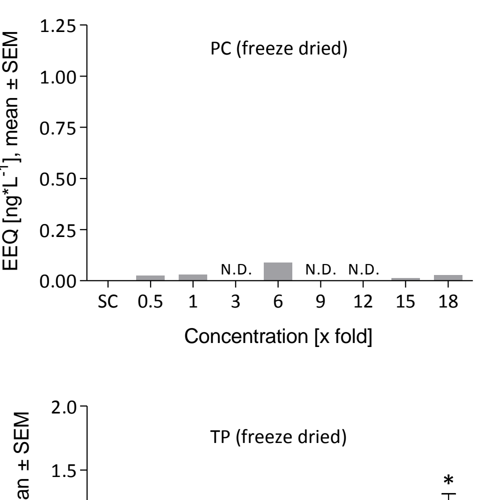


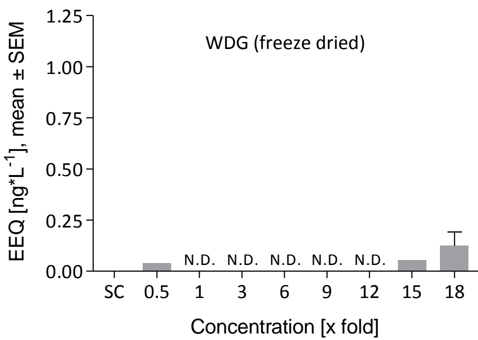
Figure 5.4 Estrogenic activity of VectoBac® TP displayed as estradiol equivalents (EEQ) after solving in water and the following treatment steps centrifugation and freeze drying in the LYES. [x fold] = x fold the recommended concentration. Bars represent mean values of three independent experiments with standard error (SEM). Data were statistically analyzed with one way analysis of variance (ANOVA) followed by Dunnett's test or non parametric ANOVA followed by Dunn's test (*=$p<0.05$). *=significant difference to the control; °=significant difference between each other. TP=Technical Powder.

The decrease was mainly caused by centrifugation as the first processing step. Estrogenicity was detectable from the three-fold concentration on whereas significant differences to the SC were detectable beginning with the six-fold concentration. Before centrifugation estrogenic activity was between 2.5±1.3 ng·L^{-1} EEQ (three-fold concentration) and 21.3±4.5 ng·L^{-1} EEQ (18-fold concentration) and dropped to 0.6±0.8 ng·L^{-1} and 15.4±2.2 ng·L^{-1} EEQ, respectively after centrifugation. The freeze drying step decreased estrogenicity further but not for all concentrations tested (see Fig. 5.4). After centrifugation losses of estrogenic activities between 74 % (three-fold concentration) and 25 % (12-fold concentration) were detected. After lyophilisation the losses compared to the activities before centrifugation were between 67 % (three-fold concentration) and 14 % (18-fold concentration).

5.4.3.2 ER CALUX®

VectoBac® TP showed a dose-dependent increase in estrogenic activity in the ER CALUX® assay while VectoBac® WDG showed no significant estrogenic activity at any of the concentrations tested (Fig. 5.5).





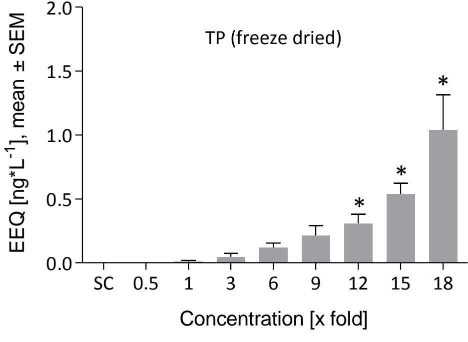


Figure 5.5 Estrogenic activity of VectoBac® TP displayed as estradiol equivalents (EEQ) in the ER CALUX®. [x fold] = x fold the recommended concentration. Bars represent mean values of three independent experiments with standard error (SEM). Data were statistically analyzed with one way analysis of variance (ANOVA) followed by Dunnett's test or non parametric ANOVA followed by Dunn's test (*=p<0.05). PC=Process Control; WDG=Water Dispersable Granules; TP=Technical Powder.

For the process control as well as VectoBac® WDG estrogenic activity below 0.2 $ng{\cdot}L^{-1}$ EEQ could be detected for some concentrations but with no dose-dependent correlation. VectoBac® TP caused estrogenicity between 0.01±0.01 $ng{\cdot}L^{-1}$ EEQ (one-fold concentration) and 1.04±0.28 $ng{\cdot}L^{-1}$ EEQ (18-fold concentration). The lowest concentration that was significantly greater compared to the SC was 12-fold.

5.4.3.3 H295R

After extraction with a PLE device significant differences in production of E2 and 21-OHP (WDG) as well as T (TP) compared to the SC were detected at concentrations greater or equal to nine-fold (Fig. 5.6).

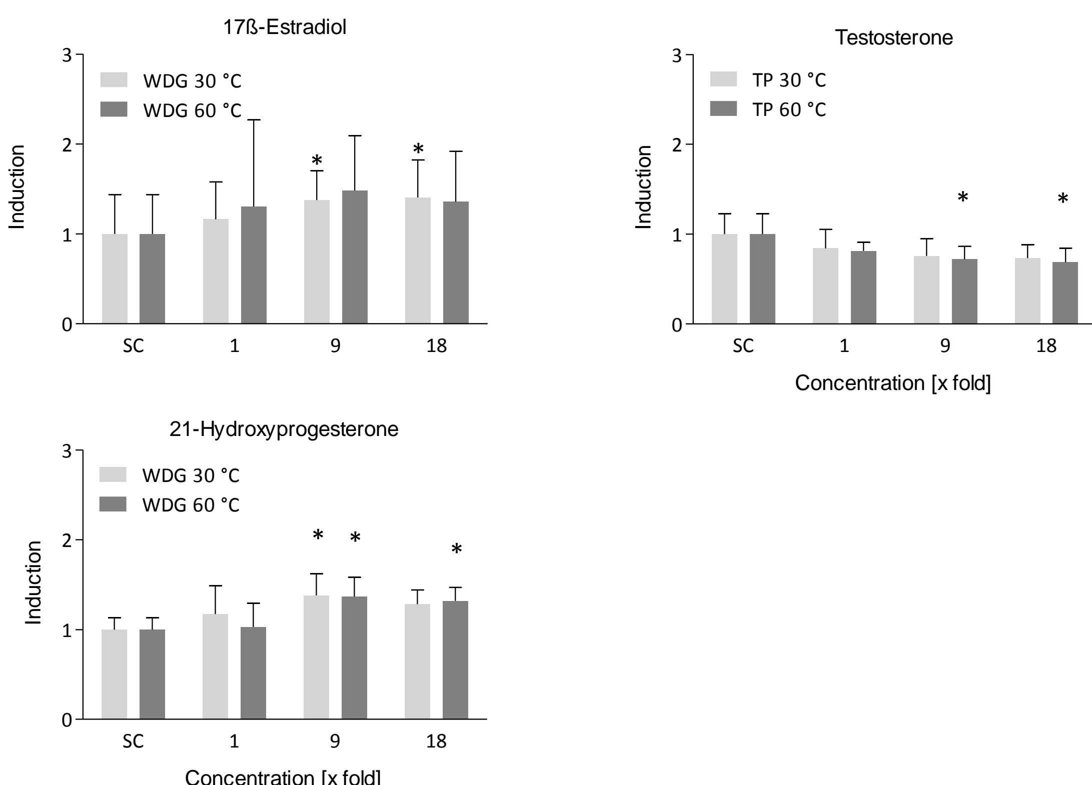


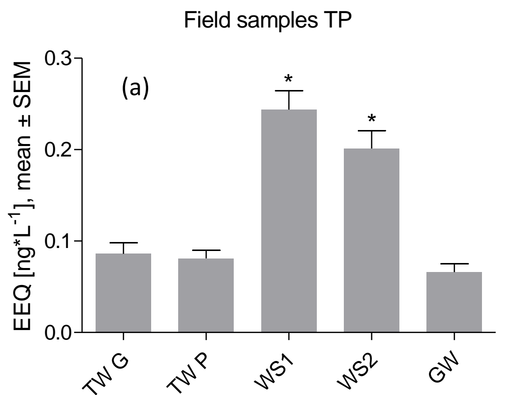
Figure 5.6 Alteration of hormone production in H295R cells after exposure to VectoBac® TP and WDG displayed as induction compared to the solvent control (SC). 30°C / 60°C = extraction temperature. Bars represent mean values of three independent experiments with coefficient of variation (CV). Data were statistically analyzed with one way analysis of variance (ANOVA) followed by Dunnett's test or non parametric ANOVA followed by Dunn's test (*=p<0.05). TP=Technical Powder; WDG=Water Dispersable Granules.

No differences could be detected for COR, ASD, PRO and 17-OHP. VectoBac® TP led to a slight but significant decrease of T production in the 9- and the 18-fold concentration after extraction at 60°C (0.7-fold induction). WDG led to a slight but significant increase of E2 production in the 9- and 18-fold concentrations after extraction at 30°C (induction 1.5- and 1.4-fold). 21-OHP production was also induced after exposure to WDG in the 9- and 18- fold concentrations after extraction at 60°C (induction 1.4- and 1.3-fold) and for the 9-fold concentration after extraction at 30°C (induction 1.4-fold). For samples that were subjected to freeze-drying and reconstitution in DCM no significant inductions could be

detected for the production of E2 and T when using ELISA. The other steroids were not tested due to the small sample size as well as a lack of an equivalent ELISA product.

5.4.4 Field samples

WS1 and WS2 showed weak but significant estrogenic activity in the ER CALUX® (Fig. 5.7 a). As there was no statistically significant difference between WS1 and WS2 but only between the surface water samples and the groundwater sample no effect of the application of TP could be detected. For WS3 and WS4 no significant differences between the samples and the tap water control could be detected (Fig. 5.7 b). Estrogenicity of the surface water samples (WS1 –WS4) was in the low ng·L^{-1} range (below 0.3 ng·L^{-1} EEQ) and did not reach 0.1 ng·L^{-1} EEQ in the groundwater sample (GW)



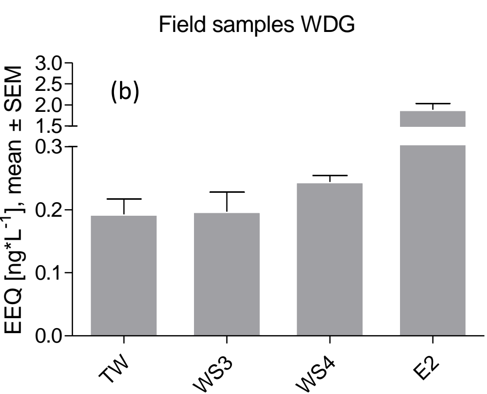


Figure 5.7 Estrogenic activities of field samples of VectoBac® TP (a) and WDG (b) applications displayed as estradiol equivalents (EEQ) in the ER CALUX®. Bars represent mean values of at least three independent experiments with standard error (SEM). Data were statistically analyzed with one way analysis of variance (ANOVA) followed by Dunnett's test or non parametric ANOVA followed by Dunn's test (*=p<0.05). TP=Technical Powder; WDG=Water Dispersable Granules; TW=Tap Water; WS=Water Sample; GW=Ground Water; E2=17ß-Estradiol Process Control (EEQ = 1.9±0.3 ng·L^{-1}).

Samples from the artificial pond showed estrogenic activities (Fig. 5.8) in the low ng·L^{-1} range (0.1 to 0.2 ng·L^{-1} EEQ). There was no significant difference in the estrogenicity calculated for the groundwater well samples before (WS5) and after (WS7 – WS10) application of TP. Only WS6, which was taken at the artificial pond directly after application of TP, showed a weak but statistically significant increase in estrogenicity relative to the process control with tap water. However, the measured activity of sample WS6 reached only 0.23±0.08 ng·L^{-1}EEQ.

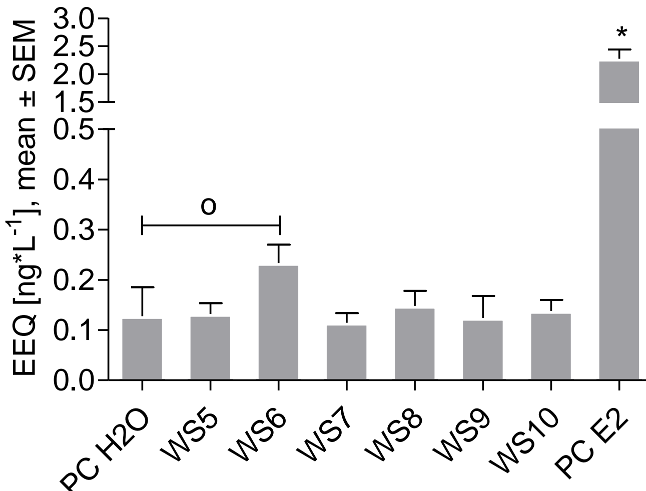


Figure 5.8 Estrogenic activity of field samples of an artificial application setup of VectoBac® TP displayed as estradiol equivalents (EEQ) in the ER CALUX®. Bars represent mean values of at least three independent experiments with standard error (SEM). Data were statistically analyzed with one way analysis of variance (ANOVA) followed by Dunnett's test or non parametric ANOVA followed by Dunn's test (*=$p<0.05$). *=significant difference to the control; °=significant difference between each other. TP=Technical Powder; PC H2O = Tap Water Process Control; PC E2 = 17ß-Estradiol Process Control (EEQ = 2.3±0.4 ng·L^{-1}); WS=Water Sample.

5.5 Discussion

In the present study the former findings on the etrogenic activity of a commercially available *Bti* formulation could be confirmed and extended by two additional formulations. Furthermore, the estrogenic activity could be linked to the active substance VectoBac® TP.

The estrogenic activity of at least one batch of VectoBac® TP could be confirmed by the LYES and the ER CALUX® assays. Results obtained with the LYES for the active substance were in the same range as the *Bti* formulations tested. Estrogenicity for the same samples detected with the ER CALUX® was in a much lower range than in the LYES. Estrogenic activity caused by the highest tested concentration of freeze dried samples was 18-fold lower in the ER CLAUX than in the LYES, respectively. This could be due to the fact that yeasts show significant structural differences compared to mammalian cells including the cell wall and different metabolic capacities (Connolly et al. 2011, Dudley et al. 2000, Legler et al. 2002, Legler et al. 1999). Such differences between the two test systems were already detected in earlier studies (Legler et al. 2002, Leusch et al. 2010, Maletz et al. 2013). But as shown in the present study even the processing of the samples reduced the estrogenic activity. Centrifugation of the diluted active substance TP already reduced the estrogenic activity by up to 74 %. Since TP is not completely soluble and substances with octanol-water partition coefficients ($\log K_{OW}$) between 2.6 and 4.0 are easily adsorbed onto solids (Khanal et al. 2006), most likely, this decrease is foremost a result of the adsorption of the estrogenic active content(s) to the remaining solids in the untreated samples. As process steps like extraction and clean-up have already shown to change the characteristics of samples (Seiler et al. 2008) the possibility to investigate unprocessed complex environmental samples is an important option to screen for the effective estrogenic potential. Investigation of the *Bti* formulations as well as the active substance VectoBac® TP and the adjuvants of product B with the LYES confirmed the applicability of the test system for non-processed samples. For investigation in test systems with mammalian cell lines some form of processing of the sample is almost always necessary to prevent the cells from contamination with microorganisms such as bacteria and yeasts which could falsify the mechanism specific results. However, to minimize the impact of this procedure on the bioassay while insuring to efficiently extract most of the active components it is crucial to identify the right extraction method for each type of samples. In case of VectoBac® TP pressurized liquid extraction with acetone did not efficiently extract the active estrogenic ingredients detected with the LYES in

the unprocessed samples. Even though further studies on the extraction of estrogenic active compounds from environmental samples confirmed the applicability of the method (Beck et al. 2008, Brix et al. 2010, Nieto et al. 2008, 2010). In contrast, freeze dried samples confirmed the previous observations regarding at least a fraction of the estrogenic activity of VectoBac® TP in native samples. The advantage of this method is the relatively low modification of the sample compared to extraction methods with solvents and high temperatures like PLE and Soxhlet (Seiler et al. 2008). Nevertheless, one observed disadvantage was that some viable bacteria spores of unknown origin in the active substances samples were able to survive the freeze drying and reconstitution process, and caused a bacterial contamination in the biotest. Therefore, a sterile filtration step prior to freeze drying (e.g. after reconstitution in DCM) could prove necessary.

Overall, based on the estrogenic activity reported in this study for the *Bti* formulations and the active substance VectoBac® TP is unlikely to have an impact on aquatic environments in the area investigated in this study. Usage of VectoBac® TP is limited on private households and manual mosquito control in areas that are difficult to access. The yearly applied amount of TP in the Upper Rhine area does not exceed 32 Kg in the entire region, and the amount per hectare is approximately 400 g (personal communication with Norbert Becker, GMCA). A minimal water depth of 10 cm would cause a maximum final concentration of 0.4 $mg{\cdot}L^{-1}$ VectoBac® TP. In most cases the water depth lies in the range of 30 cm with a final estimated concentration of 0.13 $mg{\cdot}L^{-1}$. The lowest concentration tested was 2.2 $mg{\cdot}L^{-1}$ (0.5-fold concentration), which had not significant estrogenic activity in the LYES (0.4 $ng{\cdot}L^{-1}$ EEQ) and the ER CALUX® (<0.1 $ng{\cdot}L^{-1}$ EEQ) assays. Results obtained with samples collected from the field as well as the artificial pond system support this conclusion. Neither the investigated surface water samples (WS1 and WS2) nor the groundwater samples (WS5 and WS7-WS10) caused a significant increase of estrogenic activity. For comparison, the detected estrogenic activity of the TP treated surface water sample was 2-times lower than the recommended environmental quality standard for E2 (0.4 $ng{\cdot}L^{-1}$) of the European Union Water Framework Directive (EC 2012) and 2.5 times lower than the lowest observed effect concentration (LOEC = 0.5 $ng{\cdot}L^{-1}$) on the reproduction of the aquatic standard test organism *Pimephales promelas* (Miles-Richardson et al. 1999). It can be concluded that the dilution of VectoBac® TP in the treated water bodies is sufficiently great to prevent estrogenic effects on biota or a contamination of the groundwater. Data also showed, that even under lab storage

conditions (±4°C in dark) estrogenic activity of the *Bti* formulations is attenuated relatively fast. It can be assumed that under natural conditions with higher temperatures, exposure to UV radiation and the presence of microorganisms the decrease would occur even faster (Leech et al. 2009, Raman et al. 2001). Nevertheless, the confirmed estrogenic activity of the three solid formulations as well as the active substance VectoBac® TP needs to be considered when estimating risk scenarios for accidental release of high amounts of these compounds such as can occur during spills. Therefore, storage and transport has to be carried out with appropriate caution. Until now the source of estrogenic activity is not verified, but it is suggested that it could be linked to the fermentation slurry, which is based either on fish meal or soy (personal communication with the manufacturer). Soy products contain high concentrations of phytoestrogens such as daidzein and genistein (isoflavones) and have already shown to exert estrogenic activity in both yeast estrogenic screens and ER CALUX® (Behr et al. 2011, Dip et al. 2008, Kalita & Milligan 2010). Behr et al (2011) detected estrogenic activity of tofu up to 1.5 $ng \cdot g^{-1}$ EEQ in the LYES. Kalita & Milligan (2010) calculated relative potencies of 0.1 % for genistein and 0.02 % for daidzein in comparison to E2, which was set to 100 %. Therefore, the soy based slurry could have contributed to the estrogenic activity of the samples. In case of the fish meal a certain amount of steroids can be expected as well. Concentrations of estrogens detected in whole fish seem to be relatively low (Hartmann et al. 1998) but studies with commercial fish meals showed high estrogen contents up to 1.5 µg E2 and 0.5 µg E1 in 100 g (Pelissero et al. 1989). Depending on the fish meals used for slurry, this component could also be accountable for the estrogenic activity of the samples.

Results from the H295R Steroidogenesis Assay also indicated that the application of *Bti*-based products is unlikely to cause adverse effects to aquatic ecosystem. For VectoBac® WDG there was a slight but significant decrease of E2 as well as 21-OHP production for the two greatest concentrations (9x and 18x) tested but the effect was very weak with induction factors not exceeding 1.5-fold. For the one-fold concentration no differences relative to the SC could be detected. For TP there was a slight but significant decrease of T production in the two high concentrations but again the inhibition was only low compared to the control. The one-fold concentration did not reveal significant differences to the control. Furthermore, those findings could not be verified with samples processed via freeze drying

where no significant differences could be detected at all. In case of an appropriate use of either WDG or TP there should be no adverse effects on steroidogenesis.

5.6 Conclusion

In the present study on potential endocrine effects of *Bti* formulations for mosquito control the former findings on the estrogenic activity of a commercially available *Bti* formulation could be confirmed and extended by two additional formulations. Furthermore, the estrogenic activity could be linked to the active substance VectoBac® TP. Nevertheless, the source of estrogenic activity remains unclear but a contribution of the fermentation slurry based on either soy or fish meal is supposed. In contrast to the measured estrogenic activity, no adverse effects on steroidogenesis could be detected.

Furthermore, data of field samples within this study did not reveal any results that the investigated active substances (TP and WDG) alone have the potential to cause adverse effects in the aquatic ecosystems concerned. Instead, our results amend the previous studies on the safe use of *Bti* products against mosquitoes even in sensitive habitats.

Acknowledgement

The authors would like to thank the Landesamt für Umwelt, Messungen und Naturschutz Baden-Württemberg (LUBW) for funding the present study. Furthermore, we would like to thank the German Association for Mosquito Control (GAMC), namely Dr. Norbert Becker, as well as Valent Biosciences for their cooperation and providing of the active ingredients. We also thank Büchi (Switzerland) and Christ (Germany) for sponsoring of the Student's Lab Fascinating Environment at the Institute for Environmental Research, RWTH Aachen University. Our gratitude also goes to BioDetection Systems, Amsterdam, The Netherlands, for supporting the investigations in the ER CALUX® within the thesis of S. Maletz. Last but not least we would like to thank Eric B Higley and Hong Ma from the Toxicology Centre, University of Saskatchewan, Saskatoon, Canada, as well as Simone Hotz for all their help in the lab. Parts of the study took place within the Undergraduate Research Opportunities-Program (UROP) of the RWTH Aachen University.

