Mechanism-specific toxicity bioassays for water quality assessment and effect-directed analysis

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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Nothing is softer or more flexible than water, yet nothing can resist it

(Lao Tzu)

Even small fish are fish

(Czech Proverb)

Summary

Biological assays have been applied to investigate freshwater quality for more than a century, and the public awareness of the threats of aquatic pollution has motivated advances in water quality regulations. In Europe, such a scenario led to the establishment of the Water Framework Directive (WFD) as a unified and harmonised framework for water protection, with the main objective to achieve a good water ecological and chemical status. Despite the recognized relevance of bioassays by scientists and national authorities, until now they are not recommended for direct application in the WFD monitoring activities. A reason for that is that there are remaining research questions that need further clarification before bioassays are integrated in water quality monitoring.

The EDA-EMERGE Marie Curie Initial Training Network, in which context the present thesis was developed, was set up to investigate and answer some of these questions. The project aimed at the assessment, monitoring and management of water quality in European river basins through different approaches, including the investigation and development of new effect-directed analysis (EDA) methods for the identification of toxicants in surface waters. For that, new bioanalytical, chemical and hyphenated methods were developed. In this thesis, mechanism-specific bioassays were newly developed, advanced or adapted for the assessment of emerging pollutants or water samples, and as guiding tools in EDA investigations.

The research questions guiding this thesis were: (i) How can mechanism-specific bioassays adequately be integrated into EDA?; (ii) How to advance aquatic relevant mechanism-specific bioassays?; (iii) Are mechanism-specific bioassays able to properly evaluate emerging pollutants as single compounds and as mixtures?; and (iv) How to efficiently apply bioassay battery approaches and what are their benefits for the water quality assessment? In order to answer these questions, the overall objectives of this thesis were: (1) To adapt bioassay protocols and develop respective testing strategies for application as guiding tools in EDA studies; (2) To develop aquatic relevant mechanism-specific bioassays utilizing zebrafish early life stages and zebrafish liver cell lines; (3) To evaluate the effects of emerging pollutants as single chemicals and as mixtures on aquatic organisms and *in vitro* bioassays; and (4) To apply and evaluate bioassays and bioassay battery approaches to investigate water sample extracts and emerging aquatic pollutants. These objectives were explored in complementary studies focusing on bioassay development and adaptation, followed by the application of bioassays to evaluate diverse aquatic pollutants and water samples, and

ultimately leading to a comprehensive multi-organism and multi-mechanism aquatic toxicity assessment approach. In parallel, other activities were developed in the context of the project, including an intensive training in EDA-related methods and a joint monitoring study for evaluating water samples from different European river basins in bioassays and chemicals analysis.

Initially, a literature review provided an overview of EDA investigations that applied bioassays with zebrafish as guiding tools, with mechanism-specific bioassays being identified as particularly useful for EDA investigations. Subsequently, mechanism-specific assays with zebrafish models were developed in the context of this thesis. One study focused on the development of a new method to evaluate chronic, delayed toxicity using zebrafish early life stages, which also identified early endpoints that can potentially predict later effects. Another study developed protocols to evaluate micronucleus occurrence in a zebrafish liver cell line and zebrafish larvae as a robust genotoxicity endpoint, and applied the methods to investigate genotoxic compounds. Further, the effects of neuroactive and neurotoxic compounds on the behavioural response of zebrafish larvae following a light-dark transition stimulus were also investigated.

Additionally, antiandrogenicity and the induction of the p53 protein pathway were assessed by using respective reporter gene cell-based assays. A testing strategy utilizing the p53 assay and a bioassay for cell viability assessment was applied to investigate genotoxic compounds as single exposures and mixtures. Antiandrogenicity assessment of surface water samples identified a particularly active sample, which was selected for a follow-up EDA investigation. Since only a limited sample volume was left, downscaled methods of dosing and exposure procedures had to be developed and validated using model (anti)androgenic compounds. Afterwards, the developed tools were applied in the EDA study.

Finally, an interlaboratory study involving different collaborating partners was organized within this PhD project. A basic bioassay battery containing organism-level and *in vitro* mechanism-specific assays was applied to investigate a pristine water extract spiked with emerging pollutants as single chemicals or mixtures. This study is expected to support and promote the use of a basic bioassay battery for water quality monitoring.

In summary, this thesis developed new and improved existing bioassays and bioassay testing strategies for future mechanism-specific toxicity investigations of aquatic emerging pollutants, chemical mixtures or water samples; or as guiding tools in effect-directed analysis.

Zusammenfassung

Biologische Tests werden seit mehr als hundert Jahre angewandt, um die Wasserqualität zu untersuchen, und das öffentliche Bewusstsein für die Gefahren durch Wasserverschmutzungen hat Fortschritte in der Trinkwasserverordnung herbeigeführt. In Europa kam es so zu Etablierung der Wasserrahmenrichtlinie (WRRL) als gemeinsamer Rahmen für den Gewässerschutz mit dem Ziel, einen guten ökologischen und chemischen Zustand zu erreichen. Trotz der anerkannten Bedeutung von Biotests durch Wissenschaftler und nationalen Behörden, werden sie bis heute nicht in den WRRL Überwachungsmaßnahmen für die direkte Anwendung empfohlen. Ein Grund dafür ist, dass offene Forschungsfragen bestehen, die zuerst einer Klärungen bedürfen, bevor Biotests in die Überwachung der Wasserqualität integriert werden können.

Die EDA-EMERGE Marie Curie Initial Training Network, in dessen Zusammenhang diese Arbeit entstanden ist, wurde gegründet, um einige dieser Fragen zu untersuchen und zu beantworten. Das Projekt war ausgerichtet auf die Bewertung, Überwachung und das Management der Wasserqualität in europäischen Flussgebieten, und schloss die Untersuchung und Entwicklung von neuen, Wirkungsorientierten Analyse (Effect-Directed Analysis - EDA) Methoden mit ein, zur Identifizierung von Schadstoffen in Oberflächengewässern. Dafür wurden neue bio- und chemisch-analytische und kombiniert-analytische („hyphenated“) Methoden entwickelt. Mit dieser Arbeit wurden Mechanismus-spezifische Biotests verbessert, neu entwickelt oder angepasst für die Bewertung von neuartigen Schadstoffe und Wasserproben, auch als Leitinstrumente für EDA-Untersuchungen.

Diese Doktorarbeit wurde von den folgenden Forschungsfragen geleitet: (i) Wie können Mechanismus-spezifische Biotests angemessen in eine EDA integriert werden?; (ii) Wie können aquatisch relevante, Mechanismus-spezifische Biotests weiterentwickelt werden?; (iii) Sind die Mechanismus-spezifischen Biotests in der Lage, neuartige Schadstoffe als Einzelverbindungen und als Mischungen angemessen zu bewerten?; und (iv) wie kann man Biotest-Batterien am effizientesten einsetzen und welche die Vorteile haben sie für die Wasserqualitätsbeurteilung? Um diese Fragen zu beantworten, ergaben sich für dieser Arbeit die folgenden übergeordneten Zielstellungen: (1) Anpassung von Biotest-Protokollen und Entwicklung entsprechende Teststrategien für die Anwendung in EDA-Studien; (2) Entwicklung von aquatisch relevanten Mechanismus-spezifischen Biotests mit frühen Lebensstadien des Zebrabärblings und mit Zebrabärblings-Leberzelllinien; (3) Bewertung der

Schadstoffwirkungen von neuartigen Schadstoffen, als einzelne Chemikalien und in Mischungen, auf Wasserorganismen und in den *in-vitro*-Biotests; und (4) Anwendung und Bewertung von Biotests und Biotest Batterien für die Untersuchung von Wasserprobenextrakte und neuartige Wasserschadstoffen. Diese Ziele wurden anhand von komplementären Studien zu Biotest-Entwicklung und Anpassung verfolgt, anschließend mit Anwendungsstudien für die Biotests zur Bewertung diverser Wasserschadstoffen und Wasserproben untermauert, was schließlich zu einem umfassenden Multi-Organismus und Multi-Mechanismus Bewertungskonzept für aquatische Toxizität führte. Parallel dazu wurden im Rahmen des Projekts andere Aktivitäten durchgeführt, darunter ein intensive Fortbildungen und eine gemeinsame Monitoringstudie zur Bewertung von Wasserproben aus verschiedenen europäischen Flusseinzugsgebieten mittels Biotests und chemischer Analyse.

Zunächst gab eine Literaturstudie einen Überblick über EDA Untersuchungen, die Biotests mit Zebrabärblingen eingesetzt hatten und Mechanismus-spezifische Biotests als besonders nützliche Assays für EDA identifiziert haben. Anschließend wurden Mechanismus-spezifische Tests mit Zebrabärblingen im Rahmen dieser Doktorarbeit entwickelt. In einer Studie wurde eine neuen Methode zur Bewertung von chronischer verzögerter Toxizität in den frühen Lebensstadien des Zebrabärblings entwickelt,, wobei auch Endpunkte identifiziert wurden, die möglicherweise spätere Effekte vorhersagen könnten. Eine weitere Studie entwickelte Protokolle zum Nachweis von Mikrokernbildung in einer Zebrabärblings-Leberzelllinie und in Zebrabärblingslarven als einen robusten Endpunkt für Genotoxizität,. Diese Methoden wurden anschließend für die Untersuchung von gentoxischen Stoffe verwendet. Zuletzt wurde die Wirkung von neuroaktiven und neurotoxischen Chemikalien auf die larvale Verhaltensantwort der Zebrabärblinge nach einem Hell-Dunkel-Übergangs-Stimulus untersucht.

Ergänzend wurden die antiandrogene Wirkung und Induktion des p53-Proteins mit einem Reportergen-Zellassay untersucht. Diese p53-Tests wurden angewandt, um genotoxische Chemikalien einzeln und in Mischung zu untersuchen. Die erzielten Ergebnisse unterstützen zukünftige Anwendungen dieser Methode für die Untersuchung von Wasserproben. Die Untersuchung der Antiandrogenität von Oberflächenwasserproben identifizierte eine besonders aktive Probe, die dann für EDA-Folgeuntersuchung ausgewählt wurde. Da nur ein begrenztes Probenvolumen übrig ware, mussten spezielle Dosierung- und Expositionsverfahren für kleine Volumina entwickelt und mit (anti)androgenen Modelsubstanzen validiert werden. Anschließend wurden die entwickelten Methoden in der EDA-Studie angewendet.

Schließlich wurde im Rahmen des Projekts ein Ringtest mit den verschiedenen Kooperationspartnern organisiert. Dazu wurde eine einfache Biotest- Batterie mit organismischen und *in vitro* Mechanismus-spezifische Tests ausgewählt für die Untersuchung von mit neuartigen Schadstoffen als Einzelstoffe oder Mischungen gespikten Wasserextrakten. Von dieser Studie wurde erwartet, dass sie die Verwendung eine Biotest-Batterie zur Überwachung der Wasserqualität stützen und fördern würde.

Zusammenfassend werden mit dieser Doktorarbeit neue und verbesserte bestehende Biotests und Bioteststrategien für zukünftige Mechanismus-spezifische Toxizitätsuntersuchungen für neuartige aquatische Schadstoffen, Mischungen oder Wasserproben entwickelt und bereitgestellt. Diese verbesserten Bioassays können bestimmend zukünftige EDA Untersuchungen leiten.

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1.1 Bioassays in water quality assessment

1.1.1 Historical perspective of bioassay application in water quality assessment

Biological assays and toxicity tests have been applied to investigate freshwater quality for more than a century. According to the review by Huhn (1989), the first report on the use of fish acute toxicity tests for the evaluation of spiked river water dates back to the 19th century, while *in situ* and laboratory studies with fish to assess stream water contamination by mining activities were reported in the 1920s - 30s. Systematic investigations of river water quality through field and laboratory work and involving tests with different species of fish and invertebrates, including *Daphnia magna*, were already developed in the 1930s (Fig. 1.1) (Ellis 1937).

Figure 1.1: Already in the 1930s, bioassays with fish and invertebrates were applied to evaluate stream water and aquatic pollutants by the United States Bureau of Fisheries. Investigations utilized "laboratory trucks" for field work (left); and temperature-controlled cabinets for tests with Daphnia magna (right). From Ellis, 1937.

Important advances were made towards the integration of bioassay evaluation for the assessment of industrial wastewaters and aquatic pollutants in the next years, motivated in great part by the public concern on threats to aquatic systems (Cairns 1966). For instance, protocols for fish toxicity assessment gave origin to a standard method to evaluate industrial effluents (ASTM 1960), and the importance of tests with other organisms such as algae and invertebrates was also promoted (Patrick et al. 1968). As discussed by Cairns (1966), the 'growing public appreciation of the economic value of water resources' was an important factor to improve the degree of protection of water bodies and consequently to promote the regulatory application of bioassays in industrial wastewater assessment. Consequently there was the development or improvement of water protection regulations in different countries for controlling aquatic pollution. Germany was among the first countries to promote the application of bioassays for the assessment of wastewater effluents through the

implementation of the Wastewater Act (Abwasserabgabengesetz, (1976). Also in the United Kingdom similar approach was developed, with the recommendation of a fish toxicity test for the regulatory evaluation of effluents (HMSO 1969).

After the advent of the European Union in the 1990s, the adoption of the Water Framework Directive (WFD) in 2000 established a common framework for water protection within the different member states, having as main objective to achieve good water ecological and chemical status (European_Union 2000). Its implementation promoted and continues promoting important advances for the protection of water bodies in Europe. Within the WFD, bioassays provide the input for the derivation of environmental quality standard (EQS) values, which will guide the verification of the chemical status assessment (EC 2011). However, despite their recognized relevance by many scientists and national regulatory agencies, until nowadays bioassays are not yet recommended for direct application in the WFD monitoring activities. This situation is fortunately on the verge of change, due to different initiatives demonstrating the multiple benefits of bioassay integration in water quality assessment.

1.1.2 Overview on available bioassays for water quality assessment

Experimental models from varied levels of biological organization can be applied to evaluate water quality and aquatic pollutants, and many tests are described as specific procedures such as OECD guidelines and ISO standards. Methods with organisms from different trophic levels and ecological niches are available, like tests with primary producers (e.g. microalgae, macrophytes); invertebrates (e.g. primary consumer filter-feeders daphnids, interstitial nematodes); and different life stages of fish (ranging from early embryos to adults) (OECD 2004, 2011, Hoss et al. 2012, OECD 2013b, Feiler et al. 2014). Also many *in vitro* (i.e. sub-organism level) methods, which are often performed on the cellular or molecular levels, have recognized value for testing aquatic pollutants and samples. Those include assays with a great diversity of cell-lines from different tissues and organisms, as well as cell- or yeast-based reporter gene assays, and tests with bacteria species (Bopp and Lettieri 2008, OECD 2012c, d, Reifferscheid et al. 2012, ISO 2013).