Chapter 6

Investigation of potential endocrine disrupting effects of mosquito larvicidal *Bacillus thuringiensis israelensis* (*Bti*) formulations– a microcosm study

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This chapter will be submitted to *Aquatic Toxicology*

6.1 Abstract

In this microcosm-study an approach was made to examine a possible effect of VectoBac® TP on an aquatic community of invertebrates including an indicator-species for estrogens (*Potamopyrgus antipodarum*). The results indicated an influence on the primary production of the systems, as composition of phytoplankton and periphyton-communities seemed to be changed. On the level of zooplankton communities no distinct effects were observed, however a slightly higher abundance of *Daphnia magna* was detected in treatments with a concentration of 4 $mg \cdot L^{-1}$ *Bti*. An endocrine activity of VectoBac® TP could not be clearly stated, as conditions in the microcosms seemed not to be sufficient for an unaffected development of *P. antipodarum* (probably due to quantity and quality of available nutrients). According to the data of this study a further observation seems reasonable as effects on the community level were observed, but their exact nature remains unclear. Furthermore an impact on the reproduction of *P. antipodarum* should be examined more closely as clues on a possible estrogenic activity were noticed due to a significantly higher reproduction at the beginning of the test, and the observation of a very high abundance of snails, six weeks past the treatment in the former 20 mg·L-1 *Bti*-treatments.

Keywords: *Bacillus thuringiensis israelensis*, microcosm, endocrine disruption, *Potamopyrgus antipodarum*

6.2 Introduction

In the need for specific and potent insecticides, the use of *Bt*-toxins from various strains of *Bacillus thuringiensis* became more and more popular over the last decades. One of those strains, *Bacillus thuringiensis var. israelensis* (*Bti*) is used since the 1980s as larvicide against mosquito larvae. It is considered to have a minor impact on the environment, as it is a biological insecticide with a narrow range of target organisms. Hence, *Bti* is widely distributed for mosquito control (Boisvert & Boisvert 2000, Kroeger et al. 1995). A routine examination of ground water samples from a *Bti* application area in South Germany indicated an estrogenic activity in the Yeast Estrogenic Screen (YES). A further investigation of different *Bti* products as well as the two active substances VectoBac® TP and VectoBac® WDG with the lyticase assisted Yeast Estrogenic Screen (LYES) substantiated the former detected estrogenic activity (see Chapter 5). Therefore, a further investigation to estimate the possible ecological impact on treated areas seemed necessary.

Since ecotoxicology is a multidisciplinary field which combines the knowledge of chemical fate, single-species toxicology and organism interaction, data of different scientific approaches needs to be integrated to predict a potential risk of substances introduced to the environment. Hence, the extrapolation of observed effects from the laboratory to the environment is one of the biggest difficulties in this discipline. Accordingly, mesocosms and microcosms have been proven to be reliable tools to predict the effect of a suspicious substance to the environment (Taub 1989).

The microcosm as well as the mesocosm is a system that mimics natural conditions. According to Taub (1997) the main features of this systems are the possibility to get information on direct trophic-level effects as well as the retrieval of information on: (1) indirect trophic-level effects, (2) compensatory shifts within trophic levels, (3) responses to chemicals within the context of seasonal patterns that modify water chemistry and birth- / death- rates of populations, (4) chemical transformations by some organisms that cause effects in other organisms, and persistence of parent and transformation products.

In the present investigation the possible impact of the larvicide VectoBac® TP on aquatic communities has been studied. Potential changes in the composition and abundance of algae and zooplankton populations have been revealed by use of aquatic microcosm systems. In addition, an approach to integrate the indicator-species *Potamopyrgus*

antipodarum (Stange et al. 2012, Wagner & Oehlmann 2011) into this system with the goal to deliver information on a possible endocrine disruption of higher organisms was made.

6.3 Material & Methods

6.3.1 Setup of the microcosm test systems

The microcosm study was performed in 13 aquaria with the volume of 20 L and the size of 0.4 x 0.25 x 0.25 m each. All aquaria were placed on isolating mats (thickness 0.5 cm) and filled with 1500 ml of pure silica sand as well as 17 L of dechlorinated (10 days at room temperature) tap water. After six days two fluorescent lamps (LIFE-GLO, T5 H0, 39 W, Hagen, Germany) were placed directly on each basin and two liters of sieved (mesh-width 120 µm) mesocosm-water (provided by gaiac mesocosm facility, Aachen, Germany) and 300 ml of a well grown (one month old) *Cryptomonas obovoidae*-culture in COMBO-medium (according to Kilham et al (1998)) were added. One day later 50 ml of a *Desmodesmus subspicatus*-suspension and one microscope slide well covered with periphyton (from a laboratory pre-culture) were added to each aquarium. The suspension was made from a well grown *D. subspicatus*-culture in Khul-medium (Kuhl & Lorenzen 1964) from which the algae were separated by centrifugation (4000 rpm, 10 min) and the resulting pellet was resuspended in tap water. One day later each aquarium was inoculated with a well grown *Brachionus calyciorus*-culture in COMBO-medium , additionally one glass pipette was attached to the back wall of each aquarium for aeration. The aeration results in a slight turbulence in the water column which should support the growth of the immobile algae *D. subspicatus*. The day after, 25 individuals of the copepod *Mesocyclops leuckarti* were introduced to each basin. Three days later, 30 individuals of the cladoceran *Daphnia magna*, all carrying eggs, were added to each aquarium. Two days after that, 20 synchronized larvae of the chironomid *Chironomus riparius* were added to every basin. Nine days later, 75 individuals of the mud snail *P. antipodarum* were inserted to each test aquarium. Only fecund snails with an appropriate size of 3 to 5 mm shell heights were inserted. Then, 19 days after the introduction of the *C. riparius* larvae to the systems, the aeration was removed and all basins were covered with a thin mesh fabric to avoid escaping of hatched *C. riparius* imagines. The treatment of the microcosms was started 35 days after the beginning of the microcosm installation (Table 6.1).

Tab 6.1 Timetable of the microcosm installation

Day	Operation / Addition of
0	1500 ml silica sand and 17 L dechlorinated tap water
1	2 L sieved (120 µm) mesocosm water (Gaiac e.V.) and 300 ml *C. obovoidae*-culture
2	50 ml *D. subspicatus*-suspension and 1 microscope slide covered with periphyton
3	50 ml *B. calyciorus*-suspension and installation of aeration
4	25 adults of *M. leuckarti*
7	30 adults of *D. magna*
9	20 juveniles of *C. riparius*
18	75 adults of *P. antipodarum*
28	End of aeration and covering of the aquaria with mesh fabric
35 (0)	Start of the treatment with 4 and 20 mg·L^{-1} VectoBac® TP (application biweekly for a duration of eight weeks) or 40 ng·L^{-1} 17ß-estradiol as positive control (weekly for nine weeks)

(0) = day 0 of the treatment

6.3.2 Treatment of the microcosms

Of the 13 aquaria installed, four served as negative control (NC), eight as treatment with VectoBac® TP and one as a positive control (PC). Treatment was accomplished with a high concentration (4 aquaria containing 20 mg·L^{-1} TP) and a lower concentration (4 aquaria containing 4 mg·L^{-1} TP). While the NCs were not treated at all, 40 ng·L^{-1} 17ß-estradiol (E2) were added to the PC. The application of E2 was carried out once a week for a duration of nine weeks. For the *Bti* treatments two dispersions with VectoBac® TP (Valent BioSciences® Corporation, Libertyville, USA) and dechlorinated tap water were made. Hence 1520 mg and 304 mg of VectoBac® TP were added and the mixture was stirred at least for 90 min until a good dispersion was achieved. Subsequently, 150 ml of the dispersions were applied to each of the eight corresponding treatments. This application was carried out biweekly for a period of eight weeks. 19 ml of a nutrient solution (344 mg $NH_4Cl \cdot L^{-1}$ and 66 mg $KH_2PO_4 \cdot L^{-1}$) were added twice a week. This corresponds to a nitrogen and phosphor addition of 0.09 mg N·L^{-1} and 0.015 mg P·L^{-1}. Once a week water evaporation from the systems was compensated by addition of dechlorinated tap water, resulting in a total volume change of less than 10 %. All aquaria were kept at a temperature of 16°C±4°C with a light / dark interval of 16 / 8 hours and a light intensity right beneath the water surface between 120 and 150 µE·s^{-1}·m^{-2} throughout the whole experiment.

6.3.3 Sampling

6.3.3.1 Water samples

Samples for the determination of phytoplankton and zooplankton abundance and the determination of the abiotic parameters pH, temperature, conductivity, and oxygen saturation were taken weekly. The first samples were taken right before the start of the treatment, on day 0, and the last samples were taken ten weeks after start of the treatment, on day 56. The samples were taken with a sampling-tube of an approximate length of 40 cm and 2 cm in diameter. For sampling, the pipe was inserted vertically in the water column without dipping into the sediment and removed quickly out of the water while the top hole was closed with the thumb (in total 20 times per aquarium). The microcosm water collected this way was pooled in a 1.5 L beaker as a mixed sample. Each single sample consisted of approximately 50 ml. 30 ml of each of the mixed samples were taken and stored in a 30 ml glass vial. The phytoplankton samples were measured on the same day to determine algae classes and the correspondent amount of chlorophyll a. The measurement was performed with a fluorescence analytic kit (Sensor bbe FluoroProbe, bbe Moldaenke GmbH, Schwentinental, Germany). Afterwards temperature, pH, conductivity, and oxygen saturation were measured in the 1.5 L beaker.

Subsequently, to get zooplankton samples, the water of the mixed sample was poured gently through a sieve (mesh-width 55 µm) right back in the microcosm it was taken from. The remains on the sieve were rinsed out with fixating solution (70 % ethanol with 40 $ml \cdot L^{-1}$ glycerol and 40 $g \cdot L^{-1}$ saccharose) and were transferred into a 15 ml glass vial. Zooplankton samples were stored in dark at 16°C. For determination of the taxa and the correspondent abundance the samples were transferred into a counting dish and examined completely with an inverse microscope (CK2, Olympus, Hamburg, Germany). This was repeated until all zooplanktic organisms in the sample were determined and counted. The determination of the rotifers was performed with the determination key in Koste (1978). For the dominating groups of zooplankton, the cladocerans, the copepods, and the rotifers, abundances were calculated from the volume of the samples.

6.3.3.2 Documentation of the hatching of *C. riparius*

The basins where checked regularly for hatched *C. riparius* imagines. The animals and the exuviae of the pupae were removed and the number of individuals and the date of discovery were documented.

6.3.3.3 *Potamopyrgus antipodarum*

To determine the reproductive activity of *P. antipodarum* 20 individuals were collected from each aquarium 14, 42, and 56 days after beginning of the treatment. The snails were stored in 15 ml glass vials and covered with fixating solution. According to Schmitt et al. (2013) snails were measured on a scale paper under a binocular (SMZ1500, Nikon, Chiyoda, Japan) and their length and aperture height were documented. Subsequently they were cracked carefully with a forceps. The shell was removed and the brood pouch opened gently with dissecting needles. All embryos were removed and the number of embryos with shell and without shell was documented. At the last day of sampling the number of juvenile snails sitting on the aquaria glass walls was documented. Therefore all individuals on one of the side walls and on the front wall were counted.

6.3.3.4 Periphyton determination

Periphyton was measured 64 days after beginning of the treatment. Therefore a fluorescence analytic kit was equipped with a glass fiber sonde (Sensor bbe FluoroProbe, BenthoFluor Kit, bbe Moldaenke GmbH, Germany). This sonde was held from the inside of the aquarium onto the glass wall and the corresponding area on the outside of the aquaria was covered with a black cardboard. For each basin two representative areas on the glass walls were chosen and measured.

6.3.4 Statistical analysis

All data were statistically analyzed for significant differences between the treatments and the NC with SigmaStat 3.5 (Systat Software, San José, USA) and presented as mean ± standard deviation (SD). All data were tested for normal distribution using the Kolmogorov-Smirnov test as well as for homogeneity of variance. Zooplankton data was checked by one-way analysis of variance (ANOVA) analysis followed by Dunnett's test, if data distribution was normal and variance was equal. In cases were a one-way ANOVA

could not be performed, a Kruskal-Wallis test followed by Dunn's Multiple Comparison test was used. The data of the fluorometric algae determination was analyzed via t-test. For data with unequal variance and no normal distribution a Mann-Whitney test was performed. The results of the *P. antipodarum* reproduction were analyzed by the use of Kruskal-Wallis test followed by Dunn's Multiple Comparison test. Differences were considered significant at $p<0.05$.

6.4 Results

6.4.1 Temperature, conductivity, pH, and oxygen saturation

The water parameters temperature, conductivity, oxygen saturation, and pH were measured every seven days from day 0 to day 56 of the treatment. Over the whole test period the temperature within a treatment group never differed for more than 3°C in a total range between 14.9 and 19.5°C. Throughout the test the mean conductivity measured for the different treatment groups was between 250 and 334 µS·cm^{-1}. The oxygen saturation found in the different basins generally increased over the time of treatment and never was lower than 6.9 mg·L^{-1}. Over the duration of the test mean pH values were between 7.58 and 9.95 for all treatments and steadily increased for the duration of the test.

6.4.2 Phytoplankton

In general the abundance of phytoplankton was very low throughout the microcosm study. The class of chlorophyta showed the highest abundance at the beginning of the test when the concentration of chlorophyll a never undercut 0.44 µg·L^{-1} in all treatments for the first seven days (Fig. 6.1). After day seven the concentration of chlorophyll a never exceeded 0.25 µg·L^{-1} in all treatments except for the PC on day 56 with a concentration of 0.83 µg·L^{-1}.

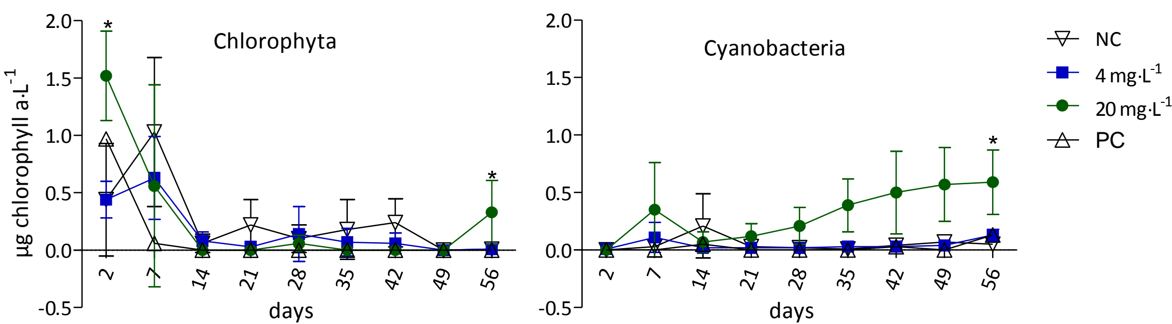

Figure 6.1 Mean concentration of chlorophyll a ± standard deviation related to green algae (Chlorophyta) and cyanobacteria for the different *Bti*-treatments (4 and 20 mg·L^{-1}) and the controls (NC = negative control; PC = positive control with 17ß-estradiol) from day 2 to day 56. * = significant differences to the NC (t-test, when t-test failed Mann-Whitney test was performed).

The class of cyanobacteria could not be detected after 36 hours except for the 4 mg·L^{-1} treatment with a very low concentration of 0.01 µg·L^{-1} chlorophyll a. After day seven

cyanobacteria were found in all treatments throughout the duration of the test. While concentrations in the NC and 4 $mg \cdot L^{-1}$ treatment stayed in a range between 0.01 to 0.07 $\mu g \cdot L^{-1}$ chlorophyll a and showed only one momentary peak after 14 days for the NC (0.21 $\mu g \cdot L^{-1}$ chlorophyll a) and two momentary peaks for the 4 $mg \cdot L^{-1}$ treatment after seven days (0.11 $\mu g \cdot L^{-1}$ chlorophyll a) and 56 days (0.13 $\mu g \cdot L^{-1}$ chlorophyll a) chlorophyll a concentrations from cyanobacteria in the PC and the 20 $mg \cdot L^{-1}$ treatment were steadily increasing from day 14 on. Both treatments had a peak after 7 days with 0.35 $\mu g \cdot L^{-1}$ (20 $mg \cdot L^{-1}$ *Bti*) and 0.8 $\mu g \cdot L^{-1}$ (PC) chlorophyll a before on day 14 low peaks with concentrations of 0.07 $\mu g \cdot L^{-1}$ (20 $mg \cdot L^{-1}$ *Bti*) and 0.06 $\mu g \cdot L^{-1}$ (PC) could be found. For the following duration of the test both treatments showed increasing cyanobacteria abundance with a chlorophyll a concentration of 0.59 $\mu g \cdot L^{-1}$ (20 $mg \cdot L^{-1}$ *Bti*) and 0.53 $\mu g \cdot L^{-1}$ (PC) on day 56 (Figure 1). A comparable development of blue algae to the PC was monitored for the 20 $mg \cdot L^{-1}$ group. On day 14 a concentration of 0.35 $\mu g \cdot L^{-1}$ chlorophyll a was measured that dropped to 0.07 $\mu g \cdot L^{-1}$ on day 14 and then increased steadily to a final concentration of 0.59 $\mu g \cdot L^{-1}$ on day 56.

6.4.3 Periphyton

At the beginning of the treatment with *Bti* the periphyton covered most of the fine sandy sediment as a very thin layer and had only started growing on the glass walls of the aquariums. With further proceeding of the test the periphyton covered almost all glass walls completely until the end of the study. In all treatment-groups, except for the PC, the dominating periphyton class was the green algae (Chlorophyta). Their mean chlorophyll a content was 1.51 $\mu g \cdot cm^{-2}$ for the NC, 1.59 $\mu g \cdot cm^{-2}$ for the 4 $mg \cdot L^{-1}$ treatment, and 1.08 $\mu g \cdot cm^{-2}$ for the 20 $mg \cdot L^{-1}$ treatment (Fig. 6.2). In the PC no chlorophyll a from green algae was found.

The second dominant class was the blue algae (Cyanobacteria) which represented 0.26 $\mu g \cdot cm^{-2}$ chlorophyll a in the NCs, 0.72 $\mu g \cdot cm^{-2}$ for the 4 $mg \cdot L^{-1}$ group, 0.64 $\mu g \cdot cm^{-2}$ for the 20 $mg \cdot L^{-1}$ treatment, and 0.32 $\mu g \cdot cm^{-2}$ chlorophyll a as the most dominant class in the PC. The least represented class, the Diatoms, were found in the negative controls with an average amount of 0.22 $\mu g \cdot cm^{-2}$ chlorophyll a, 0.2 $\mu g \cdot cm^{-2}$ in the 4 $mg \cdot L^{-1}$ treatment, 0.25 $\mu g \cdot cm^{-2}$ in the 20 $mg \cdot L^{-1}$ treatment, and 0.14 $\mu g \cdot cm^{-2}$ in the PC.

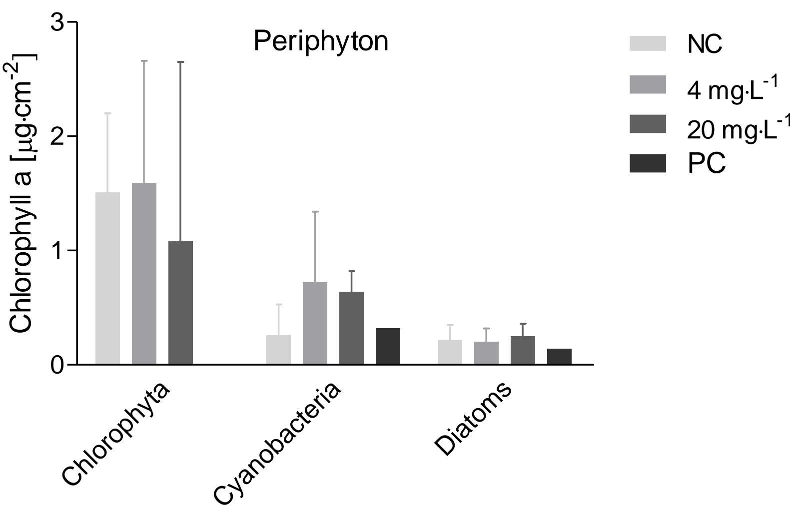

Figure 6.2 Amount of periphyton given as mean ± standard deviation of measured chlorophyll a detected 67 days after start of the treatment with *Bti* (4 and 20 mg·L^{-1}) and the controls (NC = negative control; PC = positive control with 17ß-estradiol).

6.4.4 Zooplankton

With exception of one measurement within the crustacea at day 42, the abundance of zooplankton organisms showed no significant differences compared to the NC (Fig. 6.3).

In case of *D. magna* (crustacean) abundance was very similar for all treatments through the first 28 days and oscillated in a range between 16 and 60 individuals·L^{-1}. Only the PC had a higher abundance of 87 individuals·L^{-1} at the first sampling (day 0) within this period. Even though densities did not vary very much at this time, the daphnids started to develop ephippia after 14 days of the treatment in the 20 mg·L^{-1} treatment, and within the next week for all other treatments. After day 28 densities in the 4 mg·L^{-1} Bti-treatment increased to abundances from 74 to 88 individuals·L^{-1} until the end of the test whilst abundances in the other treatments stayed in a range between 31 to 64 individuals·L^{-1}.

Abundances of *M. leuckarti* were relatively similar over all treatments in a range between 41 and 516 individuals (nauplii). The number of individuals reached a peak (305 to 516 individuals) at day 14 in all treatments and declined afterwards until day 28 (20 mg·L^{-1} *Bti* and PC), day 35 (4 mg·L^{-1} *Bti*) and day 42 (NC). For the PC a second peak was reached at day 42 with 416 individuals whereas abundances in the higher *Bti*-treatment increased until day 49. At day 56 a slight decrease could be detected for both treatments. Both the NC as well as the lower *Bti*-treatment showed increasing abundances until day 56.