Evaluated endpoints in the organism- and *in vitro* levels can cover non-specific effects, such as impairment of survival or cellular viability, respectively (OECD 2013b, Riss TL 2013). In addition and very importantly, mechanism-specific endpoints for different toxicity modes-of-action can also be evaluated. Among those, toxicity mechanisms or pathways that involve endocrine disruption, genotoxicity and neurotoxicity can produce effects that are both ecologically-relevant as well as present concerns for human health. Endocrine disruption,

which can interfere with animal development and reproduction, can be measured as organism-level reproductive effects in e.g. fish or invertebrates (OECD 2009, 2012b) or by means of *in vitro* assays to identify interferences with endocrine (van der Burg et al. 2010, Kunz et al. 2015) and aryl hydrocarbon (Eichbaum et al. 2014) receptor binding. Genotoxicity, which causes a range of effects such as impairment of offspring survival and tumour development, can also be evaluated by organism-level and *in vitro* bioassays (Kosmehl et al. 2006, Fassbender and Braunbeck 2013b, Brinkmann et al. 2014a, OECD 2014). In the recent years, scientific knowledge and public awareness on the risks of neurotoxicity and behavioral effects are motivating the integration of respective endpoints and methods to evaluate neuroactive and neurotoxic aquatic pollutants such as many pharmaceuticals and pesticides (Stehr et al. 2006, Painter et al. 2009).

Zebrafish early-life stages are particularly interesting organisms for aquatic ecotoxicology. Embryos and early larvae are well studied models that present the organism-level complexity, being possible to use these early stages to substitute experiments with adult fish (Embry et al. 2010). In this way, methods can contribute to refine and reduce investigations with adult fish, in agreement with the 3Rs principles of alternative (eco)toxicology methods (Kandárová and Letašiová 2011). Further, zebrafish up to 5 days post fertilization (dpf) are not protected under the European regulation (Strahle et al. 2012), which facilitates experimental planning. The comparability of results from survival tests using adult or early embryos and larvae stages lead to the development of the Fish Embryo Toxicity (FET) test, which is recommended to be used as often as possible in replacement to acute toxicity tests with adults (Nagel 2002, Knobel et al. 2012, OECD 2013b). Moreover, sublethal endpoints can also provide indications of involved mechanisms of toxicity, and of the occurrence of chronic or delayed toxicity (Villeneuve et al. 2014, Di Paolo et al. 2015a).

1.1.3 Effect-based assessment in the context of the Water Framework Directive

An important step towards the integration of bioassays and biomarkers in water quality assessment in Europe was taken with the publication of a technical report on aquatic effect-based monitoring tools in the context of the WFD. The report was developed by the Chemical Monitoring and Emerging Pollutants group, a sub-group of the Working Group on Chemical Aspects under the WFD Common Implementation Strategy. Therefore the activity involved stakeholders from several member states, and in addition it was supported by different scientific experts. The main objective of the report was to identify bioassays and biomarkers

that can be used within the surveillance, operational and investigative WFD monitoring programmes (Carere et al. 2012, Wernersson et al. 2014, 2015).

The motivation for such an activity was that, although there is a clear demand and interest for the application of bioassays and biomarkers in water quality assessment, and in fact that already happens on the national and regional levels, there is a lack of regulatory basis for bioassay application within the WFD. Therefore bioassay monitoring of water bodies is neither a requirement nor there are clear guidelines on how to integrate obtained results in environmental management. Not surprisingly, environmental authorities often decide to focus their efforts on the performance of the legally needed chemical and ecological status assessments. Even if the EQS values that guide chemical status assessment are developed with basis on bioassay results (EC 2011), these are verified for a very limited number of pre-selected chemicals and require the previous knowledge on which chemicals to measure. While biological aspects such as effects at the population and community levels are considered in the ecological status assessment, such indicators are not of direct use to verify the biological relevance of chemical status in each water bodies.

Bioassays on the other hand make it possible to evaluate the combined effects of chemical mixtures that are often present in water samples, ultimately helping to link the chemical and ecological status assessment. Further, although bioassays deal with lower biological organizational levels, they produce in comparatively much shorter periods of time measurable responses that can provide immediate input to the assessment of water quality (Fig. 1.2).

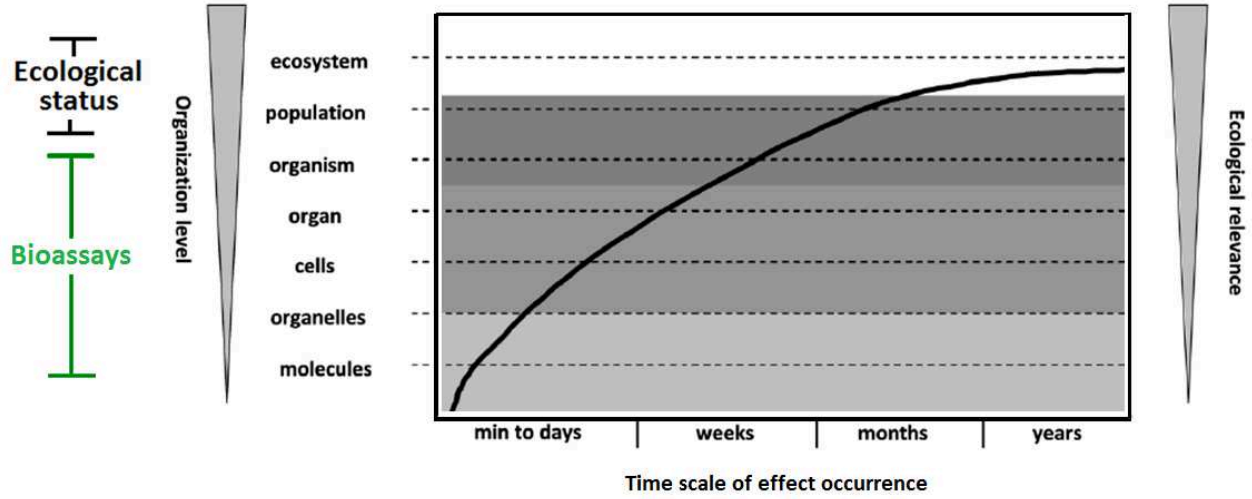


Figure 1.2: While the ecological status assessment deals with effects at the ecosystem and population levels, bioassays evaluate effects on the organism- and lower levels of biological organization (left Y-axis). Although the ecological relevance also decreases (right Y-axis), the manifestation of effects in bioassays occur in the time scale of minutes up to weeks, while the ecological status assessment

evaluate effects that take months up to years to develop (X-axis). Adapted from Wernersson et al. (2014).

The report discussed the aspects of the application of bioassays not only for surface water quality but also for sediments; their use for the prioritisation of water bodies for further monitoring; and as early warning systems, particularly when applied as bioassay batteries. Following the report publication by the European Commission (Wernersson et al. 2014), it was also published in a manuscript format in an open access peer-reviewed journal (Wernersson et al. 2015). In this way its content is freely available to the different water quality stakeholders and the public, making possible to extensively promote effect-based tools for water quality assessment. That is a very important aspect since, nowadays as in the past, public awareness continues to motivate advances on water protection issues (EC 2012).

1.1.4 Bioassays as guiding tools in effect-directed analysis of surface waters

Effect-directed analysis (EDA) is an investigative approach aimed at identifying the main chemicals responsible for a complex sample toxicity or bioactivity that cannot be fully explained by the compounds measured through target analysis. The method integrates sequential chemical fractionations, applied to reduce the sample complexity, and bioassay evaluation, which will identify bioactivity occurrence and ultimately guide the EDA investigation. Target and non-target chemical analysis is then applied to identify potential active compounds, which will also be submitted to bioassay evaluation to confirm their contribution to the sample bioactivity (Fig. 1.3) (Brack 2003, Brack et al. 2007).

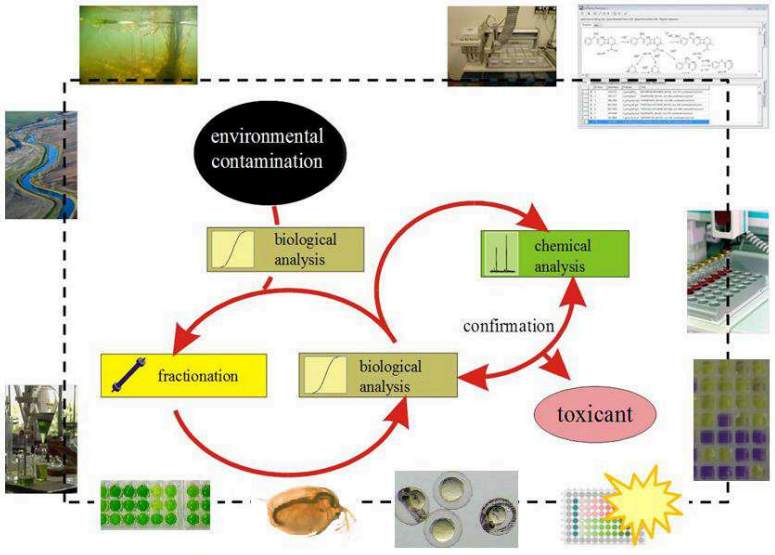


Figure 1.3: Schematic representation of the effect-directed analysis investigative approach. Bioassays are responsible for crucial investigative steps involving biological activity analysis, including identification of active samples and respective fractions, and confirmation of the activity of candidate chemicals identified in chemical analysis. Adapted from the EDA-EMERGE project website (http://eda-emerge.eu).

EDA of environmental matrices represents a relatively young research filed, with the first studies being developed in the late 1970s and early 1980s (Schuetzle and Lewtas 1986). Along the years, the increasing recognition of the relevance of EDA for water and sediment quality monitoring led to the development of the Marie Curie Initial Training Network (ITN) EDA-EMERGE, in which context the present thesis was carried out. Its overall aim were to advance new EDA tools and to train involved scientists in the interdisciplinary methods for EDA application to water quality assessment (Brack et al. 2013). One main objective of the ITN was to develop and improve bioassay methods for their application in EDA of surface waters. Recently, there was the publication of a detailed guidance paper (Brack et al. 2016) to support the planning and development of successful EDA case studies, which discussed diverse aspects of bioassay selection and design for application in EDA.

1.2 Research needs for the application of bioassays in water quality assessment

In order to promote the application of bioassay for the assessment of water quality, further investigations are needed to advance and optimize remaining research aspects.

1.2.1 Bioassay development and adaptation for application in EDA

Bioassays must be adapted or developed to attend EDA specific requirements (Brack et al. 2016), while it is desirable to simultaneously maintain assay characteristics such as sensitivity and specificity. Extensive chemical fractionation results in sample amount limitation that in its turns restricts the volumes that can be investigated in bioassays, consequently requiring downscaling of procedures while maintaining high-throughput potential. Additionally, development of appropriate testing strategies according to bioactivity, assay and sample composition can improve the guidance of the study. That involves for example decision on adequate concentration ranges in different testing phases in order to properly identify the bioactivity. Further, the method blanks should also be evaluated regarding the occurrence of non-specific and specific bioactivities that could interfere with the investigation. In both cases, complementary methods or endpoints might be needed. Reduced cell viability can lead to false negatives in reporter gene cell-based assays measuring antagonistic activity (Huang et al. 2014). In contrast, *in vitro* genotoxicity tests recommend inclusion of cytotoxic concentrations in the evaluated range (OECD 2014).

These bioassay and testing strategy improvements are crucial for the role of EDA in supporting environmental risk assessment. That is valid particularly for emerging contaminants, for which none of few specific mechanism-specific bioassays are recognized

even though there is clear indication of their significant effects in the environment. In this sense emerging pollutants present a specific challenge to both EDA and mechanism-specific assays, requiring methods that are specific to their bioactivity and also the establishment of relative potencies to model compounds. The development of such bioassays and EDA approaches will improve their respective contributions as exposure assessment and causation lines of evidence in weight-of-evidence frameworks (Chapman and Hollert 2006, Brack et al. 2007, Hecker and Hollert 2009).

1.2.2 Mechanism-specific toxicity assessment in water quality

As mentioned above, bioassay ability to evaluate mechanism-specific endpoints makes it possible to evaluate effects that present ecological and/or human health relevance. Ecotoxicology often makes use of bioassays originally developed for human risk assessment, and one of the reasons for that is simply the lack of adequate methods utilizing more relevant experimental models such as fish cells or embryos. That is not an optimal situation, since interspecies differences such as in toxicokinetics and toxicodynamics aspects can make it very challenging to extrapolate result interpretation in the context of potential ecological effects. Therefore the development of methods utilizing relevant aquatic organism models is highly desirable for water quality assessment. Methods with fish cell lines can measure endpoints traditionally measured through mammal cells, and fish embryos can be applied to investigate mechanisms of toxicity that are routinely studies using rodents (Kosmehl et al. 2006, Bopp and Lettieri 2008, Brinkmann et al. 2014a).

1.2.3 Bioassay battery approach for water quality assessment

Testing strategies applying bioassay batteries are based on the concept that an overview on potential toxic effects to different organisms and through diverse mechanisms cannot be provided by one test only. In aquatic systems, sensitivity to different toxicants can vary between organisms from different taxa and ecological niches. Further, some mechanisms of toxicity such as endocrine disruption and mutagenicity present additional risks that should be investigated by specific bioassays. Multi-taxa toxicity assessment is already applied for EQS derivation within the WFD, which requires evaluation of acute and chronic data for a primary producer alga or macrophyte; daphnids or another invertebrate; and fish (EC 2011). Similar approach has also been proven to be of relevance for the ecotoxicological characterization of wastes, in addition to genotoxicity and mutagenicity assays (Pandard et al. 2006, Gartiser et al. 2009, Römbke 2009). Estrogenicity assessment has already been recommended the

monitoring of water bodies, which is reinforced after the recent inclusion of estrogenic pharmaceuticals in the WFD watch list (Hecker and Hollert 2011, Loos 2012, EC 2013).

Still, a basic battery for the assessment of water quality on the regulatory level is not available yet. Currently there are ongoing activities for the establishment and validation of low volume, high-throughput bioassay batteries for water quality assessment and monitoring activities (Brack et al. 2013, Altenburger et al. 2015, Brack et al. 2015, Neale et al. 2015, Schulze et al. 2015). An important initiative was the bioassay battery interlaboratory study developed in the context of the NORMAN network (*www.norman-network.net*), as presented in Chapter 10 of this thesis, which organization was taken over as part of this thesis development. The demonstration of the performance and usefulness of a basic test battery will further promote the inclusion of bioassays in water quality monitoring (Wernersson et al. 2015).