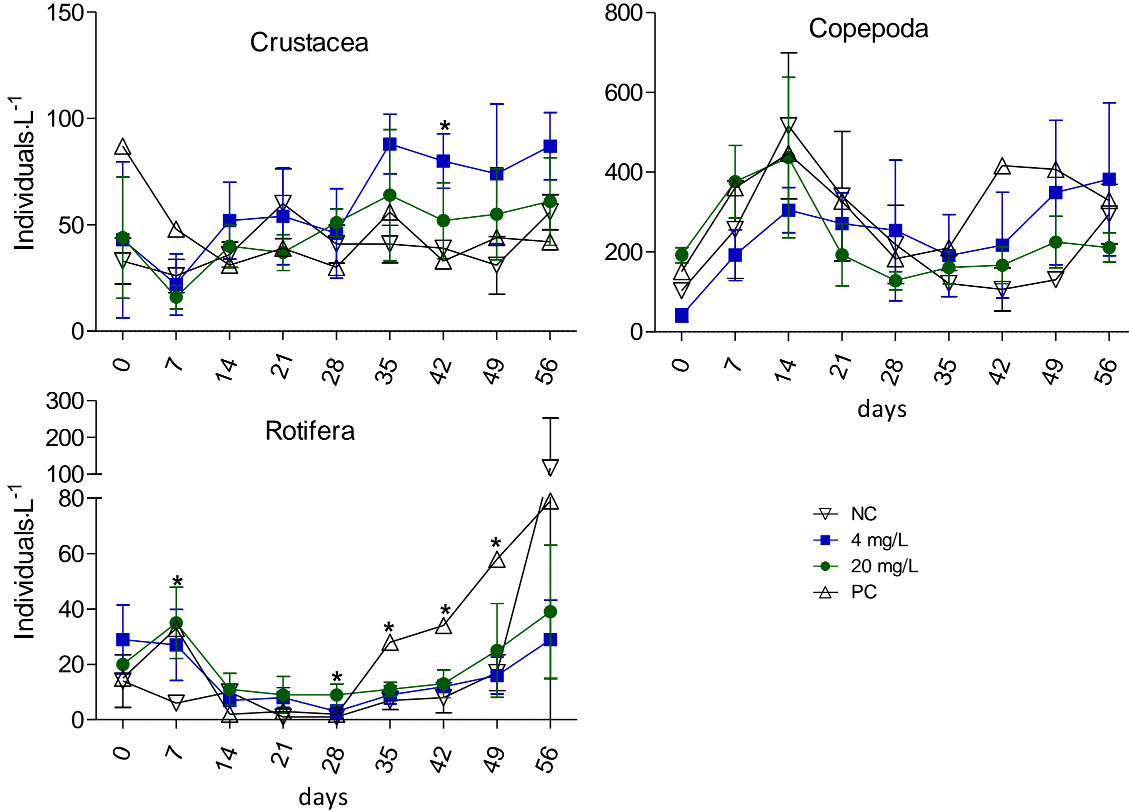

Figure 6.3 Mean values of abundance ± standard deviation of *Daphnia magna* for the *Bti*-treatments (4 and 20 mg·L^{-1}) and the controls (NC = negative control; PC = positive control with 17ß-estradiol) over the whole study period (56 days). * = significant differences to the NC (1way ANOVA followed by Dunnett's test).

With exception of day 7, where the number of individuals in the NC was much lower (6) than in the other treatments (± 30), abundances of the rotifers were in a similar range until day 28. For the higher *Bti*-treatment significant differences were detected for days 7 and 28, but taking the standard deviations into account the difference seems not to be that high. From day 35 on abundances in the PC strongly increased up to 79 individuals at day 56 with significant differences to the NC from days 35 to 49. Abundances of the *Bti*-treatments both increased in a lower scale up to 29 (4 mg·L^{-1}) and 39 (20 mg·L^{-1}) individuals, respectively. Abundances in the NC also increased slightly until day 49 but showed a strong increase on day 56 with 117 individuals per liter. In case of the rotifers also the composition of the community shifted throughout the study. While the genus *Cephalodella spec.* could be found at all sampling times, the genus *Lecane spec.* was mainly observed for the first and the last seven days of the test. From day 42 on, the new genus Mytilina spec. occurred with increasing density.

6.4.5 Reproduction of *Potamopyrgus antipodarum*

The reproduction of *P. antipodarum* in general showed a high variability (Fig. 6.4).

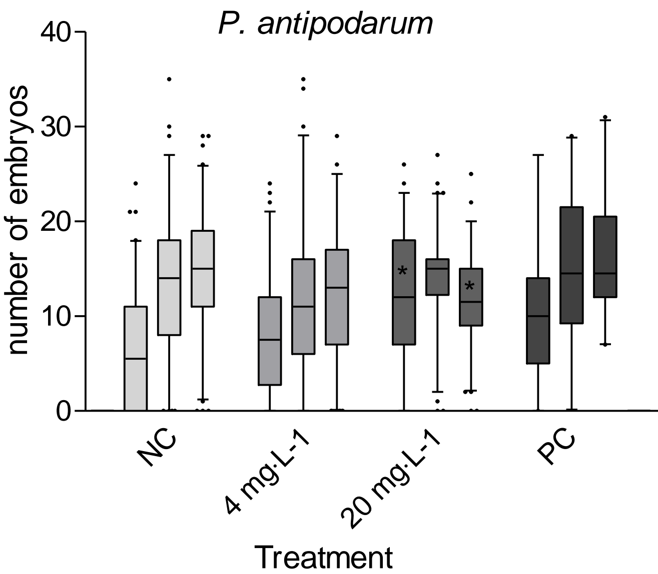

Figure 6.4 Reproduction of *Potamopyrgus antipodarum* for the different Bti-treatments (4 mg·L^{-1} and 20 mg·L^{-1}) and the controls (NC = negative control; PC = positive control) after 14, 42 and 56 days. Data are given as box plots (25 to 75 percentile), including the median (—), whiskers (5 to 95 percentile) and outliers (·); * = significant differences to the NC (Kruskal-Wallis test followed by Dunn's Multiple Comparison Test).

This is due to the fact that for almost all replicas, treatments and sampling times, animals which did not reproduce at all and animals with a relatively high offspring could be found. The median of the embryos in the NC increased from 5.5 over 14 to 15 at day 56 while the median of the PC increased from 10 to 14.5 at day 42 and remained static at day 56. In the lower *Bti*-treatment the number increased from 7 over 10.5 to 13 embryos on day 56 while in the higher treatment the number increased from 12 to 15 on day 42 and decreased afterwards to 11.5 on day 56.

The distribution between younger embryos without a fully developed shell and older embryos with a shell at day 14 was 88 % to 12 % for the NC, 81 % to 19 % for both the 4 mg·L^{-1} *Bti*-treatment and the PC and 84 % to 16 % for the 20 mg·L^{-1} *Bti*-treatment (Fig. 6.5). At day 42 this ratio was 75 % to 25 % for the NC, 77 % to 23 % for the 4 mg·L^{-1} *Bti*-treatment and 74 % to 26 % for the 20 mg·L^{-1} *Bti*-treatment and the PC, whilst for day 56 this ratios changed to 69 % and 31 % for the NC and the 4 mg·L^{-1} *Bti*-treatment, 66 % and 34 % for the 20 mg·L^{-1} *Bti*-treatment and 75 % and 25 % for the PC.

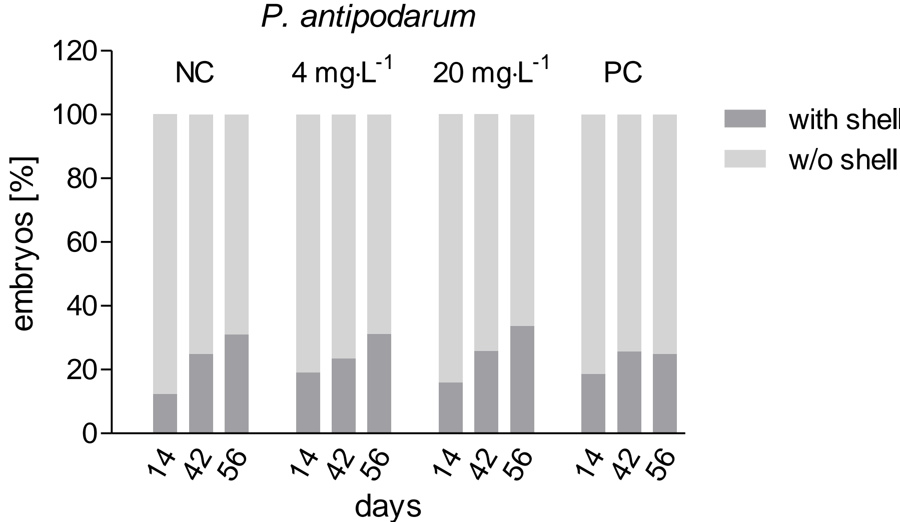

Figure 6.5 Distribution in percent between younger embryos of *Potamopyrgus antipodarum* without a fully developed shell and older embryos with a shell for the *Bti*-treatments (4 mg·L^{-1} and 20 mg·L^{-1}) and the controls (NC = negative control; PC = positive control) after 14, 42 days and 56 days.

Counting of juvenile snails at the end of the study lead to a mean of five snails for the NC, a mean of two snails for the 4 mg·L^{-1} *Bti*-treatment, a mean of ten snails for the 20 mg·L^{-1} *Bti*-treatment and 19 snails for the PC.

6.4.6 Hatching of *Chironomus riparius* larvae

The development of the *C. riparius* larvae seemed to be very unequal in between the different microcosms and even within the same treatment groups. Already in the equilibration phase differences in the development of larvae could be observed. Some larvae were already in third or fourth instar, whilst other larvae seemed to be still in second instar. Therefore, some imagines had already hatched before the beginning of the test and the application of the test substance. Hatching of imagines occurred in seven of thirteen microcosms preliminary to the start of the study. The day after the application of *Bti* in seven of eight aquaria of the *Bti* treatments dead larvae were collected. The dead larvae were laying on the sediment surface and the color of their mid-section had changed from red to dark-grey while there was also no more tension in this part of their bodies. After the application of *Bti* no more larvae could be observed in the treated microcosms, and hatched imagines could only be found the day after and no more during the further study in those treatments. Only in some of the NCs and the PC a few imagines could be seen

throughout the following weeks. After the beginning of the test some pupae were seen, also in one of the 4 $mg \cdot L^{-1}$ *Bti*-treatments, but most of them did not finish the metamorphosis and died before the imagines could hatch.

6.5 Discussion

6.5.1 Phytoplankton

The abundance of the green algae in general was very low throughout the duration of the study (Fig. 6.1). Except for the 20 mg·L^{-1} *Bti*-treatments with a concentration of 1.52 µg chlorophyll a·L^{-1} after 36 hours, all abundances were at a level that is comparable to a clear-water phase (Lampert et al. 1986). The decline within the first 14 days of the study in all treatments was probably due to grazing by zooplankton, as zooplankton densities in general were relatively high.

The statistical analysis of the data showed a significant higher abundance in the 20 mg·L^{-1} *Bti* treatments compared to the NC after 36 hours and 56 days, while almost no green algae were found between day 14 and 49 in the 20 mg·L^{-1} *Bti*-treatment.

Since the decline in the abundance was very steep within the first two weeks and after 49 days a sudden increase was monitored, it cannot be excluded that the 20 mg·L^{-1} *Bti*-dosage affected the greenalgae respective the composition of their community. The concentration of chlorophyll a from cyanobacteria was below the level of detection throughout the test for the NC and the 4 mg·L^{-1} *Bti*-treatment, whilst a constant increase could be seen for the PC and the 20 mg·L^{-1} *Bti*-treatment from day 14 on (Fig. 6.1). Even though concentrations were still very low in those treatments for most of the duration of the test, a tendency could be seen and significant differences between the NC and the 20 mg·L^{-1} *Bti*-group were detected for the final sampling on day 56. Therefore an effect on the phytoplankton community can be assumed. Boisvert & Boisvert (2000) suspected the application of *Bti* to have an impact on the primary production of green algae. It is thinkable that after a first adverse influence on the algae an adaption to the substance occurred and therefore a later increase in the population was possible, and / or that a change in the community structure to less susceptible species was performed. A comparable development could be seen in the PC. Also Hense et al. (2008) reported a shift in phytoplankton communities from green algae to cyanobacteria after the application of 17α-ethinylestradiol to freshwater microcosms as well.

An examination of one of the phytoplankton samples of the 20 mg·L^{-1} *Bti*-treatments from day 56 showed that some of the cyanobacteria were originating from the periphyton. This was probably due to grazing of the daphnids, as they were observed feeding on periphyton which was also demonstrated by Siehoff et al. (2009). Since green algae were almost

absent in the 20 mg·L^{-1} *Bti*-treatment from day 14 to day 49 this seems very likely. But also "normal" grazing on phytoplankton by large grazers like *D. magna* thus can foster a shift to the class of cyanobacteria and therefore intensify the abundance of preexisting blue algae (Haney 1987).

6.5.2 Periphyton

The results of the periphyton indicate that in the treatments containing *Bti* the growth of periphyton from the class of the cyanobacteria might be favored. The dominant periphyton group found in the NC and the 4 mg·L^{-1} treatment for all replicas (except for one) were green algae. This observation could not be confirmed for the PC and the 20 mg·L^{-1} treatment, where green algae were only dominating the periphyton community in one of the four replicas of the *Bti*-treated microcosm. Therefore, observations on a shift from green to blue algae through high concentrations of *Bti* or estrogens, respectively, could be confirmed.

6.5.3 Zooplankton abundance

The data of *D. magna* abundances illustrates that for the first four weeks of the study, population densities in all treatments were in the range of approximately 20 to 50 individuals·L^{-1} (Fig. 6.3). According to Carvalho & Hughes (1983) the observed development of ephippia occurs mainly due to three factors: a limitation in food (<0.05 mg·individual^{-1}), high abundances (300 to 400 individuals·L^{-1}), and a light / dark ratio of less than 12 : 12 hours. As population densities of the daphnids were not that high between day 14 and day 21 and the light regime was 16 hours light to 8 hours darkness, the production of ephippia was probably caused by a limitation of food. This explanation would go in hand with the fact that the abundance of green algae dropped within the first two weeks of the study for all treatments. Since the concentrations do not state information on the quality of the algae and their edible fraction is also consumed by copepods and rotifers, a limitation in food seems very likely. Especially as a status comparable to a clear-water phase could be assumed throughout the test, and population densities adjusted in a certain range.

Nevertheless, the populations of *D. magna* in the treatments were bigger than those for the controls from day 35 on. In particular the 4 mg·L^{-1} treatment showed abundances that were twice as high as those of the NC for day 35 to 49 (with a significant higher density at

day 42). This could be a hint on a possible use of the applied *Bti* as food source by the daphnids (Geller & Müller 1981). Duchet et al. (2010) reported a slightly higher fecundity for *D. magna* and thus slightly higher growth rates of the populations compared to the negative control in a laboratory experiment with VectoBac® 12AS, but no higher abundances. Therefore a favorable effect of relatively low *Bti* concentrations on *D. magna,* which is constrained by an additional antagonist effect in higher *Bti* concentrations, seems possible.

Abundances of *M. leuckarti* and the rotifers are correlating throughout the study which is related to the fact that rotifers serve as food for the copepods and therefore are directly accountable for their reproductive outcome (Hansen & Santer 1995). On the one hand, the decline in the abundances of the rotifers is due to the predation pressure of *M. leuckarti*. On the other hand Gilbert (1988) could show that grub competition of daphnids has a negative influence on rotifers. The limitation in food therefore could also have played a role. Nevertheless, there was a significant increase of rotifer abundance in the PC from days 35 to 49. Further studies could not detect increases of density after exposure to 17ß-estradiol (Gallardo et al. 1997, Preston et al. 2000), but they used much higher concentrations ranging from 10 $\mu g \cdot L^{-1}$ up to 50 $mg \cdot L^{-1}$. Effects in the present study could therefore be caused by low-dose effects of E2 (Vandenberg et al. 2012). The strong or rather very strong increase of abundances of the PC respectively the NC after 56 days was not significant due to the large standard deviation.

6.5.4 Reproduction of *Potamopyrgus antipodarum*

The reproduction of *P. antipodarum* revealed no clear tendency for the different treatments (Fig. 6.4). The NC, the 4 $mg \cdot L^{-1}$ treatment, and the PC seemed to reach a maximum in reproduction at day 42, as embryo numbers increased between day 14 and 42, and did only change marginally until day 56. The reproduction of the snails in the 20 $mg \cdot L^{-1}$ group did undergo the most severe change, since reproduction increased between the first two samplings and declined afterwards for the final sampling at day 56.

The fact that only slight differences in the number of embryos between the PC and the NC were observed might be a hint that a concentration of 40 $ng \cdot L^{-1}$ E2 for this test system is not high enough and / or a more frequent application of E2 would have been needed. Min (2011) showed that 48 hours after an application of E2 to wetland microcosms only 10 % of

the applied E2 could be recovered in the water phase, due to sorption to the sediment. Jürgens et al. (2002) reported that in rivers bio- and photodegradation of E2 form only one estrogenic active compound, the short living estron (E1), in comparison to the degradation of 17α-ethinylestradiol. Anyhow the PC did show a higher initial reproduction as the NC and had slightly more offspring at the later sampling points. Aside from that the PC was the only treatment to be found in that all snails showed reproduction (Fig. 6.4), even if sample sizes were smaller than for the other treatments as there was only one replica. The standard operation procedure of the test system does not include a positive control (Schmitt et al. 2013) but in order to classify potential endocrine effects the authors of the present investigation strongly recommend such. Therefore, for further investigations a higher concentration of E2 with a more frequent application or the use of a more potent as well as stable positive control substance such as 17α-ethinylestradiol (Jobling et al. 2003) seems appropriate.

The reproduction of the NC and the 4 $mg \cdot L^{-1}$ treatments was very even and revealed a similar number in embryos, from day 42 on. The 20 $mg \cdot L^{-1}$ group showed a relatively high initial reproduction, which exceeded that of the PC, and differed significantly from that of the NC. Even though embryo numbers were not higher (day 42) or even lower (day 56) towards the end of the test as in the other groups, the reproduction was more even among the snails in the 20 $mg \cdot L^{-1}$ treatments, as the smaller boxes of the Whiskers-blot indicate (Fig. 6.4).

The observation that the reproduction of *P. antipodarum* increased between the first two samplings for all treatments is a clue on a previous limitation in food, especially as the periphyton coverage at the start of the test was much lower than towards the end. Broekhuizen et al. (2001) have discovered that a high contamination with fine sediment of the food has a negative influence on *P. antipodarum* reproduction. Additionally zooplankton abundances were relatively high at the beginning of the study and daphnids showed signs of food limitation (epphipia), and thus were grazing on periphyton, a competition on food for this two species can be assumed (Fig. 6.3). After the biomass from periphyton increased food supply increased as well and reproduction was better. The decline in the embryo number for the 20 $mg \cdot L^{-1}$ group at day 56 could be connected to a change in quantity respective quality of food as well. Siehoff et al. (2009) stated that grazing on periphyton by *D. magna* can reduce periphyton thickness and change the

composition of periphyton communities towards less edible species (e.g. blue algae). In the 20 $mg \cdot L^{-1}$ treated microcosms cyanobacteria represented the most evenly distributed class of periphyton and therefore were probably ingested mostly by the snails, exceptionally as according to Aberle et al. (2005) *P. antipodarum* tends to ingest the better distributed food source under competition rather than the preferred one. The increased ingestion of blue algae especially of toxin forming species can have a huge impact on the reproduction of snails and thus could be considered as the main reason for a decline in embryo number (Lance et al. 2007). This could have been also a reason for the remote increase in the reproduction for the PC between day 42 and day 56, as in those samples cyanobacteria were also the predominant and best distributed class in periphyton. But as cyanobacteria abundance did not reach the same level like in the 20 $mg \cdot L^{-1}$ group the impact on reproduction might have been less strong than in those treatments.

The increase of embryo numbers without a fully developed shell over the whole study period in the *Bti*-treatments as well as the NC could be a hint for reaching a maximum of reproduction after 56 days indicating that on later sampling dates the total embryo numbers would have been stagnating or even (further) decreasing. Nevertheless, for the PC no such trend could be observed indicating that the applied low dose of E2 still could have a slight impact on the reproduction of P. antipodarum. It also might be associated with the difference in the diet of the snails though, as the higher numbers of embryos without fully developed shells were mainly observed in those microcosms that showed a high abundance and a more equal distribution of cyanobacteria.

Overall, the results indicate that under the conditions provided for the duration of the study, a specific conclusion on the endocrine activity of VectoBac® TP cannot be stated. An increased reproduction for high doses of *Bti* (20 $mg \cdot L^{-1}$) could be observed after 14 days, but not along the further treatment. One of the main factors that influenced the reproduction of *P. antipodarum* and made the results equivocal was probably the quantity and quality of food, which according to our data could have been indirectly influenced by the addition of VectoBac® TP (e.g. shift in composition of phytoplankton- and periphyton-communities).

6.5.5 Summary

The collected data revealed an effect of high concentrations (20 mg·L^{-1}) of VectoBac® TP on the community of phytoplankton that could not be observed in a fivefold lower concentration of 4 mg·L^{-1} *Bti*. Though, a similar effect in the two treatments was detected for the composition of periphyton communities. For both phytoplankton and periphyton a shift from green algae (Chlorophyta) to blue algae (Cyanobacteria) occurred, thus the exact nature of these changes could not be clarified. A promoting effect on cyanobacteria seems likely but the role of zooplanktic organisms like *D. magna* in this alteration remains unclear. The application of VectoBac® TP showed no impact on zooplankton communities. A slightly higher abundance of *D. magna* was seen for the 4 mg·L^{-1} concentration, but differences to the NC were only significant for one sampling day. Since an increase in fecundity and growth rates for *D. magna* under the influence of VectoBac® 12AS was already reported, an effect on *D. magna* populations in the present investigation also cannot be excluded.

A clear endocrine activity of VectoBac® TP could not be stated by the use of *P. antipodarum*. Even though the data of the reproduction indicated a significantly higher number of embryos in the 20 mg·L^{-1} samples of day 14, this observation could not be certified along the rest of the study. After 56 rather a significant decrease in reproduction could be observed. Differences between the treatment-groups seemed to be more likely associated with quantity and quality of the available food, which might be indirectly related to the application of VectoBac® TP (e.g. community shifts in phytoplankton and periphyton). Nevertheless a longer treatment time or a post-sampling might have led to more hints on an endocrine effect as six weeks after the end of the study very high densities of *P. antipodarum* were observed in the former 20 mg·L^{-1} treatments. Therefore, a longer study period and observation seem to be necessary.

6.6 Conclusion

Based on the data of this microcosm study it is possible that the application of high concentrations of VectoBac® TP, or the accidental release of high amounts, to an aquatic fresh water system might have an impact on the community structure of algae and invertebrates. But a shift in the ecosystem structure in routinely treated areas seems unlikely, since maximum concentrations of VectoBac® TP utilized for mosquito control in the investigated areas do not exceed 0.4 $mg \cdot L^{-1}$ (personal communication with Dr. Norbert Becker, German Association for Mosquito Control, Speyer, Germany) and therefore are 10 respective 50 times lower than the concentrations used in this study.

Acknowledgement

The authors would like to thank the Landesamt für Umwelt, Messungen und Naturschutz Baden-Württemberg (LUBW) for funding the present study. Furthermore, we would like to thank the German Association for Mosquito Control (GAMC), namely Dr. Norbert Becker, as well as Valent Biosciences for their cooperation and providing of the active ingredients.

Chapter 7

Effectiveness of advanced sewage treatment processes to eliminate endocrine activity of hospital effluents – Applicability of three *in vitro* assays

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This chapter has been published in *Water Research*

7.1 Abstract

A broad range of anthropogenic micropollutants enters the aquatic ecosystems via various paths. The main sources are sewage effluents, industrial plants, agricultural holdings and surface runoff. Among those micropollutants the class of pharmaceuticals is of increasing concern. Due to the possible adverse effects on wildlife and humans, degradation and removal of pharmaceuticals and their metabolites during wastewater treatment is an important task. The here presented study was part of a proof of concept study at a medium sized country hospital in western Germany that investigated efficiency of advanced treatment processes to remove toxic potencies from highly contaminated sewage prior to discharge in the public sewerage system. Specifically, the efficiency of the advanced treatment processes membrane bioreactor (MBR) and ozonation to remove endocrine disruptive potentials was assessed. Estrogenic effects were characterized with the two receptor-reporter *in vitro* transactivation assays, Lyticase Yeast Estrogen Screen (LYES) and Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER CALUX®). In addition, the H295R Steroidogenesis Assay (H295R) was utilized to detect potential disruption of steroidogenesis. For raw sewage estrogenic activity (determined as estrogen equivalents (EEQs)) up to 35.8±8.7 ng·L^{-1} EEQ (1x concentration) was determined by use of the LYES. After treatment by MBR and ozone the activity was significantly reduced to 1.2±0.4 ng·L^{-1} EEQ and 2.3±0.3 ng·L^{-1} EEQ, respectively. Results were confirmed by use of ER CALUX® which detected activities of 0.2±0.11 ng·L^{-1} EEQ (MBR) and 0.01±0.02 ng·L^{-1} EEQ (ozonation). In contrast, treatment with ozone resulted in higher estradiol production and aromatase activity at 3x and higher concentrations in H295R cells. It is hypothesized that this is partly due to formation of active by-products during ozonation. Substance-specific analyses demonstrated efficient removal of most of the measured estrogenic compounds by ozonation. A comparison of the ER-mediated responses measured by use of the LYES and ER CALUX® with those from the chemical analysis using a mass-balance approach revealed estrone (E1) to be the main compound that caused the estrogenic effects. Overall, treatment of sewage by use of MBR successfully reduced estrogenicity of hospital effluents. However, after ozonation, effluents should undergo further investigations regarding the formation of endocrine active metabolites. The results obtained as part of this study demonstrated applicability of the used *in vitro* assays for monitoring of endocrine-modulating potency of treated sewage.

Keywords: membrane bioreactor, ozonation, endocrine disruption, H295R, LYES, ER CALUX®

7.2 Introduction

Pharmaceuticals are designed to cure and prevent diseases as well as to enhance quality of life for humans and domestic animals. However, beside their beneficial properties these substances bear the risk of unintended harmful effects on non-target organisms in the environment and on humans. Chronic exposures as well as the presence of mixtures of pharmaceuticals and their metabolites in effluents might pose risks to aquatic organisms. Of particular toxicological concern are pharmaceuticals that were designed to exert their biological activity at small concentrations, like the synthetic hormone 17α-ethinylestradiol (EE2) (Caliman & Gavrilescu 2009, Cooper et al. 2008, Escher et al. 2011).

The two main sources of pharmaceuticals in the environment are municipal sewage treatment plants (STPs) and livestock breeding in agriculture. In addition, effluents from industrial plants, surface runoff and hospitals are known to be sources for pharmaceuticals in aquatic ecosystems (Escher et al. 2011, Fent et al. 2006, Fick et al. 2010, Moltmann et al. 2007, Ort et al. 2010, Sim et al. 2011, Ternes et al. 2004, Verlicchi et al. 2010).

Previous studies have demonstrated that elimination of pharmaceuticals during conventional wastewater treatment is not always sufficient (Daughton & Ternes 1999, Heberer 2002, Kasprzyk-Hordern et al. 2009, Ternes et al. 1999, Zwiener et al. 2001): In STP effluents concentrations of EE2 as great as 42 $ng \cdot L^{-1}$ have been detected (Ying et al. 2002). In general, median concentrations do not exceed 3 $ng \cdot L^{-1}$ (Heberer 2002) but due to the fact that the lowest observed effect concentrations for aquatic organisms are as little as 1 $ng \cdot L^{-1}$ (endpoint: reproduction of Pimephales promelas) (Caldwell et al. 2008) there is the potential for adverse effects on certain biota in receiving water bodies. For a few smaller rivers the sewage proportion sums up to 90 % in dry season (Brooks et al. 2006).

Therefore, different secondary wastewater treatment steps, such as membrane bioreactors (MBR), adsorption techniques like activated charcoal (AC) and advanced oxidation processes like ozonation (O3), are currently being evaluated with respect to their efficiency to reduce concentrations of micropollutants in STP effluents. The MBR technique combines biological sewage treatment with membrane filtration (Chapman et al. 2004) which has been shown to significantly decrease concentrations of most of the tested pharmaceuticals. Ozone reacts directly over oxidation or via hydroxyl radicals with micropollutants (Huber et al. 2005, Huber et al. 2004) which have been shown to be efficient chemical pathways to remove the estrogenic potential of pharmaceuticals from waste-water (Gunnarsson et al. 2009, Hu et al.

2007, Laven et al. 2009, Rosal et al. 2010, Snyder et al. 2007, Stalter et al. 2010a, Stalter et al. 2010b, Zwiener & Frimmel 2000, Zwiener et al. 2002). However, it has been also demonstrated that treatment with ozone can form toxic transformation products (Petala et al. 2008, Stalter et al. 2010a, Stalter et al. 2010b).

The present study is part of a larger proof of concept study to evaluate effectiveness of advanced sewage treatment methods with regard to the removal of pharmaceuticals from hospital sewage before discharge into the public sewerage. Specifically, the efficiency of a full scale membrane bioreactor (MBR) was investigated. In addition, four different advanced treatment methods – nanofiltration (NF), reverse osmosis (RO), ozonation (O_3) and granulated activated carbon filtration (GAC) – were adopted (Pinnekamp et al. 2009).

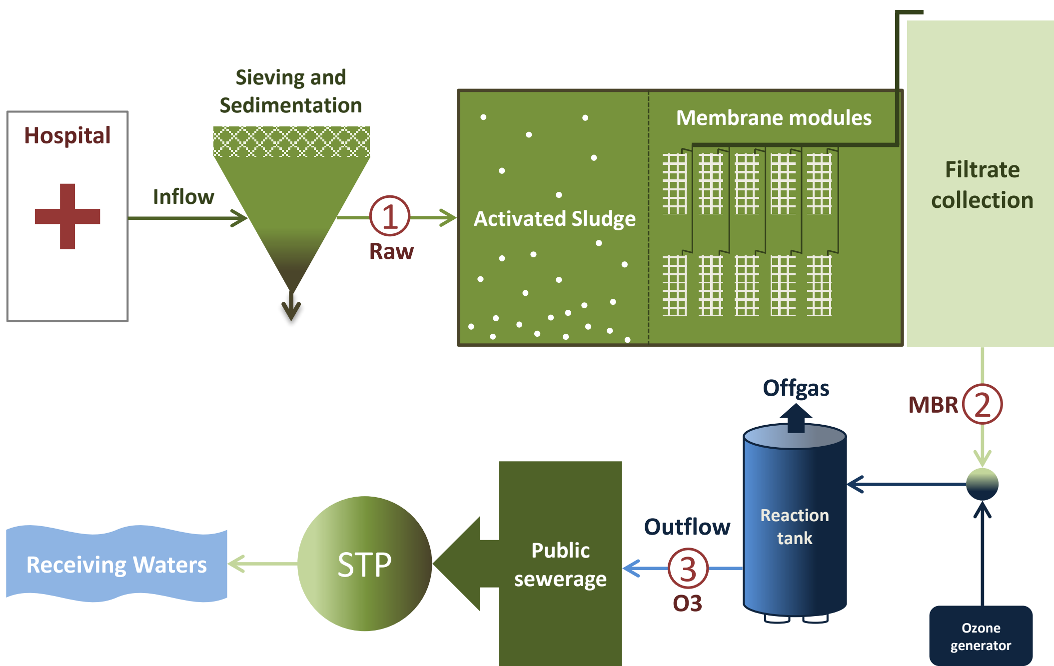


Figure 7.1 Water treatment cycle at Hospital and city of Waldbröhl. MBR=membrane bioreactor; NF / RO=nanofiltration / reverse osmosis; O3=Ozonation; GAK=granulated active carbon; STP=municipal sewage treatment plant; 1-3=sampling sites for testing of endocrine activity. (redrawn and adapted from(Pinnekamp et al. 2009)).

The objective of this study was to determine efficiency of the MBR and the subsequent ozonation to remove endocrine-active chemicals from hospital effluents (Fig. 7.1). Endocrine activities were assessed by use of three *in vitro* bioassays: Two receptor-mediated, transactivation assays, the Lyticase Yeast Estrogen Screen (LYES) and Estrogen Receptor mediated Chemical Activated LUciferase gene eXpression (ER CALUX®) assay, and the H295R Steroidogenesis Assay (H295R) that detects effects on the steroid synthesis pathway (see

also chapter 1.4.3.2 and 1.5). Previous studies have determined the applicability of the selected assays for the evaluation of endocrine activity in (waste-)water samples (Gracia et al. 2008, Hecker & Hollert 2009, Kase et al. 2009, Leusch 2008, Leusch et al. 2010). As previously reviewed by Hecker & Hollert (2011) combined use of receptor-mediated and non-receptor-mediated-methods is necessary to enable an objective assessment of endocrine potentials in complex samples. Grund et al (Grund et al. 2011) demonstrated that the combination of receptor-mediated and non-receptor-mediated assays such as the LYES and the H295R was appropriate for a holistic evaluation of potential endocrine activity of complex environmental samples. Furthermore, *in vitro* assays for detection of estrogen receptor binding are part of the Endocrine Disruptor Screening Program (EDSP; US Environmental Protection Agency) as well as the OECD Conceptual Framework for the Testing and Assessment of Endocrine Disrupting Chemicals. This also applies to the H295R Steroidogenesis Assay (Hecker et al. 2011). Both LYES and ER CALUX® are currently part of a survey of the German Institute for Standardization (DIN, NAW 119), whereas the H295R has recently been validated as an OECD test guideline (OECD 456).

7.3 Material & Methods

7.3.1 Chemicals

All chemicals were at least reagent grade and purchased from Sigma Aldrich (Schnelldorf, Germany). The SteadyLite® kit was purchased from Perkin Elmer (Wiesbaden, Germany). Cell culture media were obtained from Sigma Aldrich (Schnelldorf, Germany), Invitrogen (Darmstadt, Germany) and Otto Nordwald (Hamburg, Germany). Standards for the implemented bioassays were prepared by the actual laboratory in charge.

7.3.2 Sewage treatment methods

The sewage treatment plant was equipped with mechanical pre-treatment, aeration and nitrification and installed directly at a middle sized county hospital in western Germany. Membranes of the bioreactor were directly included in the activated sludge basin which ensured a microfiltration of the biologically treated sewage. The filtration basin contained 5 membrane modules with an overall surface of 1600 m^2, which was connected to the activated sludge tank (Pinnekamp et al. 2009). Three MBR samples (A, B, C) were collected directly before ozonation. Subsequently to MBR treatment, the waste water was treated with two concentrations of ozone over different duration times: AO (7.5 $mg \cdot L^{-1}$ / 7.5 min), BO (7.5 $mg \cdot L^{-1}$ / 15 min) and CO (12.5 $mg \cdot L^{-1}$ / 7.5 min) (Pinnekamp 2009). Ozone was produced on-site by a generator using air feed gas (ITT WEDECO, OCS GSO 30 / AS 12) and afterwards injected into the MBR-treated wastewater stream. Ozone destruction in the off-gas was achieved by a catalytic ozone destructor. Ozone was measured on-site using mobile ozone analyzers (Orbisphere 410 / 510, Hach, Germany). Directly after ozonation three samples with different ozone concentrations and duration times (AO, BO, CO) were collected.

7.3.3 Sampling

Samples of sewage were collected by personnel of the Institute of Environmental Engineering (ISA), RWTH Aachen University, Germany. Sampling was conducted with cooled automatic sampling devices. Composite samples from the membrane bioreactor as well as untreated sewage samples were collected flow-proportionally over a period of 24 h on three different dates (A, B, C). Due to the operational mode of the ozonation unit, samples after this treatment step were collected over a period of 8 h (AO, BO, CO). Based

on the experience from previous investigations the difference in sampling durations of pre- and post-treatment samples (24 hours versus 8 hours) should not significantly affect the sample composition (Pinnekamp 2009).

7.3.4 Preparation of extracts

Samples were extracted by use of solid phase extraction (SPE) with Oasis HLB cartridges (Waters) according to previously described methods by Bratkowska et al. (Bratkowska et al. 2010) and Cahill et al. (Cahill et al. 2004) at the ISA. Briefly 2000 ml of treated sewage were concentrated by passing approximately one third of the sample through one of three separate cartridges and dried under a constant stream of nitrogen. For untreated sewage 500 ml were extracted. Extracted substances were desorbed from cartridges with methanol and pooled. Extracts were dried under a gentle stream of nitrogen and redissolved in ethanol for dosing in bioassays and in methanol for chemical analyses (Pinnekamp et al. 2009). Final concentrations of original samples in the resulting extracts were 1,000-fold for the raw sewage and 2,000-fold for treated samples. Prior to use in assays samples were stored at -20°C. Because of the relatively large dilution factor of 1,000-fold for the ER CALUX® and H295R assays, extracts were concentrated as much as 33,000-fold by repeated drying under nitrogen and reconstitution in a smaller amount of solvent.

7.3.5 LYES

For principle and general implementation of the LYES see chapter 1.4.3.2 and 1.5.1 as well as chapter 2.2.2. In the present investigation, yeast cells were exposed to a dilution series (33x, 10x, 3.3x, 1x, 0.33x and 0.1x concentration) of the extracted waste water samples. Samples were at least tested in independent duplicates.

7.3.6 ER CALUX®

For principle and general implementation of the ER CALUX® see chapter 1.4.3.2 and 1.5.2 as well as chapter 2.2.3. In the present investigation, cells were exposed to a dilution series (33x, 10x, 3.3x, 1x, 0.33x and 0.1x concentration) of the extracted waste water samples. Samples were tested in independent duplicates.

7.3.7 H295R

For principle and general implementation of the H295R see chapter 1.4.3.2 as well as chapter 2.2.4. In the present investigation, the cells were exposed to a dilution series (33x, 10x, 3.3x, 1x, 0.33x and 0.1x concentration) of the extracted waste water samples as well as the two reference substances Forskolin and Prochloraz. The amount of estradiol (E2) and testosterone (T) was determined in an enzyme-linked immunosorbent assay (ELISA). Furthermore, Aromatase enzyme activity was measured using a tritiated water (^{3}H)-release assay. Due to the small amount of the samples only one measurement with three internal replicates could be conducted.

7.3.7.1 ^{3}H-release assay for measurement of aromatase activity

The ^{3}H-release assay was conducted according to Higley et al. (2010). After incubation to the samples for 48 h cells were washed twice with 250 µl PBS. Subsequently, 250 µl supplemented medium containing 54 nM 1β-^{3}[H]-androstenedione (Perkin Elmer, Boston, USA) were added to each well. Cells were incubated at 37°C under a 5 % CO_2 in air atmosphere for 1.5 h. To stop the reaction plates were put onto ice for 5 min. 200 µl medium per well were transferred to a 1.5 ml vial (Eppendorf, Hamburg, Germany) containing 500 µl chloroform. Each vial was vortexed for 15 s and spun at 11 rpm for 2 min. 100 µl of each supernatant were transferred to 1.5 ml vials containing 100 µl dextran coated charcoal. The vials were vortexed for 15 seconds and allowed to stand for 5 min. Subsequently each vial was spun at 11 rpm for 15 minutes. 125 µl of the supernatants were transferred to scintillation vials (Research Products International Corp., Mount Prospect, USA) containing 4 ml scintillation cocktail (Bio-Safe II, Research Products International Corp., Mount Prospect, USA). The activity was counted for 10 min in a scintillation counter (LS 6500 Multipurpose SC, Beckman Coulter, Brea, USA).

7.3.8 Viability Assay

A MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay (see chapter 2.2.1) was conducted to assure that the test items did not cause non-specific cytotoxic effects in mammalian cell lines.

7.3.9 Substance specific analysis

Parallel to the biological analysis samples were screened for selected estrogenic chemicals. The following compounds were quantified by means of liquid chromatography-tandem mass spectrometry (LC-MS^2): estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), 17ß-estradiol acetate (E2-ac), estriol (E3), bisphenol A (BPA), nonylphenol (t-NP) and medroxyprogesterone acetate (MPro-ac). E1, E2 and E3 are the three main estrogens that are produced by humans. EE2, MPro-ac and E2-ac are applied in hormonal therapy. T-NP and BPA are xenobiotics with endocrine activity. All analyses were conducted by use of a LTQ Orbitrap hybrid mass spectrometer (Thermo Electron) in the ESI(±)-MS and MSn modes (Gebhardt & Schröder 2007).

EEQs for the chemical analytical data (chemEEQs) were calculated based on the substance-specific relative estrogenic potencies (REP) for the above-described compounds determined in previous studies with the Yeast Estrogen Screen (YES), the LYES and the ER CALUX® (Tab. 7.1).

Table 7.1 Relative estrogenic potencies (REP) determined with the Lyticase Yeast Estrogen Screen (LYES) and the Yeast Estrogen Screen (YES) as well as the ER CALUX®

	Substance							
	E1	E2	E2-ac	EE2	E3	BPA	t-NP	MPro-ac
REP Yeast Screens	1.3E-01[a]	1	n.a.	7.3E-01[a]	n.a.	1.2E-04[b]	1.1E-05[a]	n.a.
REP ER CALUX®	1.2E-01[c]	1	n.a.	1.12[c]	1.3E-01[c]	1.13E-05[c]	3.7E-05[c]	n.a.

[a]Schultis & Metzger (2004), [b]Beck et al. (2006), [c]Houtman et al.(2004); n.a.=data not available; E1=estrone; E2=17β-estradiol; E2-ac=17ß-estradiol acetate; EE2=17α-ethinylestradiol; E3=estriol; BPA=bisphenol A; t-NP=nonylphenol; MPro-ac=medroxyprogesterone acetate.

7.3.10 Statistical analysis

Data were analyzed statistically by use of SigmaStat 3.5 (Systat Software, San José, USA). Results are presented as mean ± standard deviation (SD) or standard error of the mean (SEM). To enhance the comparability of the assays results were normalized to the average value of the solvent controls (SC) and are expressed as fold change relative to the SC. All data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene’s test. For data fulfilling the parametric assumptions a one way analysis of variance (ANOVA) followed by Dunnett’s post hoc test was used to determine treatments that differed significantly from the SC. When data did not meet parametric

assumptions, the non-parametric Kruskall-Wallis test followed by Dunn’s post hoc test was used. Differences were considered significant at $p<0.05$. To set a threshold (minimum fold change) from which the inductions are considered for evaluation the coefficient of variation (cv) was calculated for each assay. Threshold for the ER CALUX® and H295R was set to 1.2, for the LYES to 1.3.

7.4. Results

7.4.1 LYES

There were significant differences in the estrogenic potentials among sewage extracts from the different treatment groups when measured by use of the LYES assay (Fig. 7.2).