1.2.4 Evaluation of emerging pollutants as single-compounds and mixtures

Emerging pollutants such as pharmaceuticals and personal care products are a current priority in regulatory water quality monitoring (Loos et al. 2009, Brack et al. 2012). These are environmental contaminants currently not included in routine monitoring programmes that are known or suspected to cause adverse ecological or human health effects; and which are potential candidates for future regulation depending on their (eco)toxicity, public perception, and environmental occurrence monitoring data (NORMAN-Network , Geissen et al. 2015). While the identification of emerging pollutants in the various environmental compartments is needed to understand their occurrence and fate, it is also a challenge for analytical methods due to the high number of compounds / transformation products and often low concentrations (Geissen et al. 2015). In this context, bioassay analysis of water samples can provide effect-data that, when combined with chemical analysis, will support the identification and prioritization of chemicals based on environmental occurrence and biological effects (Brack et al. 2012). Additionally, bioassays can provide information on the mixture toxicity of environmental pollutants, which is more representative of their environmental occurrence (Petrie et al. 2015).

1.3 Aims and structure of the thesis

The research problems and needs mentioned above provided the basis to formulate the overall **research questions** and sub-questions addressed in this thesis, as follows:

(i) How mechanism-specific bioassay protocols can adequately be adapted for integration in EDA?

a. Can downscaling bioassay procedures be developed while also maintaining the assay accuracy?
b. Does the protocol adaptation interfere with respective bioassay specificity or sensitivity?
c. Are the assay quality criteria maintained after protocol modifications?

(ii) How to developed aquatic relevant methods that are also mechanism-specific bioassays?

a. Are zebrafish early life stages adequate aquatic organism models for the establishment of mechanism-specific bioassays?
b. Does a zebrafish-derived cell line represent an adequate *in vitro* aquatic model for the establishment of mechanism-specific bioassays?

(iii) Are bioassays able to evaluate the bioactivity of aquatic emerging pollutants as single compounds and as mixtures?

a. What are the respective contributions of organism-level and *in vitro* assays for the assessment of emerging pollutants as single exposures or as mixtures?
b. Do mechanism-specific bioassays maintain their accuracy even when non-specific toxicity is present in evaluated samples?
c. Does the presence of a water extract matrix interfere with bioassay evaluation of emerging pollutants and their mixtures?

(iv) How to efficiently apply and what are the benefits of bioassay battery approaches for water quality assessment and EDA investigations?

a. What is the contribution of organism-level tests for bioassay battery approaches?
b. What is the contribution of mechanism-specific methods for bioassay battery approaches?
c. Do the different methods that compose a bioassay battery present complementary sensitivity and specificity?

In order to answer these research questions, the overall **objectives** of this thesis were:

- To adapt bioassay protocols and develop respective testing strategies for application as guiding tools in EDA studies;
- To develop aquatic relevant mechanism-specific bioassays utilizing zebrafish early life stages and zebrafish liver cell lines for application in water quality assessment and EDA;

- To evaluate the effects of emerging pollutants as single chemicals and as mixtures on aquatic organisms and *in vitro* bioassays.
- To apply and evaluate bioassays and bioassay battery approaches to investigate water sample extracts and emerging aquatic pollutants;

Such objectives were explored in complementary studies along this thesis by focusing on bioassay development and adaptation, followed by application of bioassays to evaluate diverse aquatic pollutants and water samples, and ultimately leading to a comprehensive multi-organism and multi-mechanism aquatic toxicity assessment (Fig. 1.4).

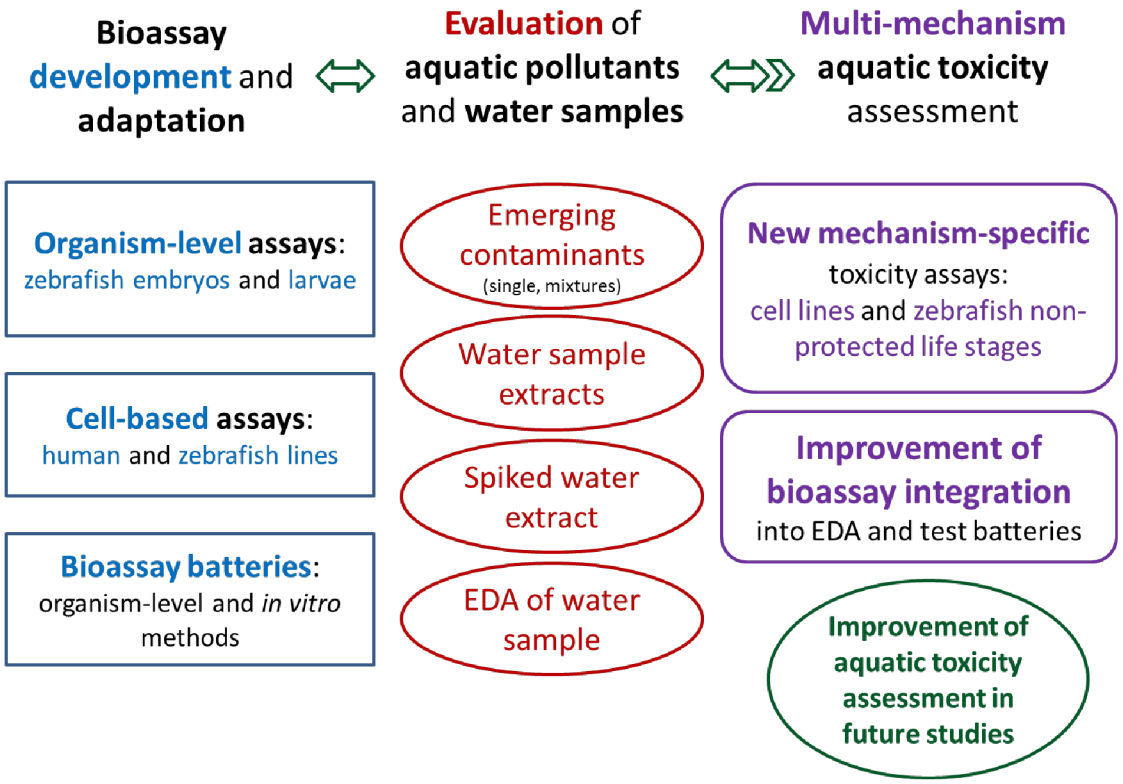


Figure 1.4: The thesis research questions and objectives were investigated in complementary studies that focused on aspects of bioassay development and adaptation (blue boxes in left), followed by application of bioassays to evaluate diverse aquatic pollutants and water samples (detailed in red central boxes), leading to a comprehensive multi-organism and multi-mechanism aquatic toxicity assessment (right purple boxes).

The outcomes are presented in the following Chapters of this thesis.

Chapter 2 presents a brief overview on the EDA-EMERGE project, in which context the thesis was developed; followed by the report on the common integrated study that applied an EDA monitoring strategy to investigate surface water samples from different European river basins. A bioassay battery integrating aquatic organism and *in vitro* mechanism-specific assays was applied to evaluate water sample fractions collected using a large-volume solid phase extraction instrument, which were also submitted to chemical analysis.

Chapter 3 summarizes and discusses the outcomes of a literature review that aimed to contribute for the optimal application of zebrafish bioassays in EDA. A critical overview of previous EDA investigations that applied zebrafish bioassays is provided, with the discussion of the potential contribution of such methods for EDA and proposition of testing strategies to improve future studies. The mains findings regarding experimental models, exposure setups, and evaluated endpoints are discussed in terms of their contribution for the development of successful EDA studies, and potential improvements for future studies.

In **Chapter 4**, zebrafish early life stages are utilized as test organisms in the development of a new bioassay, the prolonged fish embryo toxicity test, proposed as a method to investigate delayed toxicity along the fish larval development. PCB126 was utilized in exposure tests as a model chemical; and chemical analysis of exposure solutions and of embryos and larvae allowed the discussion in terms of aqueous and internal concentration values. Following early chemical exposure during the non-protected stages (i.e. between days 0 and 5 dpf), the larvae were then reared in clean water until 28 dpf. Survival, morphological and behavioral endpoints were assessed along the embryonic-larval development; and analysis of growth and metamorphosis parameters were scored by the end of test. Outcomes are discussed in the context of the ecological relevance of delayed effects, and on the potential use of early endpoint assessment to identify risks of later effects.

Chapter 5 presents the development and outcomes of an environmental monitoring study that investigated surface water samples collected at more than 30 locations near wastewater treatment plants in the catchment of the Saale and Mulde Rivers, which are among the most polluted tributaries of the Elbe River. Water sample extracts were evaluated regarding antiandrogenic activity utilizing an androgen receptor reporter gene cell-based assay; and regarding cell viability utilizing three complementary colorimetric methods. Additional results were obtained with the evaluation of samples through target chemical analysis. Mass-balance analysis estimated how much of the identified bioactivity could be explained by the measured compounds. As an outcome, a particularly highly active sample was identified and selected for further EDA investigation. Since limited amount of the water sample was available for the complete study, downscaling of bioassay steps were required, as described in the next chapter.

Chapter 6 describes the development of downscaling exposure and dosing procedures in an androgen receptor reporter gene cell based assay, in order to integrate assay in the EDA investigation of a limited-volume water sample. After the development phase, the

downscaling methods were applied to evaluate androgen receptor agonistic (5α-dihydrotestosterone) and antagonistic chemicals (flutamide, bisphenol A, 1-hydroxypyrene, triclosan) with results being discussed in terms of assay acceptance criteria and in comparison to previous studies. Finally, the miniaturized method was applied in the antiandrogenicity EDA case study.

Chapter 7 reports the potential of genotoxic compounds as single-chemical or binary / tertiary mixtures to activate the p53 tumour suppression protein pathway, a main driver of cell fate following DNA damage, utilizing a p53 response element reporter gene cell based assay. There was also evaluation of cell viability following the different exposures, which results supported bioassay planning and interpretation. Evaluated compounds included the drug actinomycin D and the prodrug cyclophosphamide as model p53-inducers in the absence and presence of metabolic activation, respectively; and the two nitroaromatic compounds 4-nitroquinoline 1-oxide (4-NQO) and 3-nitrobenzanthrone (3-NBA). Results are discussed in terms of relevance of the assay for evaluation of chemical mixtures and water samples. Further, a test strategy for the integrated assessment of cell viability and p53 induction is discussed.

Chapter 8 presents the development of new bioassays for the assessment of micronucleus occurrence in the aquatic relevant models zebrafish liver cell line and in zebrafish early larvae. Micronucleus formation is a robust chromosome damage and also a multi-target genotoxicity endpoint that can be reliably assessed *in vivo* and *in vitro*, therefore respective zebrafish assays are highly meaningful for aquatic genotoxicity assessment. Known genotoxic chemicals and environmental contaminants (4-NQO, CPP, 3-NBA, Benzo[a]pyrene) were utilized for experimental exposures with both models. For the assay with cells, there was determination of the cell line doubling time utilizing an incubator with live cell imaging system; and cytotoxicity tests were performed in parallel. For the assay with larvae, there was development of a method to obtain whole body cell suspensions; and lethal and sublethal morphological effects were assessed. Results are discussed in terms of the values of the methods for aquatic genotoxicity assessment.

Chapter 9 presents an interlaboratory study developed in the context of the NORMAN network (www.norman-network.net), which organization was taken over as part of this thesis development. The study aimed to demonstrate the suitability of a bioassay battery to evaluate water sample extracts spiked with emerging pollutants (triclosan, acridine, 17α-ethinylestradiol, 3-NBA) as single chemicals and mixtures. Applied tests included organism

level (algae, daphnids, zebrafish embryos), estrogenicity (ER-Luc, YES) and mutagenicity (Ames fluctuation) assays. Results are discussed in comparison with previous studies considering protocol differences; in terms of test battery complementary sensitivity and specificity; and regarding advances towards the application of bioassay batteries in water quality assessment.

Chapter 10 presents the evaluating of chemicals regarding their potential to be applied in positive control conditions for behavioural assessment of zebrafish larvae in a light-dark sudden transition test. Tested compounds included the neuroactive or neurotoxic chemicals ethanol, caffeine, nicotine, α-cypermethrin and chlorpyrifos. In addition, there was interlaboratory comparison for assays performed at RWTH Aachen and at the Institute for Environmental Studies (IVM), VU Amsterdam. At RWTH behavioral tests were performed in a self-built system and evaluated using the EthoVision (Noldus) software; while at IVM the system and software from Zebralab (ViewPoint) were utilized. Results are discussed in terms of relevant parameters for behavioral assessment; in terms of comparability of results obtained in the two institutes; and regarding the suitability of test chemicals for application in positive control conditions.

Chapter 11 presents the main conclusions of the different chapters and the expected outcomes regarding advancing bioassay application in EDA of surface water and in water quality assessment.

Chapter 2: EDA-EMERGE Initial Training Network and European Demonstration Program

Part of this chapter has been published as:

Brack, W., Govender, S., Schulze, T., Krauss, M., Hu, M., Muz, M., Hollender, J., Schirmer, K., Schollee, J., Hidasi, A., Slobodnik, J., Rabova, Z., Ait-Aissa, S., Sonavane, M., Carere, M., Lamoree, M., Leonards, P., Tufi, S., Ouyang, X., Schriks, M., Thomas, K., de Almeida, A., Froment, J., Hammers-Wirtz, M., Ahel, M., Koprivica, S., Hollert, H., Seiler, T.-B., Di Paolo, C., Tindall, A. and Spirhanzlova, P. (2013).
EDA-EMERGE: an FP7 initial training network to equip the next generation of young scientists with the skills to address the complexity of environmental contamination with emerging pollutants.
Environmental Sciences Europe 25: 18. DOI: 10.1186/2190-4715-25-18

Part of this chapter is being prepared for submission to the peer reviewed journal Chemosphere as:

Schulze, T., Ahel, M., Ahlheim, J., Brion, F., Di Paolo, C., Hollender, J., Hollert, H., Kloß, A., Koprivica, S., Krauss, M., Schollee, J., Shao, Y., Slobodnik, J., Sonavane, M., Tousova, Z., Walz, K.H., and Brack, W.
A novel device for on-site large volume solid phase extraction for the targeted and nontargeted chemical as well as effect-based screening of water resources.

Part of this chapter is being prepared for submission to the peer reviewed journal Science of the Total Environment as:

Tousova Z., Oswald, P., Schulze, S., Muz, M., Hu, M., Brack, W., Krauss, M., Koprivica, S., Ahel, M., Schollee, J., Hidasi, A., Hollender, J., Suter, M., Sonavane, M., Ait-Aissa, S., Creusot, N., Brion, F., Di Paolo, C., Hollert, H., Froment, J., Almeida, A.C., Thomas, K., Tollefsen, K.E., Tufi, S., Ouyang, X., Leonards, P., Lamoree, M., Torrens, V.O., Schriks, M., Špírhanzlová, P., Tindall, P., Blaha, L., and Slobodnik, J.
European Demonstration Program on the effect-based and chemical identification and monitoring of emerging organic pollutants with adverse effect potential in European surface waters using large volume sampling tool in combination with analysis by battery of bioassays and chemical screening.