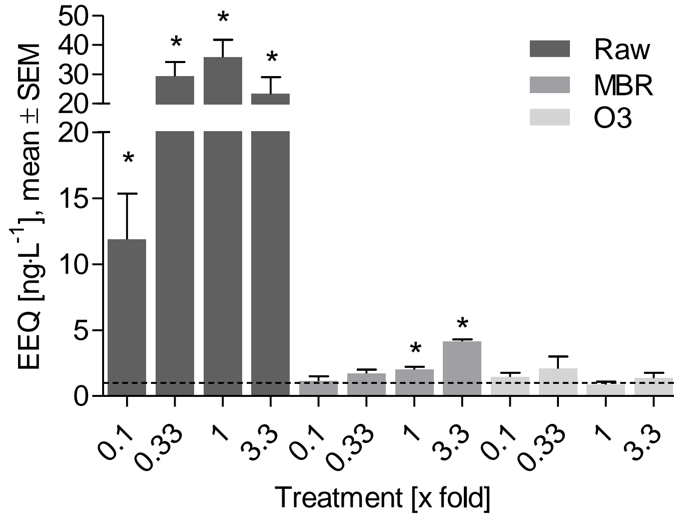
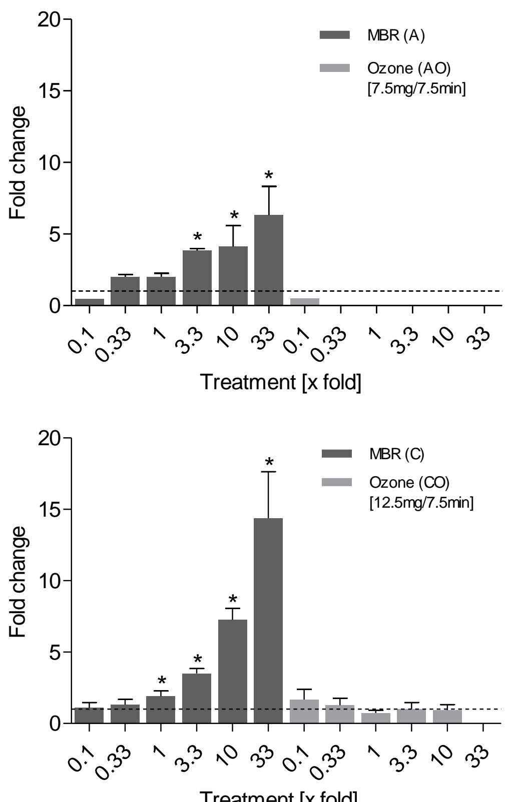


Figure 7.2 Comparison of the Estradiol Equivalents (EEQs) of the three different sewage samples Raw (raw sewage before treatment), MBR (sewage after membrane bioreactor treatment) and O3 (sewage after MBR and ozone treatment) in the LYES. Bars represent mean values of three independent sampling dates with SEM (error bars); * = significant differences to the SC ($p<0.05$).

Extracted raw sewage showed the highest estrogenic activity, which was 35.8±8.7 ng·L^{-1} in the 1x concentration (concentration of the native raw sewage). Nevertheless, raw sewage concentrations greater or equal to 1x caused significant cytotoxicity to yeast cells, and therefore, were excluded from further investigations.

Samples subjected to MBR exhibited a significant, concentration-dependent increase in estrogen potency (Fig. 7.3). However, this response was significantly less than that in untreated sewage. Estrogenicity measured in the 1x concentration of MBR effluents was 2.3±0.4 ng·L^{-1} (A), 1.6±0.6 ng·L^{-1} (B) and 2.1±0.6 ng·L^{-1} (C). Compared to the solvent controls (SC) the 1x concentration caused 2-fold inductions of estrogen potency. Significant differences to the SC were detected after exposure of cells to 33x, 10x, 3.3x and 1x concentrations of samples B and C, respectively. For sample A significant differences were detected for concentrations between 3.3x and 33x.



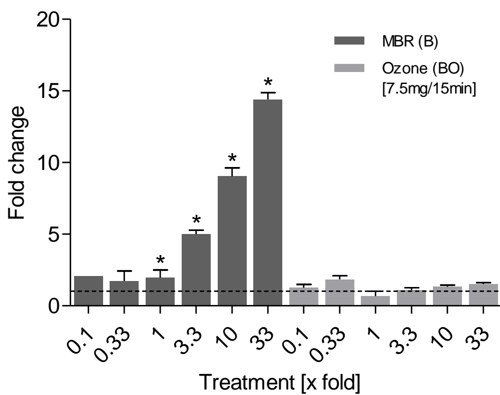


Figure 7.3 Changes of ß-galactosidase production relative to solvent controls (SC = 1, - - -) in the LYES. MBR = membrane bioreactor; A, B, C = samples after MBR treatment; AO, BO, CO = samples after ozonation; 0.1 to 33-fold = concentration of the native sewage. Bars represent mean values of two independent experiments with SEM (error bars); missing bars represent not detectable data points; * = significant differences to the SC ($p<0.05$).

After ozonation, estrogenic activity in all extracts decreased to less than 3 $ng \cdot L^{-1}$ EEQ. No significant induction of an estrogenic effect was observed at any of the extract concentrations of samples AO, BO and CO.

7.4.2 ER CALUX®

The ER CALUX® assay generally confirmed estrogenic responses detected by the LYES screen. All MBR treated samples showed a statistically significant increase in estrogenicity at concentrations greater or equal to 3.3x (Fig. 7.4).

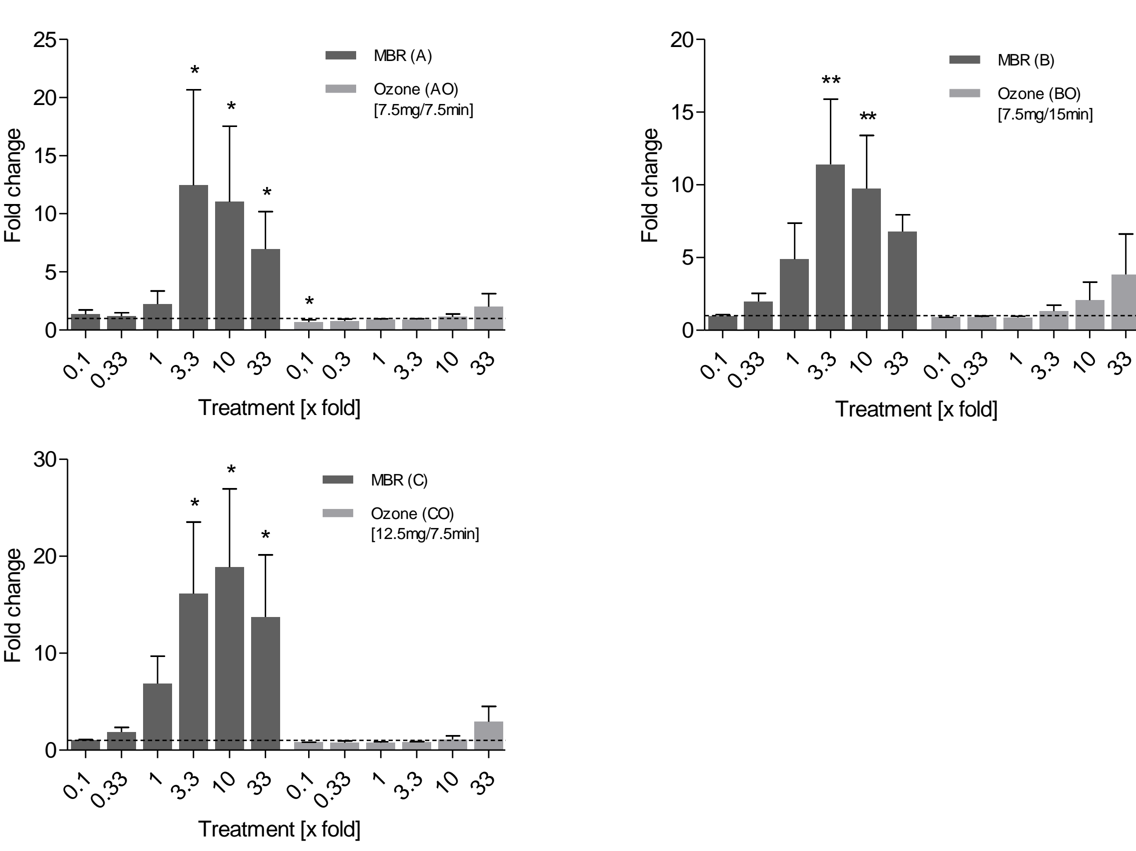


Figure 7.4 Changes of luciferase production relative to solvent controls (SC = 1, - - -) in the ER CALUX®. MBR = membrane bioreactor; A, B, C = samples after MBR treatment; AO, BO, CO = samples after ozonation; 0.1 to 33-fold = concentration of the native sewage. Bars represent mean values of two independent experiments with SEM (error bars); * = significant differences to the SC ($p<0.05$); ** = significant differences to the SC ($p<0.01$).

However, there were no concentration-dependent increases in response at greater concentrations tested, and there appeared to be a trend towards lesser estrogenicity at the greatest concentration (33x) tested. Estrogenic activity as measured in the 1x extract was between 0.37±0.09 and 1.23±0.24 ng·L-1 corresponding to average inductions between 2.2- and 6.9-fold.

There were no statistically significant inductions of estrogenicity after exposure to any of the extracts or dilutions tested for the ozone-treated samples, and EEQs were mostly below the detection limit of the assay (3.4x10-2 ng·L^{-1}) (Fig. 7.4). At the highest

concentrations (33x) there was a trend towards greater EEQs with values between 2.82±0.82 and 4.57±0.34 ng·L^{-1} and induction factors between 2.0 and 3.8-fold. The decreasing trend at greater concentrations after MBR treatment was no longer observed.

7.4.3 H295R

For the two highest concentrations no cytotoxicity could be detected in any sample

Exposure to the MBR samples resulted in significant dose-dependent increases in production of E2 (Fig. 7.5).

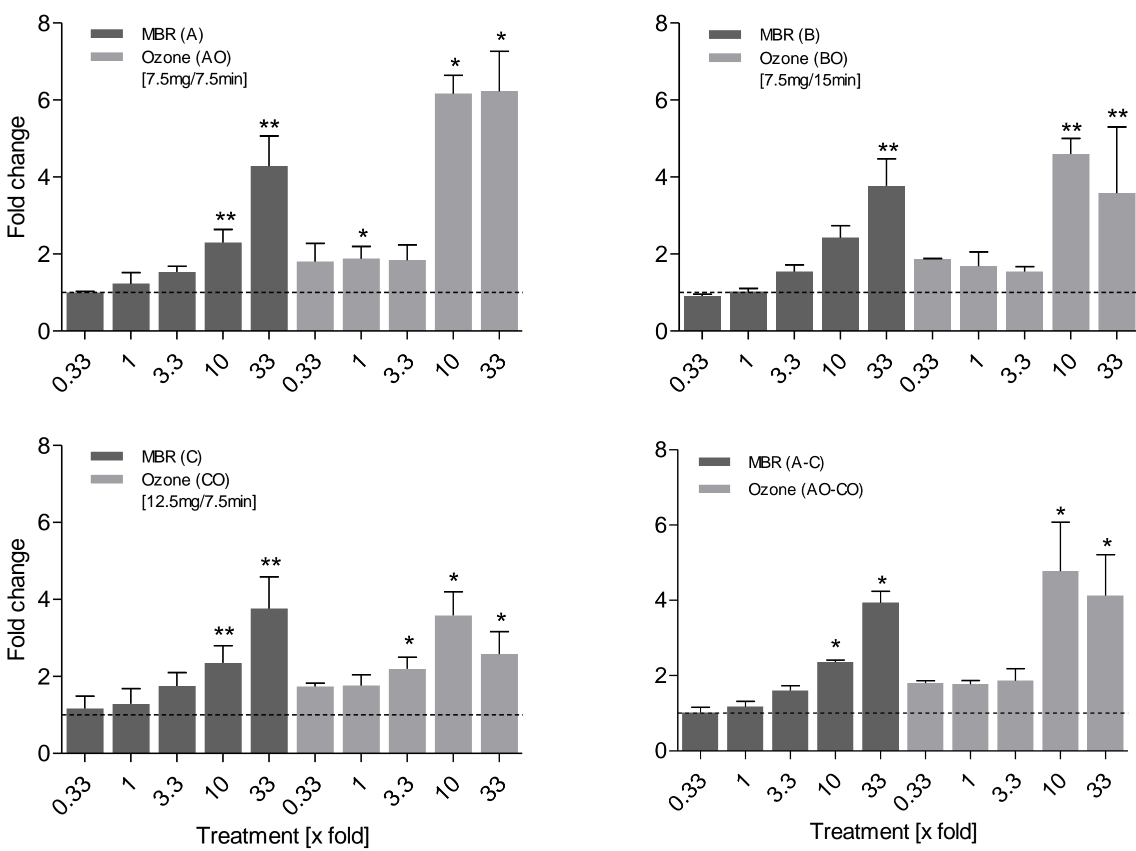


Figure 7.5 Changes in estradiol (E2) production relative to solvent controls (SC = 1, - - -) in the H295R. MBR = membrane bioreactor; A, B, C = samples after MBR treatment; AO, BO, CO = samples after ozonation; 0.33 to 33-fold = concentration of the native sewage. Bars represent mean values of one independent experiment with SD or SEM (error bars); * = significant differences to the SC ($p<0.05$); ** = significant differences to the SC ($p<0.01$).

In contrast to the estrogen receptor-mediated responses ozonation did not further ameliorate endocrine-modulating potential but resulted in greater production of E2 at to some extent even lesser concentrations with LOECS of 10x and 3.3x for samples A, B and C.

Furthermore, significant increases in fold change of E2 were observed at the 10x concentrations in the ozone treated effluent samples when compared to those only subjected to the MBR.

Similar trends could be observed for aromatase activity (Fig. 7.6). Up to concentrations of 3.3x no significant differences between MBR treated samples and samples treated by MBR and ozone occurred. At concentrations of 10-fold and greater aromatase activity significantly increased in the ozone treatment group. However, the overall induction was relatively low with a maximum induction of 2-fold.

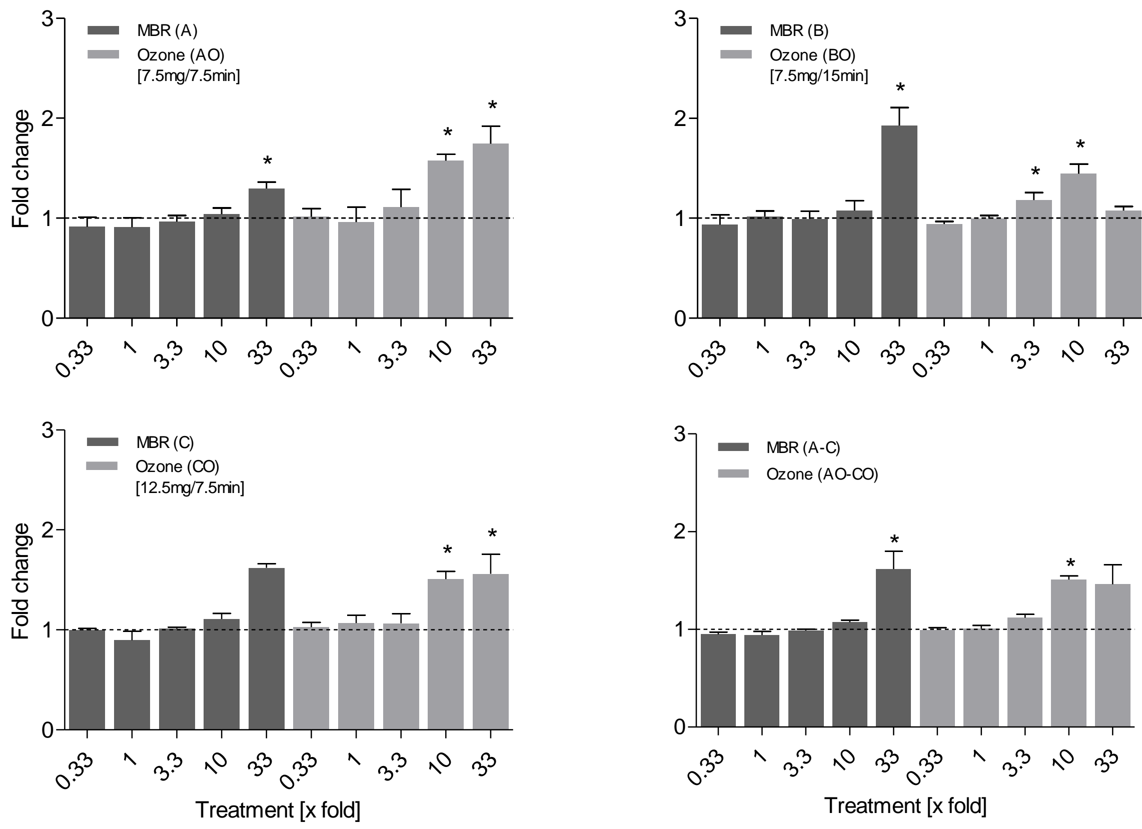


Figure 7.6 Changes of aromatase activity relative to solvent controls (SC = 1, - - -) in the H295R. MBR=membrane bioreactor; A, B, C = samples after MBR treatment; AO, BO, CO = samples after ozonation; 0.33 to 33-fold = concentration of the native sewage. Bars represent mean values of two independent experiments with SEM (error bars); * = significant differences to the SC ($p<0.05$).

The effect of the MBR samples on the production of T was weak but partly significant (Fig. 7.7). The lowest concentration caused significant increases of T production in sample A and B. For sample B even the one fold concentration had a significant effect. Therefore, the LOEC of the tested samples is 0.33 fold. Nevertheless, beginning with the 3.3 fold

concentration the effect seems to be reversed. For the ozonated samples only the highest concentration of sample C showed a significant decrease in T production.

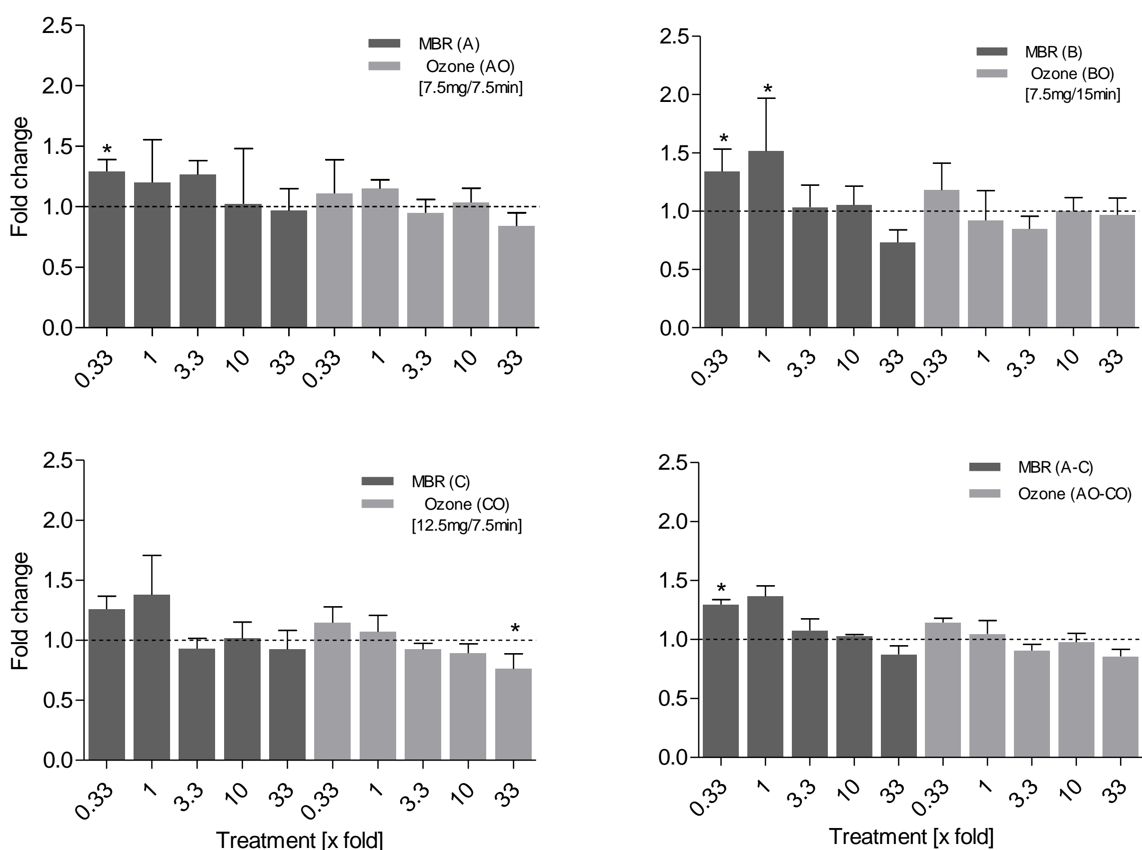


Figure 7.7 Changes in testosterone (T) production relative to solvent controls (SC = 1, - - -) in the H295R. MBR=membrane bioreactor; A, B, C = samples after MBR treatment; AO, BO, CO = samples after ozonation; 0.33 to 33-fold = concentration of the native sewage. Bars represent mean values of two independent experiments with SEM (error bars); * = significant differences to the SC ($p<0.05$).

7.4.4 Substance specific analysis

There were differences in concentrations of investigated substances as a function of treatment type (Tab. 7.2). E2, EE2 and E3 could not be detected either in influents or effluents of the ozone reactor (signals were less than the limit of quantification (LOQ)).

Concentrations of E1 were reduced from values between 12.15 $ng \cdot L^{-1}$ and 18.24 $ng \cdot L^{-1}$ in MBR samples to concentrations less than the LOQ by ozone treatment. E2-ac was not found either in the influents of MBR A and MBR B, or the effluents of AO and BO. In the CO treatment group E2-ac levels were reduced by 39 %. t-NP was reduced by 3 % in the AO treatment group. However, after being subjected to BO and CO concentrations of t-NP

decreased by 44 % and 35 %, respectively. Concentrations of MPro-ac were reduced by 95 %, 96 % and 77 % after treatment by AO, BO and CO, respectively. In contrast, BPA concentrations increased by 723 % and 822 % in the AO and BO treatment groups but were reduced concentrations less than the LOQ in the CO experiment.

Table 7.2 Concentrations of selected substances in hospital sewage water before (MBR) and after various ozone treatments (AO, BO, CO) (1xconcentration; values represent mean values of 3 replicates; < indicates the limit of quantification) (Pinnekamp et al. 2009)

Treatment	Substance [ng·L^{-1}]							
	E1	E2	E2-ac	EE2	E3	BPA	t-NP	MPro-ac
MBR A	18.24	< 5	< 5	< 5	< 5	40.67	1347.73	127.46
AO_a	< 5	< 5	< 5	< 5	< 5	293.83	1308.9	6.66
MBR B	16.6	< 5	< 5	< 5	< 5	36.96	1644.3	139.85
BO_b	< 5	< 5	< 5	< 5	< 5	303.71	912.83	5.86
MBR C	12.15	< 5	16.06	< 5	< 5	28.89	1632.78	56.33
CO_c	< 5	< 5	9.87	< 5	< 5	< 15	1054.01	13.05

a(7.5 mg·L^{-1} / 7.5 min), b(7.5 mg·L^{-1} / 15 min), c(12.5 mg·L^{-1} / 7.5 min)
E1 = estrone; E2 = 17β-estradiol; E2-ac = 17ß-estradiol acetate; EE2 = 17α-ethinylestradiol; E3 = estriol; BPA = bisphenol A; t-NP = nonylphenol; MPro-ac = medroxyprogesterone acetate

Regarding MBR treated samples, calculated chemical EEQs (Tab. 7.3 a) based on results from previous studies with the LYES / YES and the analytical data were almost similar to EEQs calculated in this study with the LYES (1-fold concentration = 2.0±0.2 ng·L^{-1} EEQ). E1 was the main substance that contributed to the theoretical EEQ. For BPA and t-NP only low theoretical EEQs were calculated. Concentrations of E2 and EE2 were both less than the LOQ. No data was available for E2-ac, E3 and MPro-ac. In case of the ozonated samples theoretical EEQs were lower than EEQs calculated with the LYES (1-fold concentration = 0.9±0.2 ng·L^{-1} EEQ). Only BPA and t-NP could contribute to the theoretical EEQs.

Regarding MBR treated samples, calculated chemical EEQs (Tab. 7.3 b) based on results from previous studies with the ER CALUX® and the analytical data were higher than EEQs calculated in this study with the ER CALUX® (1-fold concentration = 0.8±0.3 ng·L^{-1} EEQ). Again E1 was the main substance that contributed to the theoretical EEQs. For BPA and t-NP only low theoretical EEQs were calculated. Concentrations of E2, EE2 and E3 were less than the LOQ. No data was available for E2-ac and MPro-ac. In case of the ozonated samples theoretical EEQs were in the same range as EEQs calculated with the ER CALUX®

(1-fold concentration = 0.02±0.03 ng·L^{-1} EEQ). Again only BPA and t-NP could contribute to the theoretical EEQs.

Table 7.3 Calculated[a] chemical estradiol equivalents (chemEEQs) in hospital sewage samples

(a) LYES, 1-fold concentration

Treatment	Substance [ng·L^{-1}]								Total
	E1	E2	E2-ac	EE2	E3	BPA	t-NP	MPro-ac	
MBR A	2.371	< 5	n.a.	< 3.65	n.a.	0.005	0.015	n.a.	2.391
AO	< 0.65	< 5	n.a.	< 3.65	n.a.	0.035	0.014	n.a.	0.049
MBR B	2.158	< 5	n.a.	< 3.65	n.a.	0.004	0.018	n.a.	2.18
BO	< 0.65	< 5	n.a.	< 3.65	n.a.	0.036	0.01	n.a.	0.046
MBR C	1.58	< 5	n.a.	< 3.65	n.a.	0.003	0.018	n.a.	1.601
CO	< 0.65	< 5	n.a.	< 3.65	n.a.	< 0.002	0.012	n.a.	0.012
MBR A-C									2.057
AO-CO									0.036

(b) ER CALUX®, 1-fold concentration

Treatment	Substance [ng·L^{-1}]								Total
	E1	E2	E2-ac	EE2	E3	BPA	t-NP	MPro-ac	
MBR A	2.19	< 5	n.a.	< 9.2	< 0.18	0,0005	0,050	n.a.	2,24
AO	< 0.08	< 5	n.a.	< 9.2	< 0.18	0,003	0,048	n.a.	0,05
MBR B	1.99	< 5	n.a.	< 9.2	< 0.18	0,0004	0,061	n.a.	2,05
BO	< 0.08	< 5	n.a.	< 9.2	< 0.18	0,003	0,034	n.a.	0,04
MBR C	1.46	< 5	n.a.	< 9.2	< 0.18	0,0003	0,060	n.a.	1,52
CO	< 0.08	< 5	n.a.	< 9.2	< 0.18	< 0.0007	0,039	n.a.	0,04
MBR A-C									1,94
AO-CO									0,04

[a]Calculated EEQ = Relative estrogenic potency x concentration [ng·L^{-1}]; [b]"Total" calculated only from values that lay above the limit of quantification; n.a. = data not available; E1 = estrone; E2 = 17β-estradiol; E2-ac = 17ß-estradiol acetate; EE2 = 17α-ethinylestradiol; E3 = estriol; BPA = bisphenol A; t-NP = nonylphenol; MPro-ac = medroxyprogesterone acetate

7.5 Discussion

Advanced waste-water treatment with MBR and ozonation significantly ameliorated estrogen potencies of hospital sewage effluents. Significant original estrogen activity of untreated sewage was mainly reduced after treatment by MBR as determined both by the LYES and ER CALUX®. These findings are consistent with those of several studies that focused on removal of estrogen agonists through MBR treatment (Coleman et al. 2009, Holbrook et al. 2002, Hu et al. 2007). Advantages of this technique with respect to the removal of endocrine disrupting compounds (EDCs) are (1) the ability to act as a barrier to solids which usually would bind a large number of EDCs, (2) the retention capacity of the membrane itself, and (3) the extended sludge retention duration which gives time for additional biological degradation (Caliman & Gavrilescu 2009). Subsequent treatment with ozone led to a further decrease of estrogenic activity as determined with the LYES and ER CALUX® assays (EEQ < 1 $ng \cdot L^{-1}$). This is likely due to the fact that ozonation oxidizes phenolic moieties, which are critical for binding of compounds like E2, EE2 or E1 to the ER (Caliman & Gavrilescu 2009, Huber et al. 2004, von Gunten 2003). Other authors found similar reductions of estrogenic activity after treatment with ozone (Ternes et al. 2003).

Both LYES and ER CALUX® were able to detect estrogenic activity at concentrations in the $ng \cdot L^{-1}$ range. However, the concentrations which the assays were able to detect differed. With a calculated EC_{50} of 1.8±0.5 ng $E2 \cdot L^{-1}$ the ER CALUX® was able to reliably detect very low concentrations of estrogenic active substances. For samples with higher concentrations of estrogenic active substances such as raw sewage extracts the LYES with a calculated EC_{50} of 9.4±0.4 ng $E2 \cdot L^{-1}$ was more suitable. These findings correspond with findings of other studies that investigated the applicability of estrogen receptor-based *in vitro* assays for the detection of estrogenic substances (Kase et al. 2009, Leusch 2008, Leusch et al. 2010). Concentrations of EEQs determined by use of the LYES and ER CALUX® varied by as much as 10-fold. Similar variations between these two assays have been described previously (Legler et al. 2002a, Leusch 2008, Leusch et al. 2010). There are several theories regarding causes for these differences. These include: (1) Mammalian cell lines such as T47Dluc are able to metabolize estrogenic active substances and therefore minimize their effects (Legler et al. 1999), yeasts are lacking this ability (Connolly et al. 2011). (2) The yeast cell wall can act as a barrier for certain substances (Legler et al. 2002a). In a complex sample this could also apply to substances that inhibit ER binding. (3) It has been hypothesized that anti-estrogens can

act over down regulation of the ER in mammalian cell lines and that this mechanism does not occur in yeast cells (Dudley et al. 2000). It has been shown that some of these inhibitory substances, for example the selective ER modulator tamoxifen, act as ER antagonists in the ER CALUX® and as agonists in the YES (Legler et al. 2002a).

The Yeast Estrogen Screen is a well-established and common *in vitro* assay for the detection of estrogenic active substances. Its advantages are the large data base for single substances as well as mixtures, that the test is easy to perform, relatively robust and comparatively cheap (Schultis & Metzger 2004). However, structural differences between yeast cell walls and cell membranes of animal cells have been hypothesized to cause differences in the permeability for some hazardous substances, thus, resulting in a potentially limited predictability to test systems using animal cells (Kase et al. 2009, Legler et al. 2002c). Furthermore, yeasts are not able to metabolize substances in the same way like some mammalian cell lines such as the here used T47D (Connolly et al. 2011, Legler et al. 1999). In addition, conventional YES systems have limited sensitivity as characterized by a relatively great lowest observed effect level (LOEL) of as much as 10 ng·L-1 E2 as well as a method quantification limit (MQL) of as much as 5 $ng \cdot L^{-1}$ (Kase et al. 2009, Leusch 2008, Leusch et al. 2010). Therefore, the modified LYES with a LOEC of 0.5 ng·L-1 (Schultis & Metzger 2004) was used in the present study. Whereas this version of the assay is significantly faster to perform and is up to 20-fold more sensitive than the conventional YES, there are only few chemicals and complex samples that have been tested so far. One issue that has been identified in context with all YES screens is their relatively great sensitivity to cytotoxic chemicals, which limits their utilization with certain compounds (Leusch et al. 2010). This issue could also be observed in the present study. But with regard to cytotoxicity of the raw sewage extract, the LYES was less sensitive than the ER CALUX® which could not be applied to extracted raw sewage at all. Furthermore, it was shown that the robustness of the LYES in the present study was limited due to high background activities of the negative control with tap water.

The ER CALUX® is a commercially available assay with a harmonized standard operating protocol and a profound database. Amongst others, the assay has been successfully used to predict the estrogenic activity of sewage as well as surface waters (Bogers et al. 2007, Lamoree et al. 2010, Leusch et al. 2010, Murk et al. 2002, Van der Linden et al. 2008). It is easy to perform, robust and highly sensitive (LOEC $<0.5\ ng \cdot L^{-1}$, MQL $0.1\ ng \cdot L^{-1}$) (Kase et al. 2009, Leusch 2008, Leusch et al. 2010). As a human cell line, the T47Dluc cells are able to

identify samples which have the potential to interact with the mammalian estrogen receptor. Nevertheless, the transferability to aquatic organisms like fish and invertebrates is not always given. For example, the potency of E1 is similar to that of E2 in fish *in vivo* whereas the potency of E2 in the ER CALUX® is greater than that of E1. The distinctions may partly be linked to species differences regarding transcription factors as well as the regulation of ER binding and transactivation (Legler et al. 2002b, Petit et al. 1997). In the present study the ER CALUX® assay was stable and detected estrogenic potentials with great sensitivity.

Though ozonation almost completely removed the estrogenic activity from the tested effluents, the H295R steroidogenesis assay indicated that ozonation could enhance the presence of compounds that stimulate endogenous estrogen production as well as aromatase activity. It is suggested that this could be due to generation of reactive metabolites. This clearly indicates that objective assessment of endocrine activity of effluents requires inclusion of endpoints other than estrogen receptor binding activity. To date, most *in vitro* assays for the detection of endocrine disruption focus on the steroid hormone receptors. Alteration of the endogenous synthesis pathways of sex steroids represents a particularly relevant mode of endocrine disruptors because induction of the formation of e.g. endogenous estrogens such as observed in this study has the potential to potentiate estrogenic effects by increasing the endogenous estrogen pool. Changes of testosterone production were only weak but partly significant. While the low extract concentrations of MBR treated sewage caused weak but significant increases of T production the effect seemed to be reversed in higher concentrations. But only the highest concentration of sample CO caused a significant decrease of T production. These outcomes correspond with the significant increase of aromatase and estradiol production after exposure to the higher concentrated samples. Nevertheless, as already mentioned the changes in T production were only weak and there was no clear dose-dependent relationship.

The H295R Steroidogenesis Assay has been shown to be a reliable and relatively fast screening method with the potential to predict effects to higher organizational levels (see also chapter 3) (Hecker et al. 2011). However, considering that the H295R cells are of human origin (as in case of the T47Dluc cells), transferability to aquatic organisms like fish and invertebrates is not always given (Ji et al. 2010, Villeneuve et al. 2007). The increase of

estradiol production after ozonation could partly be linked to the increasing aromatase activity. The fact that ozonation of effluent increased estradiol production and aromatase activity compared to the MBR only treatment indicates that active metabolites that can stimulate aromatase activity may have been formed. One possible mechanism would be the induction of hormone-responsive adenylate cyclases. This would result in the increase of intracellular cyclic adenosine monophosphate (cAMP), which binds and up-regulates genes with cAMP responsive elements, like CYP19A1 (Higley et al. 2010, Watanabe & Nakajin 2004). It is also possible, that metabolites and conjugates of aromatase inducers occurred in the MBR-treated sewage. Ozone might have oxidized those metabolites and conjugates back to the parent substances. However, further studies are required to investigate the specific mechanisms by which this stimulation occurred. Similar differential endocrine activities of environmental samples were reported by Grund et al. (Grund et al. 2011) who found endocrine effects in sediment samples that could not be detected in the LYES but in the H295R. Furthermore, Stalter et al. (Stalter et al. 2010a, Stalter et al. 2011, Stalter et al. 2010b) demonstrated in several studies that ozone treatment of sewage reduced the estrogenicity but had negative effects on the reproduction of the oligochaete worm (Lumbriculus variegatus) as well as the development of rainbow trout (Oncorhynchus mykiss) in the fish early life stage toxicity test (FELST) which possibly could be linked to the formation of toxic metabolites during ozonation. However, it should be noted that, due to the small number of replicates (one independent replicate with three internal replicates), results obtained in this study should be confirmed by further investigations with at least two independent replicates.

Despite the suboptimal limits of quantification, that make the interpretation of the results more difficult, several conclusions can be drawn from the substance-specific analysis. Sewage is a complex mixture of compounds and it is still too little known about potential cross effects that might have affected the ozonation process. For example, a greater quantity of anti-oxidants would influence ozonation and its elimination efficiency. The increase in concentrations of BPA in AO and BO (Tab. 1) might be attributed to dissolution of BPA from polycarbonate and polyvinyl chloride (PVC) plastic parts in the ozone reactor and pipes (Howdeshell et al. 2003) as the BPA concentration in the ozonated samples decreases with operation time of the reactor (BO>AO>CO). It is also possible, that metabolites of BPA

and conjugates occurred in the influents, but were not measured. Subsequently ozone might have oxidized those metabolites and conjugates back to BPA.

Based on the results obtained during this study it became obvious that compounds such as BPA and t-NP that were present at the greatest concentrations only contributed a small proportion of the biologically determined estrogenic activities measured with the bioassays (Tab. 3). In contrast, compounds for which concentrations were consistently less than the LOQ (E2, EE2) might have represented a significant proportion of observed concentrations of EEQs since the LOQs for E2 and EE2 were in a range where effects in bioassays and on wildlife are to be expected (Caldwell et al. 2008, Legler et al. 1999). E1 is the only compound that was detectable at significant concentrations, and for which also chemical EEQs (chem EEQs) could be calculated. Concentrations of EEQs as determined by the LYES (Fig. 3) were in a similar range and directly comparable to the chemEEQ values calculated for E1 based on the chemical equivalency factors for this compound (Tab. 7.3 a), indicating that the majority of EEQs measured by this assay can be explained by this hormone. No relative estrogenic potencies are available for E2-ac and MPro-ac. E3 concentrations were less than the LOQ. Otherwise, calculated concentrations of chemEEQ as determined by use of the ER CALUX® (Tab. 7.3 b) were higher than estrogenic activity measured in the current study. This could be due to antiestrogenic substances that could be present in the complex samples. As already mentioned above, the selective ER modulator tamoxifen, acts as ER antagonists in the ER CALUX® and as agonists in the YES (Legler et al. 2002a). Therefore, it could have enhanced the estrogenic activity of the samples in the LYES while it inhibited the activity of estrogenic substances like E3 in the ER CALUX®. Again, potential effects caused by EE2 (< 9.2 ng L^{-1}), E2 (< 5 ng·L^{-1}), E2-ac (n.a.), E3 (< 0.18 ng·L^{-1}) and MPro-ac (n.a.) were not considered because chemical analytical values for these compounds were less than the method detection limit.

Concentrations of chemEEQs (Tab. 3a) were less than 0.9±0.3 ng·L^{-1} as determined in the LYES in ozonized effluents (AO-CO). However, most chemicals that could contribute to the concentration of chemEEQs were not detectable. Therefore, the inability to account for the biological effectiveness might be explained by the low sensitivity of the used analytical approach, which suggests that estrogens with greater endocrine-modulating potencies, such as EE2 and E2, could be present at concentrations less than the LOQ but in quantities potentially contributing to the positive response in the bioassay (Hollert et al. 2005). In

contrast, concentrations determined in the ER CALUX® (0.02±0.04 ng·L$^{-1)}$ were in the range of the theoretical EEQ (Tab. 3b).

The results of the instrumental quantifications as the results from the bioassays indicate that ozonation is an efficient method to remove most of estrogenic active substances. However, there was no trend towards greater efficiencies at greater ozone doses or longer ozonation times, and therefore, the recommended maximum dose and time for elimination of (xeno-) estrogens from hospital effluents based on the results of this study are 7.5 mg·L^{-1} and 7.5 minutes, respectively.

7.6 Conclusion

Results of the present study confirmed the necessity of advanced sewage treatment processes to minimize the estrogenic burden of highly charged sewages such as hospital waste-waters. The advantage of membrane bioreactors as well as the suitability of ozone treatment could be verified with regard to this specific effect. However, assessment of endocrine activities based on the sole assessment of receptor based assays would have been insufficient to objectively characterize the overall endocrine potential of the analyzed samples. In fact, advanced treatment of effluents using ozonation appeared to result in greater endogenous estrogen production, potentially due to generation of reactive metabolites by this treatment step. Therefore, a combination of receptor mediated assays such as the LYES or ER CALUX® and non-receptor mediated assays assessing effects on hormone production such as the H295R Steroidogenesis Assay to enable objective assessment of the endocrine disrupting potential of complex samples is recommended. In addition, further expansion of endocrine bioassay batteries by inclusion of androgen and thyroid hormone receptor assays is necessary to screen for potential endocrine disruptors.

Acknowledgement

The authors would like to thank the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV) for funding oh this study. Yeast cells were kindly supplied by Prof. J.P. Sumpter, of Brunel University, United Kingdom. Furthermore, special thanks to Martin Wagner (University of Frankfurt) and Stefanie Grund (University of Heidelberg) for the introduction in the LYES and H295R assays, respectively. The RWTH Aachen University Undergraduate Research Opportunity Program (UROP) provided funding for a research stay of T. Floehr in Saskatoon. M. Hecker and J. Giesy were supported through the Canada Research Program. Prof. Giesy was also supported by an at large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong, and the Einstein Professor Program of the Chinese Academy of Sciences. Furthermore the authors thank Prof. H. Fr. Schröder (Aachen University) and the team of R&D-Project Waldbröl for their support.

Chapter 8

Discussion

8.1 Suitability of the selected bioassays

One important issue of the present study was the suitability of the bioassays due to the different sample matrices investigated.

8.1.1 *In vitro* bioassays

The selected *in vitro* bioassays were suitable for the detection of endocrine activity in different sample matrices from the aquatic environment. Additionally, single substances could be investigated regarding their potential to act as endocrine disruptors.

The receptor mediated bioassay ER CALUX® was able to detect estrogenic activity in all matrices used. These included groundwater, surface water as well as sewage and therefore covered low to medium contaminated (fresh)water samples. Additionally, in case of the hetero-PAHS, the estrogenic activity of single substances which require metabolic activation could be detected. With the LYES investigation of medium to highly contaminated water samples was possible, low contaminated water samples could not be investigated due to the high limit of detection (LOD) as well as the relatively high estrogenicity of the negative control. The H295R was applicable for medium to highly contaminated samples as well as single substances. Surface water samples were not tested wherefore in this context a statement is not possible. Nevertheless, previous studies support the usefulness of the H295R even for such low contaminated samples (Gracia et al. 2008).

These findings are in accordance to further studies regarding the usefulness of the selected *in vitro* bioassays (Hecker & Hollert 2011, Kase et al. 2009, Leusch 2008, Leusch et al. 2010).

8.1.2 Reproduction test with *Potamopyrgus antipodarum*

To the best of our knowledge, the Pa-Repro was integrated into a microcosm study for the first time. Therefore, the adaption of the test organism to the artificial environment of the microcosm was not predictable. Considering the uncertainties of the present study, the suitability of the selected test species cannot be completely evaluated.

Further studies have proven the suitability of the Pa-Repro for the detection of endocrine activity in the aquatic environment (Jobling et al. 2003, Mazurova et al. 2008, Schmitt et al. 2011).

8.2 Performance of the selected bioassays

Apart from the suitability to investigate different sample matrices, the performance of the selected bioassays in the present study was examined. Overall, performance was good but differed between the test systems.

8.2.1 LYES

Performance of the LYES in the present study ranged from moderate to good.

The median EC_{50} of 82 ± 42 pM (22 ± 11 ng·L^{-1} estradiol equivalents (EEQ)) is within in the range (48-147 pM) of other studies using (nearly) the same test system (Schmitt et al. 2008, Schultis & Metzger 2004, Wagner & Oehlmann 2009). The high SD of the calculated EC_{50} in the present study is a result of different time points of measurement after exposure to the CPRG solution. For the study dealing with the cleaning performance of sewage treatment techniques (Chapter 7) optimal curve parameters (low Bottom, high Top, high goodness of fit (R^2)) were regularly reached at a point where the EC_{50} was around 40 pM. In case of the other two studies using the LYES (Chapter 4 and 5) optimal curve parameters were reached at a point where the EC_{50} was around 100 pM. This shift of the EC_{50} relating to different time points of measurement and incubation was already observed and discussed by Beresford et al (2000). Hence, for further studies a fixed time point of measurement and / or a fixed value for the EC_{50} of the standard should be set.

In contrast to chapter 5 and 7, in chapter 4 calculations for the E2 standard and the tested chemicals were performed according to Villeneuve et al. (2000). Therefore, instead of EEQs relative potency estimates (EEFs) were calculated. Data were normalized by dividing the measured values by the maximum induction of the E2 standard (E2-max.) to scale all values from zero (solvent control) to one (E2-max.). Scaled values were then plotted and the standard curve was fitted where top and bottom of the curve were set to zero and one, respectively. In case of the EEQ calculations the top of the standard curves was interpolated from the measured values. Due to these changes in the standard curve calculation, EC_{50} values differed by a factor of two. From the same raw data set Brinkmann & Maletz et al. (2014) calculated an EC_{50} of 57.7 ± 0.7 pM while in the current study, to receive the mean EC_{50} of all included LYES assays, an EC_{50} of 108 ± 25 pM was calculated. This leads again to the recommendation that standardized analysis methods should be implemented for this test system.