2.1 Introduction

The present thesis was developed in the context of the Marie Curie initial training network ITN "EDA-EMERGE novel tools in effect-directed analysis to support the identification and monitoring of emerging toxicants on a European scale" of the seventh European framework program call - FP7. A main aim of the ITN was to prepare the participant research fellows for the assessment, monitoring and management of water quality, with focus on European river basins. That was achieved through theoretical and practical training in diverse multidisciplinary analytical chemistry and biology methods, with focus on how to apply different tools to investigate aquatic contamination and effects, and on how to identify cause-effect relationships. Another aim of the project was to investigate and develop new effect-directed analysis (EDA) methods and approaches for the identification of toxicants in European surface and drinking waters. For that, method development occurred within three work packages:

(i) Bioanalytical tools: development of innovative mode-of-action-based biodiagnostic tools, including *in vitro* and *in vivo* bioassays;

(ii) Chemical tools: development of powerful fractionation and cutting edge, analytical, and computational structure elucidation tools; and

(iii) Hyphenated tools: development of hyphenated tools and automation processes to integrate the different EDA steps in a continuous workflow.

The present thesis was developed in the context of the "Bioanalytical tools" work package.

The European Water Framework Directive (WFD) (European_Union 2000) provided the regulatory background to the project. However the fact that its chemical monitoring is focused on the priority substances, which concentrations in samples often do not explain the respective bioactivity identified by bioassays, is a critical aspect. In addition, the numbers of chemical substances that potentially can reach the environment is increasing steadily, as identified by chemicals registered in the Chemical Abstract Service (www.cas.org): from 26 million chemicals in 2005, numbers increased to 72 million by June 2013 (Brack et al. 2013), and up to more than 110 million by May 2016. This continuous increase in the diversity and numbers of compounds increases the challenge to water quality monitoring, requiring approaches that can support the prioritization of chemical monitoring, such as EDA.

In this context, the EDA-EMERGE project contributed with the development and application of EDA methodologies and testing strategies, in the national and transboundary river basin levels. Further, international networking was promoted within the project

participants and collaborators. Also, the project promoted strong networking between the academic, private, and regulatory levels, and counted with an international advisory board (Brack et al. 2013).

2.2 Participant institutes

The project included participants from different countries and institutes (Figure 2.1), being:

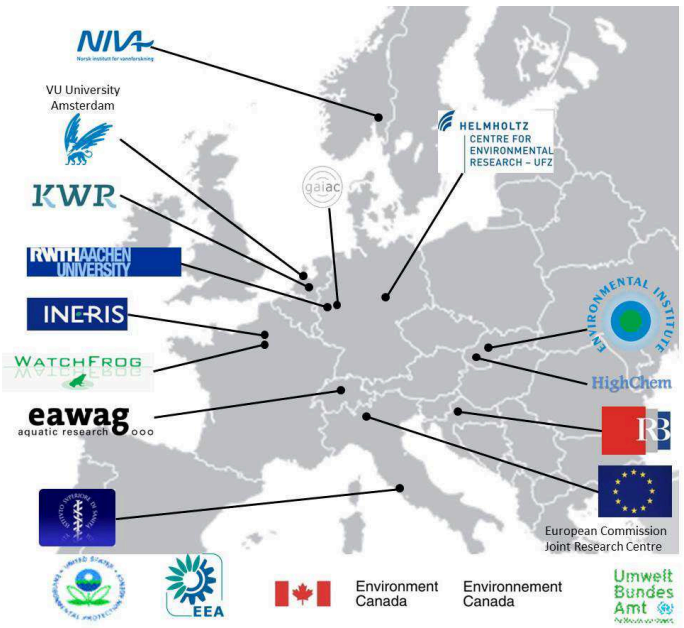


Figure 2.1: Participants of the EDA-EMERGE project (http://eda-emerge.eu).

(i) Two universities:

1) Free University of Amsterdam, the Netherlands;
2) RWTH Aachen University, Germany;

(ii) Six research centers:

3) Helmholtz Centre for Environmental Research (UFZ), Germany;
4) Institut National de l'Environment Industriel et des Risques (INERIS), France;
5) Swiss Federal Institute of Aquatic Science and Technology (Eawag), Switzerland;
6) Rudjer Boskovic Institute, Croatia;
7) Norwegian Institute for Water research (NIVA), Norway;
8) the Italian Institute of Health (ISS), Italy;
9) the JRC);

(iii) Five private companies:

10) Environmental Institute, Slovakia;

11) KWR Watercycle Research Institute, the Netherlands;
12) WatchFrog, France;
13) HighChem, Slovakia;
14) Gaiac Research Institute for Ecosystem Analysis and Assessment, Germany.

Further, there was collaboration with researchers form the NORMAN network for monitoring of emerging pollutants (Brack et al. 2012), the German Federal Environmental Agency (UBA), the US-EPA and from Environment Canada.

2.3 Training program

As mentioned, extensive training of participant fellows in interdisciplinary methods and skills required for monitoring and EDA of surface waters was a main objective of the project. At the beginning of the project, a summer-school was provided offering courses on general EDA aspects, the European regulatory framework, sampling and sample preparation, separation and fractionation, diverse bioassay methods and tools, chemical analysis, and structure elucidation. Along the project, different specialized training courses were followed (Table 2.1). During this thesis, all offered courses were followed.

Table 2.1: EDA-EMERGE Training Events.

Training event	Topic	Institute and location	Date
SC2	In vitro and in vivo assays of endocrine disrupting chemicals using zebrafish models	INERIS, Verneuil-en-Halatte, France	30-31 Jan 2013
SC3	Chemical screening, prioritization of environmental pollutants and data storage in European databases	EI, Kos, Slovak Republic	23-24 May 2013
SC5	Course on fate of emerging pollutants in the aquatic water cycle	Eawag, Dübendorf, Switzerland	2-3 Jul 2013
SC4	Special course on the water cycle and human health	KWR, Nieuwegein, the Netherlands	20 Jan 2014
SC9	Hyphenation of cell-based assays with microfractionation procedures	VU-IVM, Amsterdam, the Netherlands	21-22 Jan 2014
SC8	Biotechnology for environmental issues	Watchfrog, Evry, France	18 Mar 2014
SC1	Advanced course on preparative and analytical chromatography in EDA	IRB, Zagreb, Croatia	02-03 Jul 2014
LC2	Film Course and Statistic Course	RWTH Aachen, Germany	27-28 Oct 2014
SC7	Special course on theory and practise of gene arrays	Organised by NIVA at RWTH Aachen	29 Oct 2014

SC10	Science-based policy support with regard to emerging pollutants.	ISS, Rome, Italy	04 Dec 2014
LC3	Monitoring, Regulation, Assessment, WFD	ISS, Rome, Italy	04 Dec 2014
Final conference	EDA-EMERGE PhD student Conference	UFZ, Leipzig, Germany	29 Jun - 01 Jul 2015

SC: Specialised Course; LC: Local Training Course.

In addition, each research fellow was required to participate of a secondment research stay hosted by a partner institute to work on complementary research aspects. During this thesis, two secondments were performed. The first was developed at UFZ (Germany), with focus in (a) acquiring hands-on experience with some of the EDA chemical tools, and (b) participating of the sampling activity in the Saale river basin (Fig. 2.2A). The other secondment was developed at ISS (Italy) and had focus in deepening the knowledge in aspects of the WFD and other regulations of relevance for integration of EDA approaches and bioassay monitoring. This secondment resulted in the presentation of an overview of the project and ongoing activities at the working group Chemicals meeting of the Common Implementation Strategy of the WFD (Fig. 2.2B), held at the European Commission in Brussels.

A

B

Figure 2.2: Secondment activities included sampling in the Saale river basin (A) and presentation of the project ongoing research in a meeting at the European Commission (B).

2.4 European Demonstration Program

The European Demonstration Program (EDP) was a common activity developed by all the project partners including the development and application of a simplified EDA protocol in different sites in European river basins. The EDP utilized a novel on-site sampling device for sampling and fractionation; and obtained samples were evaluated by a battery of bioassays and by chemical analysis (Schulze et al. in preparation, Tousova et al. in preparation). A summary of the EDP approach and main activities is provided below, in order to contextualize the thesis work within the project. A detailed description of the program will soon be available as a peer reviewed publication (Tousova et al. in preparation).

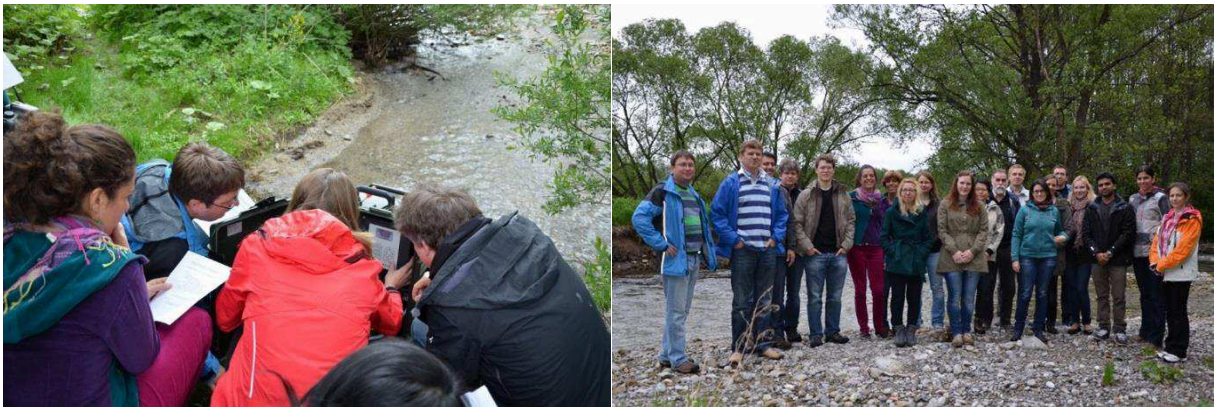
2.4.1 Sampling sites

Case studies were developed in four river basins and six countries. Sampling sites were selected considering relevant contamination by pollution sources, which was mainly wastewater treatment plants but also industrial or agriculture impact. Importantly, most of the sites were already being investigated in previous monitoring studies. Also, at least one reference, unpolluted site was evaluated in each basin. The case studies investigated the following river basins: (i) Danube (Czech Republic and Slovakia); Sava (Croatia); Emme (Switzerland); and Saale (Germany) (Tousova et al. in preparation).

2.4.2 Sampling device

A novel on-site large-volume solid phase extraction (SPE) sampling device combining the SPE with a pre-filtration cartridge (glassfiber filter, 0.63 µm, Sartopure® GF+ MidiCap, Sartorius) to separate suspended particulate matter from the water phase was utilized for water sampling and pre-fractionation. Three fractions were obtained by pressurizing the water sample through sorbent cartridges mounted in sequence as: (i) neutral polystyrene-divinylbenzene co-polymer (PS-DVB) sorbent (Chromabond® HR-X, Macherey Nagel), to capture neutral and semi-polar compounds; (ii) weak anionic exchanger based on the PS-DVB sorbent (Chromabond® HR-XAW – 3.5g), to capture acidic compounds; and (iii) weak cationic exchanger PS-DVB sorbent (Chromabond® HR-XCW – 3.5g), to capture basic compounds that are cationic at water pH ranging from 6 to 8 (Schulze et al. in preparation, Tousova et al. in preparation). One of the sampling devices and the training activity on its use are illustrated in Fig. 2.3.

A B



C D

Figure 2.3: The novel on-site large-volume solid phase extraction sampling device (A) functioning and use was presented at a training course in Slovakia (B, C, D).

2.4.4 Bioassay battery

Obtained water extracts and method blanks were evaluated for bioactivity utilizing a bioassay battery composed of nine tests (Tousova et al. in preparation), which were performed by different research fellows:

1) Algal growth inhibition assay adapted to 96-well plates (OECD 2011)
2) Fish embryo toxicity test with zebrafish (OECD 2013b)
3) In vivo thyroid activity assay with embryos of *Xenopus laevis* (Watchfrog)
4) Ames fluctuation assay (Reifferscheid et al. 2012)
5) Glucocorticoid receptor reporter gene assay (CALUX U2OS cell line)
6) Estrogen receptor (MELN cell line)
7) Androgen receptor reporter gene assay (MDA-kb2 cell line)
8) MTT cytotoxicity assay
9) Acetylcholine esterase inhibition in vitro

Standard operational procedures (SOP) were developed for the different bioassays by each responsible fellow, as part of the establishment of a simplified EDA protocol. The Ames assay and in part the FET test were performed at RWTH Aachen in the context of this thesis.

2.4.5 Chemical analyses

Water samples and method blanks were submitted to chemical analysis by state of the art GC- and LC- techniques coupled with MS, MS/MS or HRMS; complemented by GC-MS non-target screening. The list of target chemicals covered several classes of environmentally and toxicologically relevant pollutants, such as pharmaceuticals and pesticides; and their transformation products. The target compound list included also 31 WFD priority substances and three compounds from the WFD Watch list (EC 2013).

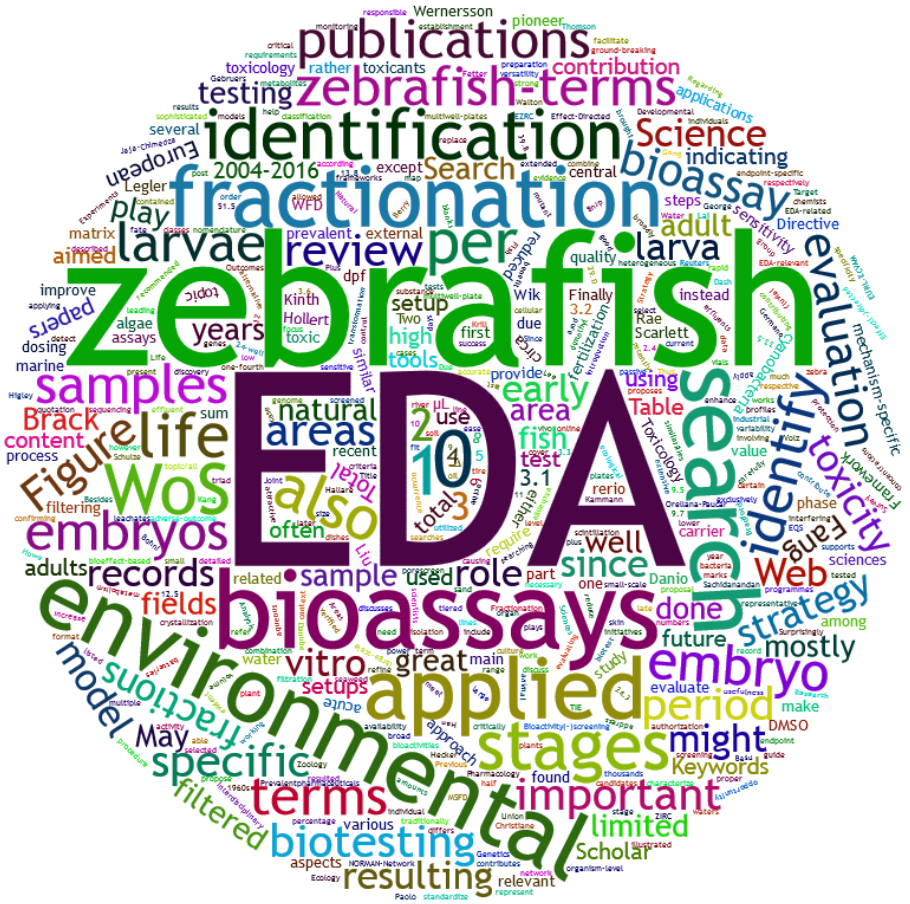
2.4.6 Outcomes

The detailed description of the EDP project and respective results is currently being prepared in manuscript format for submission to a peer reviewed publication (Tousova et al. in preparation). The EDP allowed the comparison of classical monitoring approaches with the new tools and served as a practical training of the fellows in organizing and running monitoring campaigns on a European scale.