The calculated LOD of 17.74 ± 6.73 pM (4.83 ± 1.8 ng·L^{-1} EEQ) as well as the LOQ of 38.13 ± 5.6 pM (10.39 ± 1.53 ng·L^{-1} EEQ) are also in accordance with further studies using nearly the same (Schultis & Metzger 2004) or similar (Leusch 2008) test setups. Nevertheless, regarding the often very low concentrations of estrogenic active substances in the aquatic environment (for example 0.01-1.3 ng·L^{-1} E2 in groundwater (Vulliet et al. 2008)) as well as the detected lowest observed effect concentrations (LOEC; for example 0.5 ng·L^{-1} E2 for the fathead minnow (Miles-Richardson et al. 1999)) a LOD of around 5 ng·L^{-1} is too high to detect all relevant estrogenic contaminations.

One major drawback of the here used LYES is the high background noise of the solvent (negative) control. The mean of the control was in a range between 2.23 ± 0.99 ng·L^{-1} EEQ (chapter 7) and 4.45 ± 0.49 ng·L^{-1} EEQ (chapter 5). Therefore, samples with low but eventually significant estrogenic activity could be classified as negative because measured EEQs did not differ from the negative control. As the test system is designed to investigate water samples (Wagner & Oehlmann 2009), uncontaminated reference water for the control is necessary. Internal investigations of ultrapure water (data not shown), deionized water and tap water in our laboratories revealed that tap water is the least contaminated. This is also in accordance with the investigations of Wagner & Oehlmann (2009). Nevertheless, as already shown background noise (estrogenic effectiveness) of the tap water is still quite high. Contamination of the tap water as well as the even higher contamination of the ultrapure water and the deionized water could have been caused by plastic parts of the water pipes and / or the filter systems. Krishnan et al. (1993) as well as Howdeshell et al. (2003) could show that the xenoestrogen bisphenol A (BPA) is released from polycarbonate based material (flasks and cages) into water. Additionally Wagner & Oehlmann (2009, 2011) could show that estrogenic active substances are released from polyethylene (PET) bottles. Recently, Zhang et al. (2011) could show that comparatively high amounts of BPA leached from standard plastic laboratory equipment into the test medium of the H295R. Additionally, the xenoestrogen nonylphenol (NP) which is used as stabilizer and antioxidant in plastics, including polyvinyl chloride (PVC), polyethylene, polystyrene, and rubber-based products (Snedeker & Hay 2014) has been found to leach from standard plastic laboratory equipment as well (Soto et al. 1991).

Another drawback of yeast based test systems such as the here used LYES, is the lack of vertebrate like metabolic capacities for xenoestrogens. As stated in chapter 4 the

estrogenic activity of hetero-PAHs could not be detected with the LYES because the activity was caused by metabolites only. For strains of the yeast species *Saccharomyces cerevisiae*, it is generally assumed that they lack the capability of xenoestrogen metabolism (Connolly et al. 2011). In contrast, Odum et al. (1997) stated that yeasts were capable for detecting the estrogenic potential of methoxychlor (MXC), which has only low affinity for the ERα itself (Gaido et al. 2000), due to a certain metabolic capacity. Additionally, a few other studies also detected estrogenic activity of MXC in yeast test systems (De Boever et al. 2001, Folmar et al. 2002, Gaido et al. 1997). Nevertheless, other studies could not confirm this estrogenic activity (Rehmann et al. 1999, Shelby et al. 1996). Overall, the present study supports the assumption that yeasts of the species *Saccharomyces cerevisiae* are not capable for metabolism, at least not in case of the investigated hetero-PAHS. Therefore, a metabolic activation step, e.g. by adding of the rat liver S9 fraction, to detect estrogenic active substances and samples, is strongly recommended (Okuda et al. 2011). In case of other yeast species (e.g. *Candida*) biotransformation of polycyclic aromatic hydrocarbons (e.g. phenanthrene) has already been described (MacGillivray & Shiaris 1993).

Further advantages and disadvantages of the LYES have already been discussed in chapter 7 (see section 7.5). Overall, results of the present study revealed that this test system needs some improvements regarding implementation as well as data analysis (see also section 8.3). Currently, a working group on behalf of the German Institute for Standardization (DIN) is preparing a standard operation guideline (DIN standard) which also will be transformed to a standard of the International Organization for Standardization (ISO) (Kunz et al. 2014). As a part of the standardization the performance of the test system should be improved and the outcome should be validated by an accompanying round-robin study.

8.2.2 ER CALUX®

Performance of the ER CALUX® in the present study could be evaluated as good to very good.

The median EC_{50} of 7 ± 2 pM (2 ± 0.5 ng·L^{-1} EEQ) is within the range (2-6 pM) of other studies using the same test system (Legler et al. 1999, Leusch 2008, Murk et al. 2002). Together with the mean LOD of 0.6 ± 0.3 pM (0.2 ± 0.1 ng·L^{-1} EEQ) as well as the mean LOQ of 1.2± 0.6 pM (0.3 ± 0.2 ng·L^{-1} EEQ), which are also in the range of further studies (Legler et al. 1999, Leusch 2008, Murk et al. 2002), this confirms the applicability of the ER CALUX® for measurement of estrogenic activity in the low ng·L^{-1} range. Again, the data analysis in chapter 4 revealed data which differed from the data analysis used in chapter 5 and 7. But in case of the EC_{50} of the ER CALUX® the differences were much lower than for the LYES. From the same data set Brinkmann & Maletz et al. (2014) calculated an EC_{50} of 10 ± 8 pM (3 ± 2 ng·L^{-1} EEQ) while the EC_{50} calculated for the comparison of all data in this chapter was 9 ± 4 pM (3 ± 1 ng·L^{-1} EEQ). This observation leads to the conclusion that the higher stability of the mammalian cell based test system, in contrast to the yeast cell based test system, generates more reliable data, even when different ways of analysis are applied.

In chapter 4, it could be shown that the ER CALUX® with the T47Dluc cell line is capable of transforming non-estrogenic active substances (in this case hetero-PAHs) to estrogenic active metabolites. As already described by several authors (Kuil et al. 1998, Legler et al. 1999, Piao et al. 1997, Spink et al. 1998), the T47D cell line expresses at least some metabolic enzymes (e.g. CYP1A and CYP1B as well as 17ß-HSD) and therefore is capable for metabolism of xenoestrogens. Nevertheless, to enhance the predictive character of this *in vitro* test system for effects *in vivo* (see also section 8.4) an additional biotransformation step such as the pre-incubation of the samples with rat liver fraction S9 could prove necessary (Legler et al. 2002).

Further advantages and disadvantages of the ER CALUX® have already been discussed in chapter 7 (see section 7.5). Overall, results of the present study revealed that this test system is a sensitive and reliable tool to detect estrogenic active substances in the whole aquatic environment. Additionally, also for this test system a working group on behalf of the German Institute for Standardization (DIN) is preparing a standard operation guideline (DIN standard) which also will be transformed to a standard of the International Organization for Standardization (ISO) (Kunz et al. 2014).

8.2.3 H295R

Overall, performance of the H295R in the present study was good.

Actual quality criteria of the H295R are given in the OECD guideline (OECD 2011). However, during the application of the test system in the current study prior to publication of the guideline quality criteria according to the standard operating procedure of the Aquatic Toxicology Laboratory, Michigan State University, USA (Hecker et al. 2007c) were followed. Criteria for the induction (inhibition) of estradiol or testosterone production by the standard substances prochloraz and forskolin were always met. Additionally, criteria for the coefficient of variation (CV) within and between the replicas were met for the majority of the samples. Basal hormone production of the cells was always ≥ 2000 $ng \cdot L^{-1}$ (testosterone) or ≥ 50 $ng \cdot L^{-1}$ (estradiol), respectively.

Nevertheless, data revealed for the production of testosterone in chapter 7 did not follow clear dose-response relationships but undulated through the tested concentrations. While U-shaped or biphasic dose-response relationships are frequently observed in toxicological studies (Calabrese & Baldwin 2003) undulating curves are quite problematic to interpret. So far, the source of this effect is not confessed and therefore, parts of the data revealed with the H295R in the current study are not suitable for a reliable risk assessment.

8.2.4 Pa-Repro

Overall, performance of the Pa-Repro in the present study was only moderate. This is most likely a consequence of the introduction of the species into a multispecies-microcosm system without external food supply. While the nutritional requirements for laboratory breeding in glass vessels are defined in the standard operating procedure (Schmitt et al. 2013) knowledge about the appropriate amount of periphyton cover per adult snail in the presence of competitors like D. magna is lacking. As a consequence, the food supply seemed to be limited and therefore also the reproduction of *P. antipodarum*. Prior to further studies, the nutritional requirements of the test species in complex microcosm systems have to be investigated. Additionally, the use of 17ß-Estradiol (E2) as positive control in the test system hast to be reconsidered. The absence of a significant effect on the reproduction could be due to the fast biodegradation of E2. For further studies the use of the much more resistant synthetic estrogen 17α-Ethinylestradiol (Jürgens et al. 2002) is recommended.

8.3 Data analysis

According to Wagner et al. (2013) there are three common mathematical models to derive estradiol equivalents from bioassay data: Linear interpolations, nonlinear interpolations and ratios of effect concentrations (EC_x ratio). In the present study the nonlinear as well as the EC_x ratio model to determine estrogenic activities have been applied.

In chapter 5 and 7 estradiol equivalents (EEQs) were calculated using the nonlinear model (see chapter 2.2.1.3 and 2.2.2.3). Therefore, measured values for the regarding sample concentrations were inserted into a conversion of the corresponding standard curve (Eq. 8.1 and 8.2).

$$EEQ\ (M) = 10^{\left(logEC50_{E2} - \frac{log\left(\frac{TOP_{E2} - BOTTOM_{E2}}{corrected\ OD540_{Sample} - BOTTOM_{E2}} - 1\right)}{SLOPE_{E2}}\right)}$$ **Equation 8.1 (LYES)**

$$EEQ\ (pM) = EC50_{E2}\left(\left(\left(\frac{Max_{E2}}{corrected\ RLU_{Sample}}\right) - 1\right)^{\frac{1}{Slope_{E2}}}\right)$$ **Equation 8.2 (ER CALUX®)**

The equivalent concentration of every single sample concentration tested can therefore be calculated. This method to determine EEQs has been applied in several studies for different test systems (Behr et al. 2011, Bogers et al. 2007, Brand et al. 2013, Lamoree et al. 2010, Maletz et al. 2013, Stalter et al. 2011, Van der Linden et al. 2008, Wagner & Oehlmann 2009). In case of this analysis method it has to be kept in mind that only values within the linear range of the standard curve should be considered. Additionally, this method requires a similar curve progression of standard and sample. Therefore, concentrations ranges which cover the whole dose-response relationship should be tested to confirm this similarity.

Another common analysis method to determine EEQs depends on the EC_x ratio model originally used for the calculation of TCDD equivalents for dioxin-like activity (Eadon et al. 1986). In this case, at first the estradiol equivalent factor (EEF) is calculated by dividing the ECx of the standard (E2) by the concentration of the sample which induces the same response as the ECx of the standard (Eq. 8.3). To calculate the EEQ the EEF is multiplied by the initial concentration of the sample (Houtman et al. 2006, Keiter et al. 2006) (Eq. 8.4).

$$EEF = \frac{ECx_{E2}}{ECx_{Sample}}$$ **Equation 8.3**

$$EEQ = EEF \cdot concentration_{Sample}$$ **Equation 8.4**

In most studies the EEQ is calculated for only one concentration of the tested sample. Depending on the potency of the sample, the ECs between 50 % and 10 % are calculated. Again, to achieve reliable data the curves of standard and sample have to be parallel and should exhibit equal efficacy. As this is not true for most cases a single point estimate of the EEF is not sufficient because equivalent factors at different ECs would be quite different. According to Villeneuve et al (2000) for non-parallel curves a relative equivalent factor range should therefore be applied. Therefore, multiple point estimates (EC_{20}, EC_{50} and EC_{80}) are calculated for each sample tested. The derived relative potencies (REP) are then used to reveal the $REP_{20\text{-}80}$ range (Villeneuve et al. 2000) (Eq. 8.5).

$$REP20-80\ range = minimum\ REP\ to\ maximum\ REP \qquad \textbf{Equation 8.5}$$

This method was also used in chapter 4 to determine EEF ranges for the tested hetero-PAHS. To compare the approaches a LYES *Bti* data set from chapter 5 was analyzed in both ways to calculate the EEQs as well as the EEFs (Tab. 8.1).

Table 8.1 Comparison of EEQs derived from the nonlinear as well as the ECx ratio based model

VectoBac® TP [x fold concentration]	EEQ [ng·L^{-1}] Nonlinear model	EEF_{EC50}	EEQ [ng·L^{-1}] EC_{50} ratio model
1	1,14	$3.47\cdot10^{-7}$	1,18
3	3,90		3,54
9	11,74		10,62
18	22,77		21,24

In case of the here analyzed dataset the results from the different models coincide almost perfectly for all concentrations tested. This is also true for EEQs which are derived from the EC_{20} as well as the EC_{80} ratios (data not shown). Considering the shape of the related curves this is probably a result of the similar curve progression (Fig. 8.1).

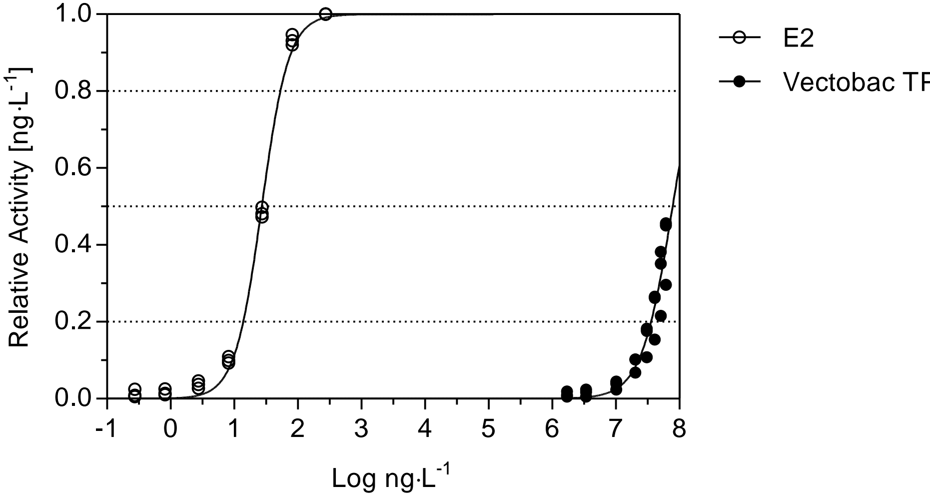


Figure 8.1 Curve progression of 17ß-Estradiol (E2) and VectoBac® TP in the LYES given as relative estrogenic activity (Bottom = 0; Top = 1).

Nevertheless, it has to be kept in mind that the TP concentrations tested did not show a whole does-response curve and therefore, data had to be interpolated from the standard curve. The maximum response was set to the maximum response of E2 which is most likely not true. However, since higher concentrations could not be tested due to the limited water solubility this assumption was inevitable.

8.4 Development of a bioanalytical test system to screen for EDCs

8.4.1 Development of the H295R

In 1998 the OECD started a program to revise existing test guidelines as well as to develop new test systems to screen for EDCs. As a result several new test systems have been developed or still are in development. The existing as well as new developed test systems are listed in the "Guidance Document on Standardized Test Guidelines for Evaluating Chemicals for Endocrine Disruption" (OECD 2012). While there are many test systems covering the field of receptor binding and / or activating only few are dealing with steroidogenic pathways (Hecker et al. 2006). Additionally, the available test systems mostly only exhibit one single endpoint. To screen a large set of substances for their potential to disrupt the endocrine system, it would be useful to have more test systems covering a set of significant endpoints. Currently, a research group of the Department of Ecosystem Analysis (RWTH Aachen University) is working on this field (data not shown).

In the current study chapter 3 describes the development and validation of the H295R Steroidogenesis assay (Hecker et al. 2011). From the first descriptions of the characteristics of the NCI-H295 cell line (Gazdar et al. 1990) to the development of the H295R Steroidogenesis assay (Hecker & Giesy 2008, Hecker et al. 2006) more than 15 years have passed. The validation and preparation of a OECD guideline for the test system took another 5 years (OECD 2011). In the meantime several research groups worked on different usages of the H295R cell line (Tab. 8.2). The number of working groups as well as different usages clearly shows that the cell line or the bioassay, respectively, is widely spread among the scientific community. Nevertheless, by now the cell line is approximately 35 years old and due to the fast increase of hormone production during cell culture it is questionable how long the initial cell line will be usable for the test system. Therefore, additional improvements in the test system seem necessary.

Table 8.2 Research groups working with the H295R cell line between 2000 and 2011

Working group	Usage	References
Aquatic Ecology and Toxicology Section, Department of Zoology, University of Heidelberg, Heidelberg, Germany	Steroid production	(Grund et al. 2011)
Center for Integrative Toxicology, National Food Safety and Toxicology Center, Department of Zoology, Michigan State University, Michigan, USA	Expression of steroidogenic genes; Steroid production	(Gracia et al. 2007, Gracia et al. 2006, Gracia et al. 2008, Hecker et al. 2006, Hilscherova et al. 2004, Zhang et al. 2005)
Centre for Coastal Pollution and Conservation, Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong SAR, China	Expression of steroidogenic genes	(Xu et al. 2006)
Department for Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Aachen, Germany	Steroid production	(Hecker et al. 2007a, Maletz et al. 2010)
Department of Basic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan	Expression of steroidogenic genes; Steroid production; Proliferation	(Furuta et al. 2008, Jaroenporn et al. 2008)
Department of Biochemistry, Hoshi University School of Pharmacy and Pharmaceutical Sciences, Shinagawa, Tokyo, Japan	Aromatase activity; Expression of steroidogenic genes; Steroid production; Steroidogenic acute regulatory (StAR) protein promoter activity	(Nakajin et al. 2001, Ohno et al. 2002, Sugawara et al. 2001, Watanabe & Nakajin 2004, Watanabe et al. 2006)
Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Fukuoka, Japan	Aromatase activity	(Fan et al. 2007)
Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Kaohsiung, Taiwan	Steroid production; Expression of steroidogenic genes	(Li & Wang 2005, Li et al. 2004a, Li et al. 2004b)
Pharmaceutical and Medicinal Chemistry, Saarland University, Saarbrucken, Germany	Enzyme inhibition	(Muller-Vieira et al. 2005)
Research Centre for Toxic Compounds in the Environment (RECETOX), Masaryk University, Brno, Czech Republic	Steroid production	(Blaha et al. 2006)
Research Institute of Toxicology / Institute for Risk Assessment Sciences (IRAS), University of Utrecht, Utrecht, The Netherlands	Aromatase activity; Expression of steroidogenic genes	(Cantón et al. 2005, Heneweer et al. 2004, Sanderson et al. 2002, Sanderson et al. 2004, Sanderson et al. 2001a, Sanderson et al. 2000, Sanderson et al. 2001b, Sanderson et al. 2003)
School of Biomedical and Molecular Sciences, University of Surrey, Guildford, Surrey, UK	Expression of steroidogenic genes	(Oskarsson et al. 2006)
State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, People's Republic of China	Expression of steroidogenic genes	(Ding et al. 2007)
Toxicology Centre, University of Saskatchewan, Saskatoon, Canada	Steroid production	(Hecker & Giesy 2008, Hecker et al. 2007a, Hecker et al. 2007c, Higley et al. 2010)
US Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, Duluth, MN, USA	Steroid production	(Villeneuve et al. 2007)

8.4.2 Further development of established test systems

As already mentioned in chapter 3 as well as paragraph 8.3 the culturing process of the H295R cell line exhibits some problems due to a relatively fast increase of steroid production through cell culturing (Hecker et al. 2011). Therefore, the cells are only usable within a short time slot (approximately 10 passages). For example, during this study basal E2 concentrations in the solvent control increased by a factor of 5 from passage three to passage seven (36 ng·L^{-1} to 180 ng·L^{-1}). Consequential, data from different approaches are only comparable by transforming the measured steroid concentrations into fold changes relative to the control. Additionally, the natural kinetics of hormone production (in this case estrogen production via conversion through aromatase) at the beginning show an increase that is more or less linear but diminishes over time and results in a steady state condition (Sohl & Guengerich 2010). Due to this, changes in steroid production after exposure to a steroidogenic substance are strongly depending on the time point of exposure and the actual growth phase. Furthermore, there is a natural limit of steroid concentration within the cells. Cells that are exposed to a steroidogenic substance at the beginning of the growth phase therefore are capable of higher increases in steroid production than cells that are in the late growth phase. Taking into account the limited available amount as well as the age of the original cell line this will sooner or later lead to severe problems as far as the unfeasibility of the test system.