2.5 High tier EDA case studies

As another collaborative aspect of the project, the developed higher tier methods from different fellows from complementary work packages were integrated in EDA case studies. In this context, Chapter 6 of this thesis describes the adaptation of bioassay procedures for integration in EDA, followed by the application in an EDA case study.

Chapter 3: Zebrafish as an integrative model in Effect-Directed Analysis



(Created with www.wordclouds.com)

Part of this chapter has been published as:

Di Paolo C., T.-B. Seiler, S. Keiter, M. Hu, M. Muz, Brack, W. and Hollert, H. (2015). The value of zebrafish as an integrative model in Effect-Directed Analysis – a review. Environmental Sciences Europe 27:8. DOI: 10.1186/s12302-015-0040-y

Abstract

Bioassays play a central role in effect-directed analysis (EDA), and their selection and application have to consider rather specific aspects of this approach. Meanwhile, bioassays with zebrafish, an established model organism in different research areas, are increasingly being utilized in EDA. Aiming to contribute for the optimal application of zebrafish bioassays in EDA, this review provides a critical overview of previous EDA investigations that applied zebrafish bioassays, discusses the potential contribution of such methods for EDA and proposes strategies to improve future studies. Over the last 10 years, zebrafish bioassays have guided EDA of natural products and environmental samples. The great majority of studies performed bioassays with embryos and early larvae, which allowed small-scale and low-volume experimental setups, minimized sample use and reduced workload. Biotesting strategies applied zebrafish bioassays as either the only method guiding EDA or instead integrated into multiple bioassay approaches. Furthermore, tiered biotesting applied zebrafish methods in both screening phase as well as for further investigations. For dosing, most of the studies performed solvent exchange of extracts and fractions to dimethyl sulfoxide (DMSO) as carrier. However, high DMSO concentrations were required for the testing of complex matrix extracts, indicating that future studies might benefit from the evaluation of alternative carrier solvents or passive dosing. Surprisingly, only a few studies reported the evaluation of process blanks, indicating a need to improve and standardize methods for blank preparation and biotesting. Regarding evaluated endpoints, while acute toxicity brought limited information, the assessment of specific endpoints was of strong value for bioactivity identification. Therefore, the bioassay specificity and sensitivity to identify the investigated bioactivity are important criteria in EDA. Additionally, it might be necessary to characterize the most adequate exposure windows and assessment setups for bioactivity identification. Finally, a great advantage of zebrafish bioassays in EDA of environmental samples is the availability of mechanism- and endpoint-specific methods for the identification of important classes of contaminants. The evaluation of mechanism-specific endpoints in EDA is considered to be a promising strategy to facilitate the integration of EDA into weight-of-evidence approaches, ultimately contributing for the identification of environmental contaminants causing bioassay and ecological effects.

Keywords: effect-directed analysis, bioassay-guided fractionation, zebrafish, embryo, larva, bioassay, *in vitro*, *in vivo*.

3.1 Introduction

Effect-directed analysis (EDA), bioassay-guided fractionation and similar approaches are testing procedures applied to identify the individual bioactive compounds contained in highly complex matrices, such as natural products and environmental samples. Bioassays play a central role in EDA, since biological activity directs the chemical fractionation and analysis steps as well as the testing strategy. Since fractionation of the sample is required to reduce the complexity of the original mixture, bioassays are needed to identify the active fractions and to guide further fractionation steps. Target and non-target chemical analysis are applied to select candidates and identify bioactive substances. Bioassays again play an important role in the confirmation phase, for biotesting of the pure substance identified as the bioactive compound (Brack 2003, Weller 2012, Burgess et al. 2013).

Therefore, bioassay selection for EDA studies has to consider aspects that are rather specific to this application. For accurate identification of bioactive fractions, the bioassays should present high sensitivity and low internal test variability, and be able to detect different chemicals that address similar endpoints or modes-of-action. Furthermore, due to limited sample amounts and large numbers of fractions to be tested, high-throughput low-volume bioassays are required (Burgess et al. 2013).

Thus, *in vitro* bioassays are often selected for EDA studies; however certain bioactivities require the organ or organism level for their proper identification, as for compounds in which metabolism plays an important role by interfering with formation or transformation of bioactive metabolites and bioaccumulation profiles (Di Paolo et al. 2010). These are the cases when bioassays with zebrafish early life stages are considered to be of great value, since they combine the organism-level endpoints with advantages of the *in vitro* format. Furthermore, biotesting strategies integrating organism-based and *in vitro* bioassays are expected to cover a broad range of bioeffects and related toxicants. This diagnostic power supports the identification of specific toxicants in EDA case studies (Wernersson et al. 2014).

The zebrafish *Danio rerio* is a model vertebrate organism broadly applied in biological sciences, being one of the most important organisms that is used in different research areas as genetics, developmental biology and ecotoxicology (Strähle et al. 2012). More recently, its versatility has also been recognized by chemists, which provides an opportunity to enhance interdisciplinary studies involving biology and chemistry (Basu and Sachidanandan 2013) as in Effect-Directed Analysis (EDA) (Brack 2003). Zebrafish exhibits characteristics that make it a very attractive research model, including small size, ease of culture, high fecundity, rapid

development, external fertilization and development, and transparency of the embryo. Bioassays with zebrafish embryos and larvae have further advantages that fit very well to EDA requirements. While these tests are relevant to evaluate acute (Knobel et al. 2012) and chronic (Volz et al. 2011) effects in later life stages, the experimental setup exhibits several *in vitro* test characteristics, including reduced volume of sample for testing and potential for high-throughput applications. Experiments with early life stages often do not require animal test authorization, and no external feeding is needed by embryos and larvae (Strähle et al. 2012).

The zebrafish success as a model organism is in great part due to the work of pioneer scientists between the late 1960s to mid-1990s, as George Streisinger, who established the first zebrafish models and performed pioneer works on its genetics and developmental biology (Streisinger et al. 1981, Streisinger et al. 1989, 1995); Charles Kimmel's description of the cellular fate map (Kimmel et al. 1990) and the stages of development (Kimmel et al. 1995) in embryos; and Christiane Nüsslein-Volhard, who performed a large-scale mutant screen to identify genes for vertebrate development control (Nüsslein-Volhard 1996, 2012). Following these ground-breaking studies, there was evident increase in the use of zebrafish in research (Kinth et al. 2013), resulting in the sequencing of its genome (Howe et al. 2013); extensive information on its genetics, genomics, phenotypic and developmental biology (Bradford et al. 2010); and the establishment of thousands of wildtype and transgenic zebrafish lines (EZRC , ZIRC).

Importantly, zebrafish embryos and early larvae might be used to replace or refine experiments with adult fish, being increasingly applied in ecotoxicology to evaluate the toxicity of chemicals, plant protection products, biocides, pharmaceuticals, wastewater effluents, various aqueous environmental samples, and for the assessment of sediment toxicity (Braunbeck et al. 2005, Hallare et al. 2011, Strähle et al. 2012, EURL-ECVAM 2014). Recently, zebrafish embryo toxicity assays have been integrated in biotest batteries in environmental monitoring programmes, as the Joint Danube Survey (Survey) and the working group on bioassays of the NORMAN network (NORMAN-Network). Fish bioassays also play an important role in the implementation of the European Water Framework Directive (WFD), since they provide data for the derivation of environmental quality standards (EQS) and might represent a sensitive taxon for substances with specific modes-of-action (EC 2011). Besides, biotests with fish are also included among recommended bioeffect-based tools for environmental assessment in the context of the WFD and the European Marine Strategy Framework Directive (MSFD) (Wernersson et al. 2014). Consequently, current and recent EU

projects are investigating the contribution of zebrafish bioassays for water quality assessment and EDA of environmental samples, with focus on specific modes-of-action, mechanism-specific endpoints and adverse-outcome pathways (Brack et al. 2013, Brack et al. 2015). Such initiatives are supported by the proposal that EDA contributes as an additional line of evidence in weight-of-evidence frameworks, such as the triad approach, ultimately leading to the identification of the contaminants responsible for the toxic effects observed in bioassays and the environment (Chapman and Hollert 2006, Hecker and Hollert 2009).

This literature review aimed to: provide an overview of previous EDA investigations that applied bioassays with zebrafish, critically evaluating their objectives, methods, biotesting strategy, and outcomes; discuss the potential contribution of further zebrafish bioassays for EDA; and propose strategies that might help optimizing the integration of such biotools into future EDA studies investigating environmental samples.

In order to meet these objectives, the literature was searched using the online tools Thomson Reuters Web of Science (WoS), ScienceDirect (SD), and Google Scholar (GS). In WoS, the terms were searched by topic (searching the fields Title, Abstract, Author Keywords, and Keywords Plus® per record) in all databases; and in SD and GS the terms were searched in all fields. The searches were done for publications in all years, except where indicated. The zebrafish-terms used for search or filtration were a combination of "zebrafish" or "zebra fish" or "*Danio rerio*". The EDA and life stage search terms are detailed below.

3.2 Zebrafish potential for EDA application

3.2.1 Zebrafish in EDA-relevant research areas

The application of the zebrafish model in EDA-related research areas was verified by search in WoS for the zebrafish-terms as keywords in topic/all databases, followed by classification per research area (Figure 3.1). The search period was limited to 2004 - 2016, to be in agreement with the publication years of EDA studies evaluated in this review. Outcomes are in good agreement with recent review that applied much more sophisticated search strategy (Kinth et al. 2013), indicating the usefulness of WoS for a first evaluation of research areas. Among the research areas strongly related to EDA, Toxicology (9.7 %) and Pharmacology (9.5 %) were each referred by circa 9 % of the publications; while Environmental Sciences & Ecology (3.6 %) and Chemistry (2.4 %) were referred by lower percentage. The prevalent research fields addressed by more than 20 % of publications were

those that traditionally apply zebrafish, as Genetics and Heredity (39.8 %), Biochemistry and Molecular biology (33.5 %), Developmental Biology (29.0 %), and Zoology (24.3 %).

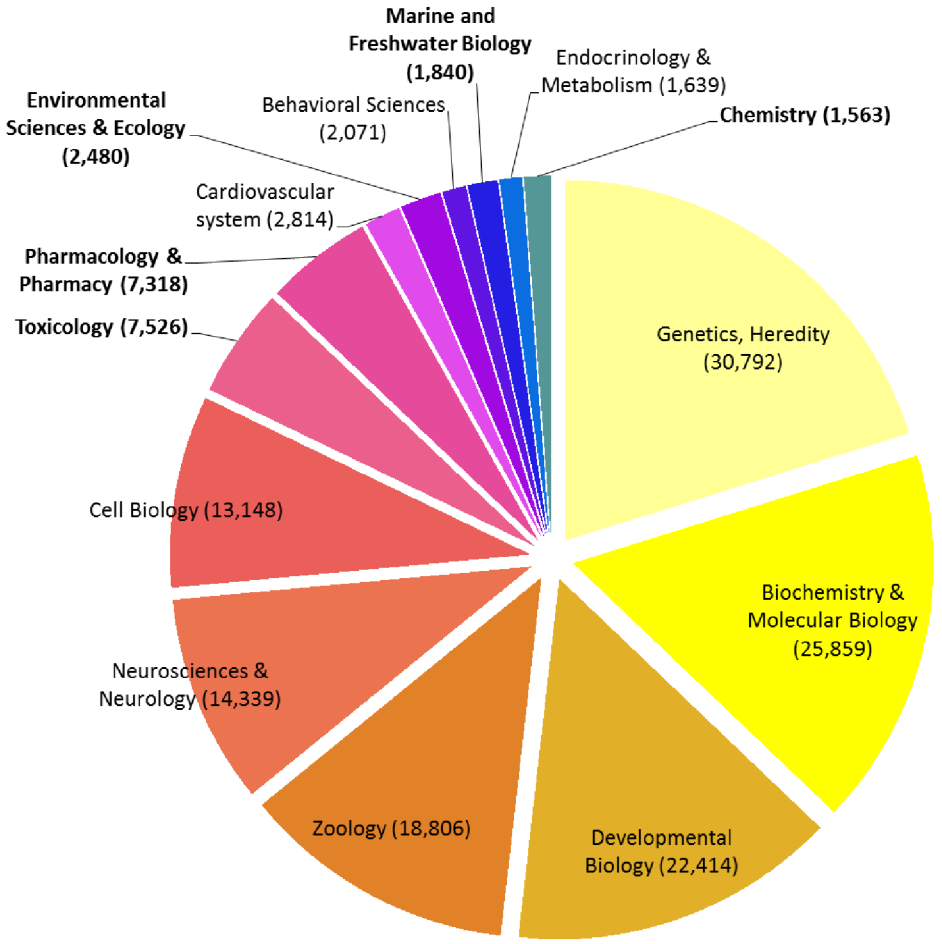


Figure 3.1: Number of records resulting from search for the zebrafish-terms, filtered by publication period (2004 - 2016) and classified according to respective research area. Total number of records per period: 77,286. Search done in May 2016 (Web of Science).

3.2.2 Life stages referred to by research studies

The use of different life stages in studies with zebrafish was estimated by search for the zebrafish-terms filtered by the terms "embryo*", "larva*", "juvenile*", "adult*"; and combinations of those. Again, the search period was limited to the publication years of reviewed EDA studies (2004-2016). As illustrated in Figure 3.2, more than half of the studies with zebrafish refer to embryos (51.5 %), and more than one-fourth of these mentioned also either adults or larvae, corresponding to 7.2 % and 8.1 % of total publications respectively. The occurrence of studies mentioning adults (13.8 %) and larvae (12.5 %) was also representative, while circa 1% only referred to juvenile life stages.

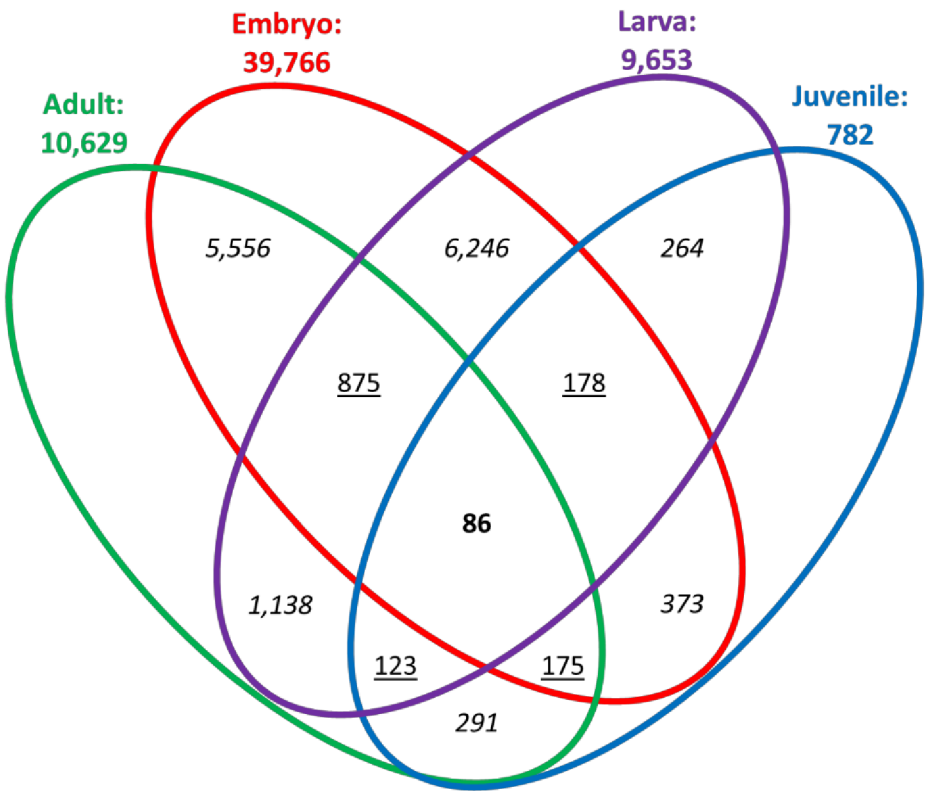


Figure 3.2: Number of records resulting from search for the zebrafish-terms, filtered by publication period (2004 - 2016) and by the terms: "embryo*", "larva*", "juvenile*", "adult*", or combinations of those. Total number of records per period: 77,286. Search done in May 2016 (Web of Science).

3.3 Previous EDA studies integrating zebrafish bioassays

Due to the heterogeneous nomenclature found in the literature, different EDA-terms as listed in Weller 2012 (Weller 2012) plus the term "fractionation" was searched in quotation marks, using the different search tools. After filtering by the zebrafish-terms mentioned above, resulting publications were screened for confirmation of the searched content. Review papers, or publications that did not include the EDA procedure or zebrafish bioassays, were excluded. Two studies that followed procedures for toxicity identification evaluation (TIE) (Wik et al. 2009, Fang et al. 2012) instead of EDA were included, since the similarities between both approaches (Burgess et al. 2013) make them relevant for this review. In total, 35 publications were found (Table 1), which were carefully evaluated for research area, objective, investigated matrix, bioassay endpoint and setup, biotesting strategy, and study outcomes.

Table 3.1: Number of records resulting from search for the EDA-terms in Web of Science (WoS); after filtering results by zebrafish terms; and after confirming that described studies indeed performed EDA. The sum of papers from WoS, ScienceDirect (SD) and Google Scholar (GS) after confirmation of content is also presented. Search done in May 2016.

Search terms	WoS, all databases by topic	WoS, filtered by zebrafish-terms (n)	WoS, confirmed content	Confirmed papers in WoS+SD+GS (n)
Bioassay(-)guided fractionation	1,991	17	11	12
Effect(-)directed analysis	242	22	3	5
Bioassay(-)guided isolation	538	11	8	9
Bioassay(-)directed fractionation	488	9	0	1
Toxicity(-)identification evaluation	364	4	1	2
Bioactivity(-)screening	109	5	2	2
Activity(-)guided fractionation	708	6	1	1
Bioactivity(-)guided fractionation	636	2	1	3
Bio(-)guided fractionation	277	1	1	1
Fractionation	227,699	108	19	25
Total *				35

*: the total number differs from the sum since some studies resulted in more than one search.

3.3.1 Research Areas and investigated matrices

Two main fields were prevalent among EDA studies using zebrafish bioassays: drug discovery from natural products, and environmental toxicology (Figure 3). Natural product studies aimed to identify bioactive compounds for pharmacological applications, investigating mostly plants (He et al. 2009, He et al. 2010, Krill et al. 2010, Crawford et al. 2011, Liu et al. 2011, Han et al. 2012, Orellana-Paucar et al. 2012, Bohni et al. 2013, Buenafe et al. 2013, Gebruers et al. 2013, Lee et al. 2013, Kang et al. 2014, Lai et al. 2014, Liu et al. 2014, Germano et al. 2015, Liang et al. 2015, Gong et al. 2016), but also extracts of bacteria (Dash et al. 2011), cyanobacteria and algae (Suyama et al. 2010), seaweed (Fitzgerald et al. 2013), and marine organisms (Rae et al. 2011). Environmental toxicology studies aimed to identify the toxic compounds in various environmental samples, including marine and fluvial sediments (Kammann et al. 2004, Higley et al. 2012, Fetter et al. 2014), soil (Legler et al. 2011), cyanobacteria and algae (Berry et al. 2007, Jaja-Chimedza et al. 2012, Walton et al. 2014), industrial effluent (Fang et al. 2012), rubber tire leachates (Wik et al. 2009), oil sand process waters (Reinardy et al. 2013, Scarlett et al. 2013), and river pore water (Fang et al. 2014). Finally, fish skin extracts were investigated in a behavioral sciences study (Mathuru et al. 2012).

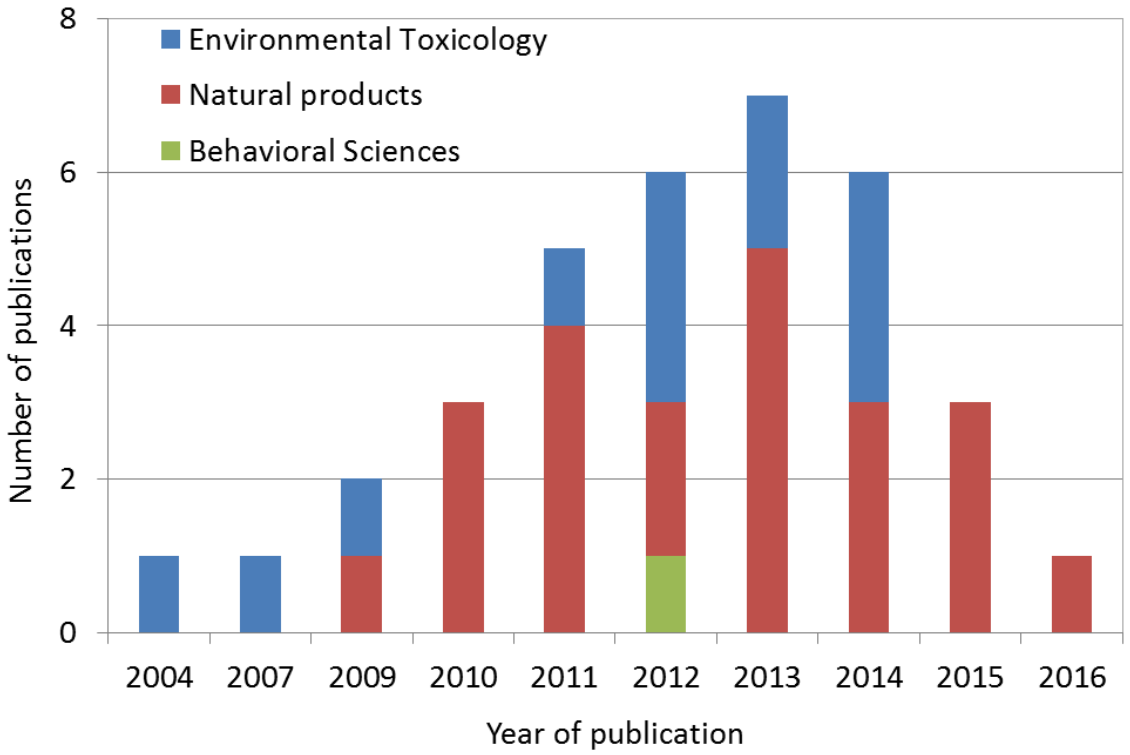


Figure 3.3: Number of EDA studies applying zebrafish bioassays that were evaluated in the literature review, identified per main research area and year of publication (Web of Science, ScienceDirect and Google Scholar, May 2016).

3.3.2 Prevalent life stages and exposure setups

EDA studies applied mostly bioassays with early embryos and larvae, following exposure to chemical extracts and fractions in multiwell-plates, often with exposure of several individuals in the same well (Table 3.2). Zebrafish up to 5 days post fertilization (dpf) were the life stages mostly applied, except for experiments that extended the assays up to 6-7 dpf (Legler et al. 2011, Rae et al. 2011, Buenafe et al. 2013, Reinardy et al. 2013, Scarlett et al. 2013) or a few studies with adults (Suyama et al. 2010, Fang et al. 2012, Mathuru et al. 2012). Environmental Toxicology studies for the most part performed exposure in 24-well plates (200 µL to 2 mL per embryo or larva) but also in crystallization dishes, scintillation vials, or beakers (450 µL to 5 mL per embryo or larva, 40 to 300 mL per adult); while natural product studies were performed exclusively in multiwell-plate setup (<100 to 250 µL per embryo or larva). The exposure of several individuals in the same well or vessel was observed for most of the studies, reflecting the need to reduce workload for EDA biotesting.

Table 3.2: Exposure vessel type, number of fish per vessel, and volume of exposure media per fish, for studies on Natural Products or Environmental Toxicology.

Exposure format	Fish per well or vessel (n)	Natural Products Medium volume per fish and respective reference	Environmental Toxicology Medium volume per fish and respective reference
96-well plates	1	100 µL (Hecker and Hollert 2009, He et al. 2010, Krill et al. 2010, Orellana-Paucar et al. 2012, Buenafe et al. 2013)	100 µL (Higley et al. 2012)
	4	<100 µL (Lee et al. 2013)	-
24-well plates	1	-	2 mL (Wik et al. 2009)
	5	n.a. (Dash et al. 2011)	200 µL (Kammann et al. 2004, Berry et al. 2007, Jaja-Chimedza et al. 2012)
	10	100-250 µL (Crawford et al. 2011, Han et al. 2012, Bohni et al. 2013)	-
	15	<100 µL (Gebruers et al. 2013)	-
	25	<100 µL (Kang et al. 2014)	-
12-well plates	10	n.a. (Rae et al. 2011, Fitzgerald et al. 2013)	-
6-well plates	25-30	n.a. (Liu et al. 2011, Liu et al. 2014)	-
Beakers, dishes, vials	5 to 40	40 mL [b] (Suyama et al. 2010)	0.45 - 5 mL [a] (Legler et al. 2011, Reinardy et al. 2013, Scarlett et al. 2013, Fang et al. 2014), 300 - 1000 mL [b] (Fang et al. 2012, Mathuru et al. 2012)

n.a.: information not available; a: bioassay with embryos and larvae, b: bioassays with adults.

3.3.3 Biotesting strategy

The EDA investigations guided only by zebrafish bioassays followed either a single test setup (e.g. (Legler et al. 2011, Liu et al. 2011, Jaja-Chimedza et al. 2012, Reinardy et al. 2013, Scarlett et al. 2013)) or a combination of methods (e.g. (He et al. 2009, Fetter et al. 2014)) to evaluate endpoints in zebrafish. Other studies applied methods with additional experimental models, mostly cell-based (e.g. (Kammann et al. 2004, He et al. 2010, Lee et al. 2013)) but also bacteria (Dash et al. 2011, Higley et al. 2012) and rodent (Orellana-Paucar et al. 2012) assays. When the application of multiple biossays aimed to evaluate distinct bioactivities, the tests were mostly performed in parallel. For instance, bioassay batteries evaluated the occurrence of different toxicity mechanisms (Kammann et al. 2004, Higley et al. 2012); or effects on different trophic levels in the two TIE studies (Wik et al. 2009, Fang et al. 2012). When instead the aim of multiple methods was to analyze different aspects of the same bioactivity or toxicity mechanism, there was prevalence of tiered approach biotesting

(He et al. 2010, Orellana-Paucar et al. 2012, Lee et al. 2013). When applied in screening phase, zebrafish bioassays aimed to identify active fractions by organism-level endpoints, which were later further investigated by additional methods with zebrafish (Fetter et al. 2014) or with other experimental models (He et al. 2010, Lee et al. 2013). As an example, zebrafish bioassays were applied to screen extracts and fractions for anti-angiogenic effects, followed by further investigations on human cells and transgenic zebrafish embryos (He et al. 2010). On the other hand, zebrafish bioassays applied only as secondary tests aimed mostly at the confirmation of bioeffect occurrence at the organism-level (Rae et al. 2011); or to evaluate the occurrence of acute toxicity in fish (Suyama et al. 2010, Dash et al. 2011).

3.3.4 Use of solvents in bioassays

In biotesting, solvents were used for transference of samples into exposure vessels or as carriers. A first approach applied acetonitrile (Jaja-Chimedza et al. 2012) or ethanol (Berry et al. 2007), including also solvent control conditions, and proceeding to solvent evaporation before adding exposure media. The use of solvents as carriers in bioassays showed prevalence of DMSO, in concentrations ranging from 0.01 % (Legler et al. 2011), 0.1% (Kang et al. 2014), 0.2 % (Hecker and Hollert 2009), 0.5 % (Liu et al. 2011, Liu et al. 2014), 11 % (Crawford et al. 2011, Orellana-Paucar et al. 2012, Bohni et al. 2013, Kammann et al. 2004) up to 2 % (Gebruers et al. 2013). In addition, ethanol was also used as carrier by a few studies, in concentrations of 0.001 % for experiments with larvae (Reinardy et al. 2013, Scarlett et al. 2013) or 0.435 % for experiments with zebrafish adults (Suyama et al. 2010).

It is relevant to mention that the different procedures for extraction, cleanup, pre-concentration, and fractionation of samples, already extensively reviewed elsewhere (Brack 2003, Weller 2012, Burgess et al. 2013), also involve the use of different solvents and chemicals. Criteria for the use of solvents in EDA studies are: low or lack of toxicity in the biotest; the capacity of the solvent to dissolve complex extracts and fractions; and the possibility to use the solvent in chemical analysis. The latter is the precondition to make sure that the chemical mixture tested in the bioassay resembles the mixture evaluated in chemical analysis. While DMSO excellently meets the first criterion, it is less suitable for dissolving complex mixtures when compared to other possible alternatives, and it completely fails the criterion related to the use in chemical analysis. Thus, the investigation of other solvents as possible carriers for exposure in zebrafish embryo testing might help to reduce possible artefacts during solvent exchange to DMSO. The evaluation of process blanks in order to exclude artefact toxicity is crucial for successful EDA and will be discussed below.