Recently, at our laboratories there have been different approaches to enhance the usability of the H295R. In a first approach, the cells were brought into suspension culture. At first the cells genomic DNA was demethylated with 5-Aza-2'-deoxycytidine (5-Aza-dc) to presumably reactivate silenced genes. Approximately 20 % of the cells were indeed brought into suspension but after a few passages insufficient proliferation and finally complete cell death were discovered. Therefore, this approach was skipped. The observed growth arrest may be caused by the reactivation of tumor suppressor genes like CDKN2A and SCGB3A1 (Fonseca et al. 2012). In a second step the standard cell culture medium (DMEM F12 / Hams) containing fetal bovine serum (FBS) was replaced by serum free or chemical defined medium. There are several drawbacks related to the usage of FBS in cell culture. First, there is the issue of animal welfare and the claim to save the lives of laboratory animals by use of alternative *in vitro* methods. FBS is a common supplement of cell culture media and includes several essential components for growth, homeostasis and

survival of the cells (Barnes & Sato 1980, Even et al. 2006). The amounts of FBS that are used for *in vitro* methods are increasing mostly due to large scale manufacturing of cell therapy processes and biotechnology (Brindley et al. 2012) and to a certain extent even to the increasing number of *in vitro* test systems that are used for toxicity testing (e.g. for REACH purposes) (Hecker & Hollert 2011). While *in vitro* methods are a sufficient way to reduce suffering of laboratory animals the collection of FBS from fetal calves after slaughtering of the pregnant cow may cause severe suffering of the fetuses. This seems to be contradictory to the 3R (replace, reduce, refine) principle (Russell et al. 1959) and is more than ethical questionable. Beside the ethical problems regarding to the use of FBS, there are also scientifically issues. On the one hand, FBS is a mixture of unknown composition which could lead to reduced reproducibility of different experimental approaches. For bioassays on endocrine disruption, for example, different steroid contents in the used FBS could influence the steroidogenesis within the cells. Therefore, in most cases stripping of FBS prior to use in the exposure medium is necessary. On the other hand, there is the risk of disease transmission because FBS could be contaminated with fungi, viruses, bacteria, mycoplasmas and prions. To overcome the problems related to the use of FBS as well as for the manufacture of biological products for human use, several serum free and chemical defined media have already been developed. Nevertheless, in contrast to media supplemented with FBS the alternative media are more cell type specific. Therefore, a special medium has to be used for most of the cell types used (Even et al. 2006, Van der Valk et al. 2004). Additionally, culture medium without FBS leads to suspension cell cultures in most cases (Barnes & Sato 1980), which was a desired side effect in the present study. Replacement of the standard culture medium of the H295R cell line by the serum-free medium ProVero™1 (Lonza, Cologne, Germany) showed sufficient cell growth as well as a suspension rate of about 20 %. Unfortunately, there were issues regarding the quality between different batches, most likely due to the use of plant derived protein hydrolysates, wherefore an alternative medium was needed. In contrast, the replacement of the standard media by a chemical defined media for biopharmaceutical production without proteins and steroids (working title: CDM5) was overall successful. The H295R cells grew in suspension with a stable cell viability of ≥95 % over 12 days without passage. Cell density increased in a linear manner from $0.5 \cdot 10^6$ cells·ml^{-1} to $2 \cdot 10^6$ cells·ml^{-1}, a reaction that was already described by Logie et al. (1999). Hence, the total cell density was higher

than achieved in adherent cultures in tissue culture flasks. With this modification it is now possible to study the effect of a substance (or a mixture as well as environmental sample) on the steroidogenesis over a time period of up to 10 days. Within this period, medium samples can be taken and analyzed at a daily rate without changing the exposure medium. Additionally, the basal testosterone (T) production was higher compared to the adherent cells (Logie et al. 1999) but basal production of E2 was much lower (approximately 50 ng·L^{-1} after 16 passages; data not shown). The quality criteria of the OECD guideline (OECD 456), however, were met by the suspension cells. The high level of T and at the same time low level of E2 could be explained by the lack of growth factors such as the epidermal growth factor (EGF). In the presence of the EGF, expression of aromatase is upregulated in H295R cells (Watanabe et al. 2006) which leads to an increased transformation of T to E2. In addition, the fast increase of steroid production over time could be decelerated (data not shown).

After the successful adaption of the H295R cell line to chemical-defined medium the next steps will be to adapt further cell lines such as the T47D cell line which is used for the ER CALUX®. Since T47D cells are human breast carcinoma cells it is possible to refer to existing protocols for the adaption of the MCF-7 cell line to serum-free media (Barnes & Sato 1979). Recently, at our laboratories, a new ELISA method to measure the steroid concentration in the exposure medium was developed (data not shown). Thereby, it was possible to replace the so far used ELISA kits from Cayman Chemicals (Cayman Chemicals Europe, Tallinn, Estonia) which are the largest matters of expense of the whole test system (expect personnel costs). Moreover, the detection of steroids in the exposure medium spares the extraction step which again not only saves costs but time and resources.

Overall, with the already adapted and further designed improvements the H295R could be significantly upgraded. For the other cell based assays used within this study, planned improvements are still in the process of development.

8.5 Comparison between *in vitro* and *in vivo* test systems

At the time the term endocrine disruptor (ED) should exclusively be used for substances that have been tested in *in vivo* test systems (WHO 2002). Nevertheless, to classify a substance as a possible ED the usage of *in vitro* test system data is permitted (Kortenkamp et al. 2011). Furthermore, since the introduction of the 3R principle (replace, reduce, refine) of Russel et al. (1959) there have been a number of approaches to replace *in vivo* with *in vitro* biotests (Hecker & Hollert 2011, Leist et al. 2008, Ukelis et al. 2008). By means of this, it is necessary to compare data obtained by *in vitro* test systems with data obtained by *in vivo* test systems to detect significant correlations and, at best, replace the *in vivo* test with an *in vitro* test.

For the yeast systems (YES and LYES, respectively) correlations for estrogenic activity of sediments and single substances have been reported with *P. antipodarum* (Schmitt et al. 2010, Schmitt et al. 2008) but the cell based system was up to tenfold less sensitive to single substances. In the present study the biological larvicide VectoBac® TP was tested with both LYES and Pa-Repro (see chapter 5 and 6). In the LYES significant increased estrogenic activity beginning with a concentration of approximately 20 $mg \cdot L^{-1}$ (6-fold concentration) TP was detected. In the microcosm study with *P. antipodarum* a concentration of 18 $mg \cdot L^{-1}$ caused a significant increase of embryo production after 14 days of exposure. Therefore, the comparability could be proofed even for the microcosm approach. Nevertheless, after 42 days embryo production decreased. But this was most likely associated with quantity and quality of the available food, which again might be indirectly related to the application of TP (e.g. community shifts in phytoplankton and periphyton). Consequently, to verify the correlation the approach should be repeated with continuous external feeding of the adult snails during exposure. Similar correlations could be observed for the investigation of riverine sediments with both the ER-Luc (Rogers & Denison 1999) assay and the Pa-Repro (Schmitt et al. 2011). Fractions containing bisphenol A and phtalates led to a significant increase in reproduction of *P. antipodarum* as well as significantly increased estrogenic activity in the ER-Luc assay. In the present study, significant differences in estrogenic activity could only be observed from the 12-fold concentration (or 41 $mg \cdot L^{-1}$) on. But since the processing of the sample led to a distinct decline in estrogenic activity (see chapter 5.4.3.1) it cannot be concluded that the ER CALUX® was less sensitive than the LYES or the Pa-Repro. So far no comparative studies with the Pa-Repro and the H29R have been performed but Gust et al. (2010) could show that the specific vertebrate aromatase inhibitor fadrozole led

to a significant decrease in the reproduction of *P. antipodarum* with a LOEC of 6.2 µg L^{-1} ($2.8 \cdot 10^{-2}$ µM). Fadrozole has also been shown to significantly inhibit aromatase activity (Heneweer et al. 2004) and to significantly decrease E2 production in H295R cells with a LOEC of 1 µM (Hecker et al. 2006). Consequential, the effect measured with the Pa-Repro was more sensitive by a factor of 36. In the present study TP had no clear effect on the H295R cells wherefore no convincing comparison was possible.

8.6 Usability of the tests systems for risk assessment and regulation

In the EU there are different legislations which cover the field of EDCs regulation. Under REACH (European Commission 2006) the relevant endpoints for environmental risk assessment are acute toxicity, chronic toxicity, bioaccumulation and endocrine disruption. Generally, the advised test systems cover OECD test guidelines for animal testing. But it is also stated that testing of chemicals on animals should only be accomplished when no reliable alternative ways of testing are possible. While at the moment there are only few validated alternative test systems (e.g. *in vitro* bioassays such as the H295R) the provided ecotoxicological information are gained from alternative methods in more than three-quarters of the in 2011 registered substances (Scholz et al. 2013). In the Water Framework Directive (WFD) assessment of the chemical status of water bodies is generally based on chemical analysis (European Commission 2000). Environmental Quality Standards (EQS) for priority substances are set for all EU member states, whereas, EQS for other pollutants are set by the member states themselves. As stated by the European Commission (EC) the list of priority substances covers substances and preparations as well as metabolites that possess carcinogenic, mutagenic and endocrine disruptive properties. But for the entirety of the enormous number of chemicals found in the aquatic environment setting of individuals EQS is almost impossible. Therefore, the European Commission elaborated a technical report on potential aquatic effect-based monitoring tools to be used as links between chemical and ecological status assessment (Hecker & Hollert 2011, Wernersson et al. 2015). In this report, use of effect-based tools is recommended for several objectives, for example: (1) Prioritization of water bodies for further monitoring; (2) Early warning systems to discover effects related to chemical substances before significant effects on population level occur; (3) Effects of mixtures and not analyzed chemicals; (4) Water and sediment quality assessment. The technical report also recommends the use of bioassay batteries to cover substances with a certain mode of action (e.g. estrogenic activity) which are not included in the chemical analysis. Two of the mentioned test systems are the ER CALUX® and the YES which are currently also standardized and validated for preparing a standard of the International Organization for Standardization (ISO) (European Commission 2014, Kunz et al. 2014). The outcomes of the present study support the usefulness of *in vitro* bioassays for screening of water samples for endocrine active samples. In addition to the in the technical report mentioned receptor reporter gene assays a test system that covers the steroidogenic

pathway as well as a *in vivo* test system to cover adverse outcomes on whole organisms and to a certain extent populations are also strongly recommended. Nevertheless, depending on the samples to be tested the test battery should be adapted (Fig. 8.2).

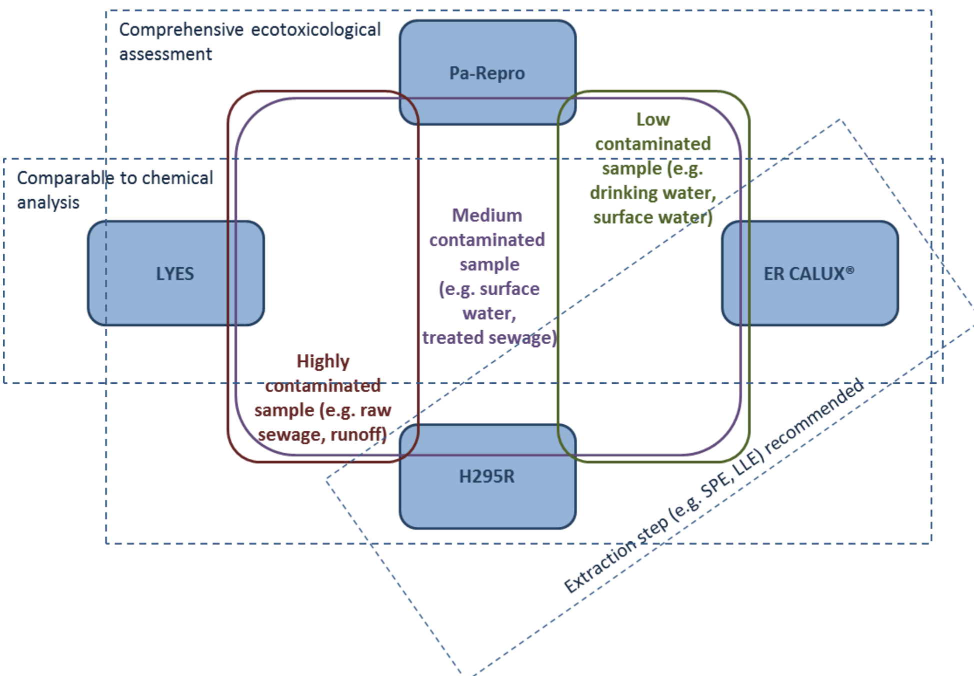


Figure 8.2 Applicability of the examined bioassays depending on the sample matrix as well as the sample preparation and statements to be made from the investigation.

Depending on the information expected from the analysis different approaches should be considered. If a comprehensive view on the ecotoxicological relevance of a sample is required it should be tested as a native sample preferably with the whole bioassay battery (Hecker & Hollert 2011). If, on the other hand, a sample is to be screened for a certain mode of action in comparison or addition to data from chemical analysis the application of receptor reporter gene assays should be sufficient (Brand et al. 2013). In this case, usage of the ERα CALUX® which depends on the cell line U2OS and has the advantage of being even more selective to ligands interacting with the estrogen receptor (Sonneveld et al. 2005, van der Burg et al. 2010) should be considered. But since the U2OS cells are lacking the ability to metabolize the ER CALUX® depending on the cell line T47D (Legler et al. 1999) should be used for a more comprehensive risk assessment.

Chapter 9

Conclusion

Water is a prerequisite for life. Only 2.5 % of earth's water resources are freshwater, from which's accessible part to date over 50 % are already used by mankind. Concurrently, accessible freshwater is not only crucial for humanity but also for the ecosystems functions in general. Even though, freshwater resources are exposed to an increasing number of (anthropogenic) pollutants like fertilizers, pesticides, pharmaceuticals, personal care products, metals and other industrial chemicals. One important and heavy discussed group of pollutants are the endocrine disruptive compounds (EDCs), which include natural as well as synthetic steroids, pharmaceuticals, pesticides, organotin compounds, polychlorinated biphenyls (PCBs) and phthalates, dioxins, phenol derivatives etc. In the last years more and more (potential) EDCs have been detected in the environment as well as organic tissues of humans and wildlife. To screen samples for known EDCs, chemical analysis is a fast and sensitive approach. However, to detect an unknown endocrine active potential of a substance or to screen an environmental sample with unknown composition, a sensitive and reliable bioassay battery is needed.

In the present study, the applicability of four different bioassays to detect EDCs in the aquatic environment was investigated. Therefore, the test systems have been applied with the different sample matrices groundwater, surface water and sewage as well as single substances. All three *in vitro* test systems could proof their suitability for the detection of EDCs. Performance of the bioassays varied within the categorizations (1) good to very good: ER CALUX®; (2) good: H295R; (3) moderate to good: LYES. While the LYES was suitable to detect estrogenic activity even in extracts from highly contaminated raw sewage as well as in native samples, it was not suitable to detect low estrogenic activity (≤ 5 $ng \cdot L^{-1}$) because of its high background activity. In contrast, the ER CALUX® was suitable to detect low to medium estrogenic activity in extracts from complex samples (e.g. treated sewage) as well as single substances. Furthermore, the here used T47Dluc cell line showed significant metabolic capacity and therefore was even applicable to detect substances that needed metabolic activation to act as xenoestrogens. Depending on the samples to be tested, it can be concluded that in most cases a combination of both receptor-mediated bioassays should be used. Highly contaminated native samples such as sewage can be analyzed by the LYES without any further extraction steps. Therefore, an investigation of unchanged samples, which is strongly recommended by the ISO standard on biotesting of samples (ISO 5667-16:1998), is possible. In addition, samples with low but potentially environmentally relevant

concentrations of EDCs such as groundwater can be investigated by the ER CALUX®. With the H295R, extracts from complex samples as well as single substances could be investigated for their potential to disrupt the steroidogenesis. Complementary to the receptor-mediated test systems, the H295R allows an investigation of samples for their effects on the vertebrate steroidogenesis. With the application of this test system a much broader analyses of the potential endocrine disrupting effects of samples is feasible. Altogether, the here used *in vitro* bioassay battery is most suitable to investigate a broad range of samples with different endpoints and sensitivities. It therefore can be recommended as a fast and relatively inexpensive screening of (water)samples prior or even alternatively to *in vivo* tests.

The suitability of the *in vivo* test with *P. antipodarum* in a complex microcosm system could not be proofed in the present study. In contrast to the standard protocol for the Pa-Repro, which was recently applied for a number of studies regarding EDCs in the environment (see also chapter 8.1.2), to the best of one's knowledge an introduction of this test species into a multispecies-microcosm without external feeding to test for EDCs was performed for the first time. As limited food supply strongly impacts the reproductive outcome of any test species, limited conclusions on the direct effect of the sample could be drawn only. Consequently, the performance of *P. antipodarum* in the microcosm study was moderate only. Nevertheless, referring to the WHO's definition a substance has to be tested in an *in vivo* study to be declared as an EDC. As test systems using vertebrates such as fish should be reduced to a minimum and the Pa-Repro has proven its suitability in previously performed studies on EDCs the test system is still the first choice for the completion of the here presented bioassay battery.

Concluding, it can be stated that the established bioassay battery is qualified for the analysis of different sample matrices with high relevance to endocrine disruption in the aquatic environment. Nevertheless, biological test systems are no ultimate but rather developing techniques which almost always could be improved. The completion of the present study therefore presents not the end of the establishing process but rather an interim goal of the ongoing research.

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Curriculum Vitae

Personal information

Name, Prenames	Maletz, Sibylle Xenia
Date of birth	27.06.1981
Place of birth	Heidelberg
Family status	Unmarried, 2 daughters
Nationality	German

Functions

Since 10/2010	Head of the cell culture laboratory at the department of ecosystem analyses of Prof. Henner Hollert (Institute for Environmental Sciences, RWTH Aachen University) Head of the research team for endocrine disrupting substances at the department of ecosystem analyses of Prof. Henner Hollert (Institute for Environmental Sciences, RWTH Aachen University)
Since 04/2009	Doctoral candidate at the Institute for Environmental Research (RWTH Aachen University, Germany) under supervision of Prof. Henner Hollert „Endocrine activity in the aquatic environment"
Since 12/2007	Scientific assistant at the Institute for Environmental Research; Establishment of several bioassays for the detection of endocrine disrupting compounds in complex environmental samples

Studies

Since 09/2008	Post gradual course "Skilled Ecotoxicologist"
10/2000 until 10/2007	Studies of biology, geography and political sciences at the University of Heidelberg, Germany
10/2007	1st state examination (teaching degree for secondary schools)
10/2006 until 11/2006	Research internship at the Institute for Environmental Chemistry & Health and Ecotoxicology (MTM Man, Örebro University, Sweden)
07/2006 until 01/2007	State examination thesis "Ecotoxicological characterization of suspended matter"

Internships

05/2007 until 07/2007	IVF lab of the Heidelberg fertility clinic, Heidelberg, Germany
03/2003 until 04/2003	Jatun Sacha Reserve, Napo Province, Ecuador

Education

06/2000	Matura at Heinrich-Sigmund-Gymnasium, Schriesheim, Germany

Qualifications

Laboratory	Management of a S1-facility for cell culture
	Cultivation of several cell lines (mammals, fish, yeast, bacteria)
	Establishment and implementation of the *in vitro* test systems (L)YES, ER CALUX®, T47D-KBluc, DR CALUX®, H295R Steroidogenesis Assay, cytotoxicity (neutral red), viability (MTT), Comet Assay, Ames Fluctuation Assay, ELISA
	Establishment and implementation of the *in vivo* reproduction test with the New Zeeland mudsnail *Potamopyrgus antipodarum*
Office	Preparation of subject-specific reports and presentations in English and German
	Microsoft Office (Word, Excel, Power Point), SigmaPlot and SigmaStat, GraphPad Prism, EndNote
Teaching	Supervision of BSc and MSc as well as diploma theses
	Supervision of practical courses
	Supervision of advanced trainings (PGC “Skilled Ecotoxicologist”)
Languages	German (native language), English (Advanced, B2), French (Basic), qualification in Latin

Projects

Leading	„Ökotoxikologische Bewertung der endokrinen Wirksamkeit von *Bacillus thuringiensis ssp. israelensis* (*Bti*)-Präparaten“ on behalf of the Landesanstalt für Umwelt, Messungen und Naturschutz Baden-Württemberg (LUBW, Germany)
Cooperation	The OECD validation program of the H295R steroidogenesis assay under leadership of ENTRIX and the Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
	„Pilotprojekt Kreiskrankenhaus Waldbröl – Eliminierung von Spurenstoffen aus Krankenhausabwässern mit Membrantechnik und weitergehenden Behandlungsverfahren“ under leadership of the Institute of Environmental Engineering, RWTH Aachen University, on behalf of the Ministerium für Umwelt und Naturschutz, Landwirtschaft und Verbraucherschutz des Landes Nordrhein-Westfalen (LANUV, Germany)
	„Strategy MicroPoll“ under leadership of the Ecotox Centre of the Eawag/EPF, ETH Zurich, on behalf of the Bundesamt für Umwelt (BAFU, Switzerland)
	„Modular Stepwise Procedure“ under leadership of the Ecotox Centre of the Eawag/EPF, ETH Zurich, on behalf of the Bundesamt für Umwelt (BAFU, Switzerland)
	„Tox-Box – Gefährdungsbasiertes Risikomanagement für anthropogene Spurenstoffe zur Sicherung der Trinkwasserversorgung“ under leadership of the Umweltbundesamt (UBA, Germany) on behalf of the Bundesministeriums für Bildung und Forschung (BMBF, Germany)

Scientific contributions

Research articles in international peer-reviewed journals

Bluhm K, Heger S, Agler MT, Maletz S, Schäffer A, Seiler TB, Angenent LT, Hollert H (2012): Assessment of the Ecotoxicological and Environmental Effects of Biorefineries: The Role of Green Chemistry in Biomass Processing and Conversion, 435-467.

Brinkmann M, Maletz SX, Krauss M, Bluhm K, Schiwy S, Kuckelkorn J, Tiehm A, Brack W, Hollert H (2014): Heterocyclic aromatic hydrocarbons show estrogenic activity upon metabolization in a recombinant transactivation assay. Environmental science & technology 48, 5892–5901.

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Heger S, Bluhm K, Agler MT, Maletz S, Schaeffer A, Seiler T-B, Angenent LT, Hollert H (2012): Biotests for hazard assessment of biofuel fermentation. Energy & Environmental Science 5, 9778-9788.

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Woelz J, Engwall M, Maletz S, Takner HO, van Bavel B, Kammann U, Klempt M, Weber R, Braunbeck T, Hollert H (2008): Changes in toxicity and Ah receptor agonist activity of suspended particulate matter during flood events at the rivers Neckar and Rhine - a mass balance approach using in vitro methods and chemical analysis. Environmental Science and Pollution Research 15, 536-553.

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Research articles submitted or to be submitted to international peer-reviewed journals

Lippmann N, Maletz S, Strauss T, Ratte HT, Hollert H (2014): A microcosm study on the endocrine activity of *Bacillus thuringiensis* var. *israelensis* (*Bti*). (To be submitted to Aquatic Toxicology)