3.3.5 Positive / negative controls and biotesting of blanks

Positive control conditions that were specific to the evaluated endpoints were often described, with exposure of the zebrafish to compounds known to cause specific effects such as anticonvulsant activity (Orellana-Paucar et al. 2012), glucose uptake (Lee et al. 2013), pro-angiogenesis (Liu et al. 2011, Lai et al. 2014, Liu et al. 2014), anti-angiogenesis (He et al. 2009, He et al. 2010), or estrogenic effects (Reinardy et al. 2013, Fetter et al. 2014). Regarding negative control conditions, most of the studies reported testing of solvent controls in same concentration as for the respective sample (Kammann et al. 2004, He et al. 2009, Wik et al. 2009, He et al. 2010, Suyama et al. 2010, Crawford et al. 2011, Legler et al. 2011, Rae et al. 2011, Han et al. 2012, Higley et al. 2012, Jaja-Chimedza et al. 2012, Orellana-Paucar et al. 2012, Bohni et al. 2013, Buenafe et al. 2013, Kang et al. 2014). Some studies have evaluated a medium only condition in addition to the solvent control (Berry et al. 2007, Krill et al. 2010, Liu et al. 2011, Reinardy et al. 2013, Scarlett et al. 2013, Liu et al. 2014).

The preparation and biotesting of blanks was described only in few of the evaluated EDA studies and in the two TIE studies. In the EDA studies, there was submission of the respective solvents (Kammann et al. 2004, Legler et al. 2011, Reinardy et al. 2013, Scarlett et al. 2013) or of HPLC-grade water (Fang et al. 2014) through the same or part of the procedures that were applied to samples (i.e. sample preparation, extraction, fractionation). The TIE studies described blank preparation by treatment of milliQ water (Wik et al. 2009) or 0.1 M KCl solution (Fang et al. 2012) in the same way as samples for all procedures. In all these studies, the prepared blanks were evaluated in bioassays in the same way as done for samples and fractions. Another strategy was the use of a fraction that showed to be negative for the evaluated effect as a blank condition (Lee et al. 2013). The exchange between elution solvents and DMSO was identified as critical step since solvent traces might interfere with bioassays; therefore blank testing was suggested to always be performed (Kammann et al. 2004).

3.4 Investigated endpoints and outcomes

The specificity and sensitivity of endpoints in identifying the bioactivity or adverse effects in fractions was considered to be a key issue for the relevance of zebrafish bioassays in EDA. Therefore, it is recommended to identify the critical aspects for endpoint assessment, to optimize bioassays accordingly, and to demonstrate the validity of the bioassay by testing known bioactive compounds (Cordero-Maldonado et al. 2013). The endpoints and bioassays

described in the different studies are summarized in Table 3, and discussed in the context of respective study objectives and outcomes.

Table 3.3: Organism-level effects and assessed endpoints, according to the research area of studies and the investigated sample matrices.

Organism-level effect	Endpoint	Research area	Matrix and Reference
Non-specific toxicity	Lethality, respiratory rate, heart rate, movement	Environ Toxicol	Sediment extracts (Higley et al. 2012), industrial effluents (Fang et al. 2012), rubber tire leachates (Wik et al. 2009), oil sand process waters (Scarlett et al. 2013)
		Natural Products	Cyanobacteria and algae extracts (Suyama et al. 2010), seaweed hydrolysates (Fitzgerald et al. 2013)
Developmental toxicity / teratogenicity	Morphological / phenotypic endpoints	Environ Toxicol	Extracts of sediment (Kammann et al. 2004), soil (Legler et al. 2011), microalgae (Berry et al. 2007), cyanobacteria (Jaja-Chimedza et al. 2012, Walton et al. 2014), river pore water (Fang et al. 2014)
		Natural Products	Extracts of bacteria (Dash et al. 2011) and plant (Gebruers et al. 2013)
Anti- or pro-angiogenesis	ISV formation and/or function in wildtype or *fli-1:EGFP* zebrafish, EAP activity of vascular endothelial cells	Natural Products	Plant extracts (He et al. 2009, He et al. 2010, Krill et al. 2010, Crawford et al. 2011, Liu et al. 2011, Han et al. 2012, Lai et al. 2014, Liu et al. 2014, Germano et al. 2015, Liang et al. 2015, Gong et al. 2016)
Anti-angiogenesis and anti-inflammatory	ISV outgrowth in *fli-1:EGFP*; leukocyte migration after tail transection	Natural Products	Plant extracts (Bohni et al. 2013)
Antithrombotic activity	Accumulation of thrombocytes in transgenic line	Natural Products	Plant extracts (Li et al. 2015)
Glucose uptake	Uptake of fluorescein-tagged glucose bioprobe	Natural Products	Plant extracts (Lee et al. 2013)
Lipid storage modulation	Uptake and metabolism of fluorescent fatty acid	Natural Products	Heterofibrin molecules from *Spongia* sp. (Rae et al. 2011)
Antioxidant effects	ROS generation, cell death	Natural Products	Plant extracts (Kang et al. 2014)
Estrogenicity	GFP induction in *tg(cyp19a1b-GFP)*	Environ Toxicol	Sediment extracts (Fetter et al. 2014)
	vtg1 gene expession by qPCR	Environ Toxicol	Oil sand process waters (Reinardy et al. 2013)
Anti-convulsant	Locomotor activity, electrographic activity, epileptiform discharges	Natural Products	Plant extracts (Orellana-Paucar et al. 2012)
	Inhibition of seizure activity; WISH for Brain c-fos Expression	Natural Products	Plant extracts (Buenafe et al. 2013)
Fear behaviour	Alarm response, olfactory bulb activation in *Tα1:GCaMP2*	Behavioural Sci	Skin extracts (Mathuru et al. 2012)

EAP: endogenous alkaline phosphatase; ISV: intersegmental vessels; *fli1:EGFP*: transgenic zebrafish line with expression of enhanced green fluorescent protein marker in endothelial cells of vasculature; ROS: reactive

oxygen species; GFP: green fluorescent protein; WISH: Whole Mount in Situ Hybridization; *Tα1:GCaMP2*: transgenic zebrafish line with expression of the calcium indicator GCaM.

3.4.1 Non-specific toxicity and lethality

Bioactive sediment fractions (Kammann et al. 2004) and components partially responsible for toxicity in oil sand process water fractions (Scarlett et al. 2013) have been identified by acute toxicity bioassays. The two TIE studies reported inconsistent acute toxicity of industrial effluents (Fang et al. 2012) and rubber tire leachates (Wik et al. 2009). One study investigating seaweed hydrolysates evaluated *in vivo* toxic potential through acute toxicity testing (Fitzgerald et al. 2013).

It may be summarized that EDA studies that focused on acute toxicity and lethality had only modest success in determining active compounds. These are unspecific responses that might occur due to exposure to very broad range of compounds; therefore fractionation typically results in the distribution of toxicity over many different fractions. However, also other unrelated factors might have been involved, as for example the high complexity of investigated matrices in the reviewed studies. Nevertheless, acute toxicity testing might be a powerful tool in TIE, when applied to evaluate highly contaminated sites with acute toxicity caused by compounds that are well characterized (Connon et al. 2012).

3.4.2 Teratogenesis and developmental toxicity

Assessment of teratogenesis and developmental effects was done in studies that identified the bioactive compounds from microalgae, cyanobacteria and plant (Berry et al. 2007, Jaja-Chimedza et al. 2012, Gebruers et al. 2013, Walton et al. 2014), river pore water (Fang et al. 2014), and developmental toxicants in soil (Legler et al. 2011). Most studies evaluated traditionally-assessed morphological endpoints, while one investigation of plant fractions focused on ectopic tail formation (Gebruers et al. 2013). One study identified embryotoxicity in sediment extracts but not in respective fractions, which was attributed to losses of active compound or of synergistic effect during fractionation (Higley et al. 2012).

An aspect shared by the successful studies was the meticulous experimental characterization of the original matrices and respective fractions regarding their teratogenic effects and developmental toxicity potential. For instance, there was determination of the optimal exposure period to identify phenotype of interest that caused minimal acute toxicity (Gebruers et al. 2013, Walton et al. 2014). Characteristic phenotypical effects were identified for the extract and respective fractions (Walton et al. 2014) and for specific fractions (Legler et al. 2011), also on a dose-dependent manner (Berry et al. 2007, Jaja-Chimedza et al. 2012).

Two studies also investigated if additive or synergistic effects occurred between different fractions (Jaja-Chimedza et al. 2012), or between AhR agonists by co-exposure to a CYP1A inhibitor (Fang et al. 2014).

3.4.3 Angiogenesis modulation

Bioassays investigating pro- and anti-angiogenesis modulation by different bioactive plants were the most frequent studies in natural products. To this end, studies applied wildtype zebrafish (Hecker and Hollert 2009, Krill et al. 2010, Han et al. 2012, Germano et al. 2015) or the transgenic fli-1:EGFP (Lawson and Weinstein 2002) zebrafish line (He et al. 2010, Crawford et al. 2011, Liu et al. 2011, Bohni et al. 2013, Liu et al. 2014, Liang et al. 2015, Gong et al. 2016), in which the zebrafish fli1 promoter drives expression of enhanced green fluorescent protein in blood vessels. In wildtype zebrafish, scoring was done through staining of the vessels (Hecker and Hollert 2009, Han et al. 2012) or quantification of endogenous alkaline phosphatase activity of vascular endothelial cells (Germano et al. 2015), while the transgenic line allowed *in vivo* observation of the embryonic vasculature. Selected endpoints evaluated specific cellular-morphological phenotypes, as intersegmental vessel formation. In these assays, the exposure start and duration were set to the most sensitive developmental windows related to the assessed endpoints.

All of the evaluated studies were successful in identifying at least one bioactive compound causing angiogenesis modulation, indicating that the identification of highly specific endpoints on the organism level might be a good requirement for the efficient use of zebrafish bioassays in EDA. Complementary, quantitative polymerase chain reaction (qPCR) showed to be a useful method to clarify the molecular mechanisms involved in phenotype development (Lai et al. 2014, Gong et al. 2016). The use of transgenic zebrafish lines is also considered to be a great asset for studies that evaluate specific morphological effects, since it can facilitate endpoint observation and increase sensitivity of bioassays.

3.4.4 Energy uptake and storage

EDA was successful in identifying known and novel insulin-mimetic compounds in plants (Lee et al. 2013) with the contribution of zebrafish bioassays to characterize glucose uptake modulation. The study applied fluorescein-tagged glucose bioprobes and measured fluorescence by microscopy imaging and microplate reader, obtaining dose and time dependent responses. Another study applied a fluorescent *fatty acid analogue* to evaluate fatty acid storage modulation in zebrafish embryos by extracts from marine sponge (Rae et al. 2011). In this case, the characterization of effects was done by extraction of zebrafish lipids

followed by thin-layer chromatography. These studies demonstrated that the use of fluorescent bioprobes is a good tool to evaluate effects on the uptake and storage capacity of zebrafish, allowing not only for qualitative but also quantitative analysis of effects.

3.4.5 Antioxidant effects

Zebrafish embryos were integrated into an EDA study that identified and purified *Aloe vera* polysaccharide with protective effects against oxidative stress (Kang et al. 2014). Tests with zebrafish bioassays provided valuable information on organism-level responses regarding the generation of reactive oxygen species and oxidative stress-induced cell death, which were observed in a dose response manner.

3.4.6 Antithrombotic activity

Antithrombotic activity of leaves of the Hawthorn plant, which is utilized for its medicinal and nutritional properties, was investigated by in vitro assays and utilizing transgenic zebrafish embryos, leading to the isolation and identification of new bioactive compounds (Li et al. 2015).

3.4.7 Estrogenicity assessment by gene expression

Estrogenic effects were investigated in extracts and fractions of oil sand process waters by vitellogenin gene expression (*vtg1*) through qPCR in zebrafish early larvae (Reinardy et al. 2013). Estrogenicity was also assessed by the use of transgenic zebrafish embryos that exhibit green fluorescence protein expression in response to aromatase (*cyp19a1b*) gene induction, with confirmation of results by qPCR (Fetter et al. 2014).

Gene expression analysis by qPCR showed to be a useful EDA endpoint in zebrafish embryos and larvae when background information allows the selection of specific biomarker genes for the studied effect, as for estrogenicity. The evaluation of sets of genes by qPCR is considered to be a promising strategy for endocrine disruption investigation, when following optimized experimental design regarding exposure intervals and evaluated zebrafish developmental stages(Schiller et al. 2013). The transgenic zebrafish embryos were also considered to be experimental models compatible with EDA, and their integration in future studies is expected to be facilitated by automated image analysis procedures (Fetter et al. 2014).

3.4.8 Neuroactivity and Behaviour

EDA was applied to identify anticonvulsant compounds present in plants, by co-exposure of evaluated samples with a convulsant compound, followed by the analysis of larvae total

locomotor activity. Effects were assessed with video-tracking and software analysis and with electroencephalogram recording analysis (Orellana-Paucar et al. 2012). Another EDA study of plant neuroactivity applied similar bioassays, in combination with larvae whole mount in situ hybridization to assess increased brain *c-fos* gene expression as an indicator of seizure onset and brain damage (Buenafe et al. 2013). Both studies identified anticonvulsant compounds, demonstrating the usefulness of the zebrafish model to identify neuroactivity in EDA. Also for neuroactivity and behaviour, the assessment of specific endpoints and setting the bioassay accordingly demonstrated to be an effective EDA biotesting approach.

The identification of neuroactive compounds extracts of a mixture of red algae and cyanobacteria was investigated by a biotest battery including *in vitro* and organism-level methods (Suyama et al. 2010). Bioassays with zebrafish adults aimed at evaluating the neurotoxic potential of the matrix. However, evaluated endpoints were non-specific acute toxicity and mortality, which provided only minor contribution to the overall study outcomes.

The bioactive compounds responsible for fear behaviour response in fish were investigated by exposure of zebrafish adults to fish skin extracts and fractions (Mathuru et al. 2012). Video tracking was used to quantify alarm behaviour by measuring swimming speed and vertical position. The study identified the bioactive compound, and proposed a new class of odorants that trigger alarm behaviour in fish. This study required the development of experimental setup and endpoint assessment that were specific to the evaluated behavioural alteration, confirming the importance of this step also for behavioural assessment.

3.5 Summary and Discussion

Over the last ten years, EDA studies guided by zebrafish bioassays have successfully identified bioactive or toxic compounds present in diverse biological matrices or environmental samples. Embryos and early larvae were the prevalent zebrafish life stages in these studies, with exposure being done in multiwell-plates, often with several individuals in the same well. In consequence, sample volume for biotesting was minimized and workload was reduced, which are important aspects in EDA. Zebrafish bioassays showed also versatility in terms of biotesting strategy, being applied alone or as part of biotest batteries; and in both screening phase as well as for further investigation of active fractions in tiered biotesting.

In spite of its limited capacity to dissolve complex matrix extracts, DMSO was the main carrier solvent applied in zebrafish bioassays. As a result, it was used in concentrations up to two orders of magnitude higher than the recommended for single compound biotesting (0.01%) (OECD 2013b). Additionally, DMSO is not suitable for chemical analysis, which restricts the characterization of samples evaluated in biotesting. Therefore the investigation of alternative carrier solvents would be an asset for zebrafish bioassays in EDA. Passive dosing methods are also promising options, as recently done in EDA investigation of sediments though the use of silicone rods for dosing of extracts and fractions in algae bioassay (Bandow et al. 2009). In fact, loaded polymer silicone cast has successfully been integrated in zebrafish embryo assay for dosing of polycyclic aromatic hydrocarbons (Seiler et al. 2014).

The EDA procedures for sample extraction, cleanup, pre-concentration, and fractionation involve the use of different solvents and chemicals. Nevertheless, while most of the studies evaluated solvent and medium control conditions, the investigation of process blanks in bioassays was reported only by a small number of studies. In addition, methods for blank preparation varied considerably between these studies. Since the biotesting of process blanks is crucial for effective EDA, there is need to improve and standardize the procedures for their preparation and biotesting in future studies.

Most of the successful EDA studies applied specific and sensitive bioassays evaluating molecular, morphological or behavioural endpoints. Some studies optimized bioassays by identifying the most adequate exposure windows and assessment setups, to maximize the specific endpoint response and minimize the interference of acute toxicity (Gebruers et al. 2013). Further improvements might be achieved by advancing methods for the analysis of endpoints. For instance, the automated analysis of morphological phenotypes in transgenic or wildtype zebrafish would reduce workload and increase reliability in EDA (Fetter et al. 2014). Also, EDA of environmental samples would benefit from the analysis of bioassay results in correlation with previously characterized responses to specific classes of pollutants. That is the case of gene expression analysis of biomarker genes for specific mechanisms and modes-of-action. When analysed in correlation with respective gene modulation by known classes of compounds (Kosmehl et al. 2012), biomarker gene responses might indicate the presence of certain classes of chemicals (Keiter et al. 2010). Similarly, EDA studies evaluating behavioural phenotypes to identify neuroactivity might rely in the near future on databases of behavioural profiles for different classes of compounds (Kokel et al. 2010, Ali et al. 2012).

Such outcomes support the idea that EDA investigations of toxic environmental samples would benefit of the application of endpoint- and mechanism-specific methods with zebrafish. That is in fact a great advantage since mechanism-specific toxicity methods with zebrafish are broadly developed, as for aryl hydrocarbon receptor mediated toxicity (Schiwy et al. 2014), genotoxicity (Kosmehl et al. 2006), and neurotoxicity (de Esch et al. 2012). Furthermore, EDA guided by such zebrafish bioassays could integrate broader environmental assessment strategies, complementing effect-based approaches (Wernersson et al. 2014) and weight-of-evidence frameworks (Hecker and Hollert 2009). In this way, EDA would support the identification of contaminants causing bioassay and ecological effects, and the clarification of links between ecosystem functioning and the responses at different biological levels (Hecker and Hollert 2009, Connon et al. 2012). Finally, the evaluation of toxic aquatic contaminants through EDA guided by zebrafish bioassays might improve the protection of water **bodies** in the context of the European WFD and MSFD (EC 2011, Wernersson et al. 2014). In conclusion, endpoint- and mechanism-specific zebrafish bioassays are considered of great relevance not only for guiding EDA studies, but also for the integration of EDA into environmental assessment and monitoring, ultimately contributing for environmental quality improvement (Wernersson et al. 2014).

3.6 Conclusions

Zebrafish bioassays have successfully guided different EDA studies, however further method developments are still needed. Alternative dosing procedures should be investigated, and process blank preparation and biotesting should be standardized. Endpoint- and mechanism-specific bioassays with embryos and larvae are considered to be the most promising zebrafish biotests for future EDA of environmental samples. When integrated into broader environmental assessment strategies, EDA guided by specific zebrafish bioassays might support the identification of compounds causing bioassay and ecological effects, ultimately contributing for environmental quality improvement.

Acknowledgements

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Chapter 4: Early life exposure to PCB126 results in delayed mortality and growth impairment in zebrafish larvae

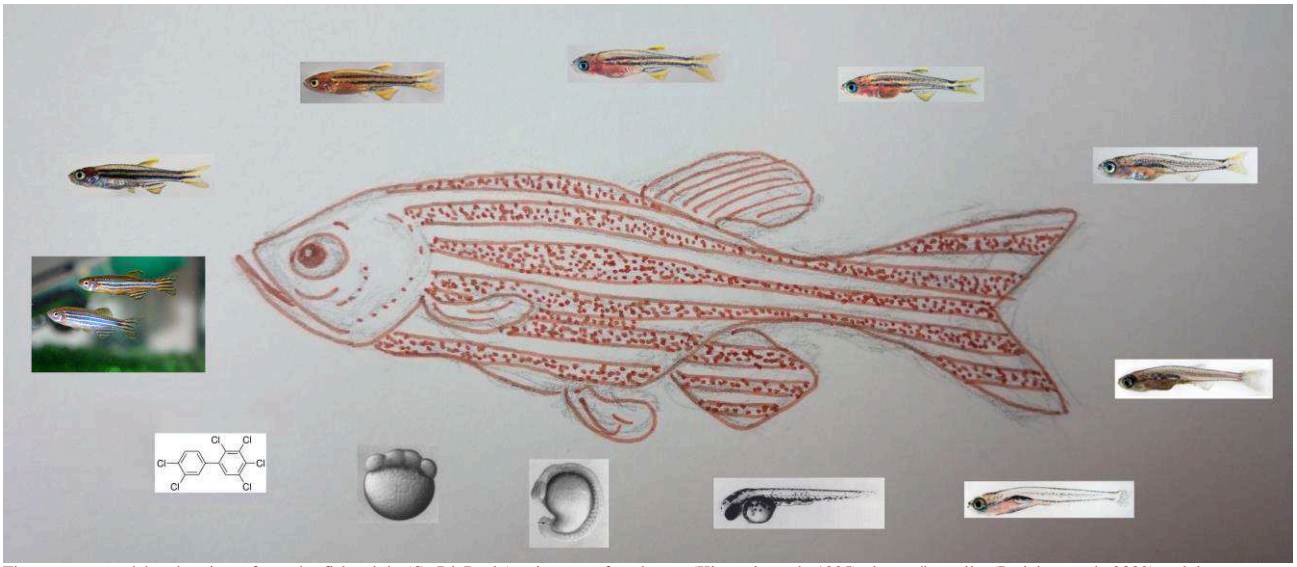

Figure composed by drawing of a zebrafish adult (C. Di Paolo), pictures of embryos (Kimmel et al. 1995), larvae/juvenile (Parichy et al. 2009), adults (Vetmeduni Vienna/ KLIVV), PCB126 molecule (Sigma-Aldrich).

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Abstract

The occurrence of chronic or delayed toxicity resulting from the exposure to sublethal chemical concentrations is an increasing concern in environmental risk assessment. The Fish Embryo Toxicity (FET) test with zebrafish provides a reliable prediction of acute toxicity in adult fish, but it cannot yet be applied to predict the occurrence of chronic or delayed toxicity. Identification of sublethal FET endpoints that can assist in predicting the occurrence of chronic or delayed toxicity would be advantageous. The present study characterized the occurrence of delayed toxicity in zebrafish larvae following early exposure to PCB126, previously described to cause delayed effects in the common sole. The first aim was to investigate the occurrence and temporal profiles of delayed toxicity during zebrafish larval development and compare them to those previously described for sole to evaluate the suitability of zebrafish as a model fish species for delayed toxicity assessment. The second aim was to examine the correlation between the sublethal endpoints assessed during embryonal and early larval development and the delayed effects observed during later larval development. After exposure to PCB126 (3-3000 ng/L) until 5 days post fertilization (dpf), larvae were reared in clean water until 14 or 28 dpf. Mortality and sublethal morphological and behavioural endpoints were recorded daily, and growth was assessed at 28 dpf. Early life exposure to PCB126 caused delayed mortality (300 ng/L and 3000 ng/L) as well as growth impairment and delayed development (100 ng/L) during the clean water period. Effects on swim bladder inflation and cartilaginous tissues within 5 dpf were the most promising for predicting delayed mortality and sublethal effects, such as decreased standard length, delayed metamorphosis, reduced inflation of swim bladder and column malformations. The EC50 value for swim bladder inflation at 5 dpf (169 ng/L) was similar to the LC50 value at 8 dpf (188 and 202 ng/L in two experiments). Interestingly, the patterns of delayed mortality and delayed effects on growth and development were similar between sole and zebrafish. This indicates the comparability of critical developmental stages across divergent fish species such as a cold water marine flatfish and a tropical freshwater cyprinid. Additionally, sublethal effects in early embryo-larval stages were found promising for predicting delayed lethal and sublethal effects of PCB126. Therefore, the proposed method with zebrafish is expected to provide valuable information on delayed mortality and delayed sublethal effects of chemicals and environmental samples that may be extrapolated to other species.

Keywords: zebrafish, early life stages, delayed effects, sublethal effects, growth, PCB126.

4.1 Introduction

The Fish Embryo Toxicity (FET) test, frequently performed with zebrafish (*Danio rerio*), is widely used by the scientific community in diverse modified versions. This assay has been demonstrated to be predictive of acute toxicity in older, juvenile and adult, developmental stages (Lammer et al. 2009, Knobel et al. 2012, Belanger et al. 2013). Based on this, the zebrafish FET has been recommended as a substitute for acute toxicity testing with adult fish (OECD 2013b). A clear advantage of this test is that it is performed with non-protected animal life stages (EC 2010), thus offering an opportunity to reduce the use of protected life stages of animals in chemical testing and environmental monitoring (Van der Jagt et al. 2004, Scholz et al. 2013). However, environmental concentrations of pollutants rarely lead to manifestation of acute toxicity, with chronic or delayed toxicity presenting a much more pronounced concern. Therefore, environmental risk assessment requires the estimates of chronic toxicity that result from long-term continuous or fluctuating exposure to chemicals at sublethal concentrations. Unfortunately, the results obtained with the zebrafish FET or any other acute toxicity test cannot be directly applied for the estimation of chronic toxicity. This is related to the fact that the available approaches for acute-to-chronic extrapolation are largely based on the use of assessment factors, which are not always reliable. Thus, in many cases direct chronic toxicity testing with protected life stages is still the only alternative. For example, between 420 and 720 (Volz et al. 2011, OECD 2013a) zebrafish are needed to perform one Fish Early Life Stage (FELS) test, following an exposure period that starts during embryonic development and continues until the control fish reach the juvenile life stage (Oris et al. 2012, OECD 2013a).

Delayed toxicity can occur following early exposure to chemicals and can be viewed as a special case of chronic toxicity. Maternal transfer and exposures during early life stages (embryos and early larvae) have been demonstrated to be particularly effective in inducing delayed toxicity (LeBaron et al. 2010) and even trans-generational effects (King-Heiden et al. 2009, Baker et al. 2014). In some cases, epigenetic modifications induced by toxic insults during critical periods of early development have been suggested to underlie the delayed toxicity mechanisms (Mirbahai and Chipman 2013). Another possible cause for delayed toxicity is through bioamplification of highly lipophilic compounds (Log Kow>5) that are not easily excreted and have a significant tendency for bioaccumulation (Daley et al. 2009, Di Paolo et al. 2010). When deposited maternally or accumulated during early life exposure in the yolk-sac of embryos, such compounds can be released during mobilization of lipids at

later developmental periods, leading to bioamplification and increased toxicity (Daley et al. 2014). Indeed, in common sole (*Solea solea*), early life stage exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenylethers (PBDEs) was shown to cause delayed mortality (Foekema et al. 2008, Foekema et al. 2014). The onset of delayed mortality coincided with the transition from yolk-sac to the free-feeding larval stage, the moment when the yolk lipids become depleted. The associated release of lipid-stored organic substances leads to a high peak in the internal exposure concentrations (Foekema et al. 2012).

PCB126, an aryl hydrocarbon receptor (AhR) agonist recommended as reference chemical in investigations of dioxin-like toxicity (Van den Berg et al. 1998), caused delayed toxic effects in sole, such as oedema, delayed metamorphosis and mortality (Foekema et al. 2008, Foekema et al. 2014). While flatfish represent a convenient test model among marine fish species, zebrafish is the freshwater fish test species adopted by several test guidelines (ISO 2007b, OECD 2013b, a). Also, it is a model species for the evaluation of chronic and trans-generational effects of dioxins in fish (King-Heiden et al. 2009, Baker et al. 2014). PCB126 was previously shown to cause typical dioxin-like toxic effects in zebrafish embryos, such as oedema, skeletal and cardiovascular malformations (Grimes et al. 2008, Seok et al. 2008), effects that are consistent with the blue sac syndrome observed in wild fish populations (King-Heiden et al. 2012). However a systematic investigation of the delayed toxicity of PCB126 in zebrafish is missing.

The first aim of our study was to investigate the occurrence and temporal profiles of delayed toxicity during zebrafish larval development and compare them to those previously described for sole, in order to evaluate the suitability of zebrafish as a model fish species for delayed toxicity assessment. The second aim was to examine the correlation between several sublethal endpoints assessed during embryonal and early larval stages and the delayed effects observed during larval development. We exposed zebrafish embryos to the model compound PCB126, and assessed (i) whether delayed mortality and other toxic effects would occur during subsequent rearing in clean water and (ii) whether the developmental time of the delayed mortality onset would correlate to that observed in sole. In addition, the concentrations of PCB126 in the exposure solutions and in fish were chemically analysed. We evaluated the comparability of effective PCB126 concentrations between zebrafish and sole, to determine the suitability of zebrafish as a general test species for assessment and prediction of delayed toxicity caused by lipophilic compounds in fish.

In practice, the general test setup for observing delayed toxicity follows that of a FELS test. The main difference is that the exposure occurs during a short period in the beginning of the test, mimicking maternal transfer or a short exposure during the early embryo-larval period, being followed by a longer-term rearing in clean water. Obvious disadvantages of such long-term tests are the time, resources and large numbers of animals required. For these reasons, there is an urgent need to develop alternative methods to substitute long-term chronic toxicity testing in general. The assessment of multiple sublethal endpoints in the FET test is considered a potentially fruitful approach to discover suitable chronic toxicity predictors. Suggested endpoints include gene expression changes (Liedtke et al. 2008, Weil et al. 2009, Schiller et al. 2013), assessment of target organ toxicity and morphological abnormalities (Nagel 2002, Lammer et al. 2009, Embry et al. 2010, Sipes et al. 2011, Volz et al. 2011), as well as behavioural alterations (Sloman and McNeil 2012). The assessment of the capacity of various sublethal endpoints assessed in the FET test setup to predict chronic toxicity in FELS tests forms already an area of active research (Villeneuve et al. 2014). However, the possible value of such sublethal effects for prediction of delayed toxicity has not been investigated in detail yet. Therefore, in this study we examined the correlation between several morphological and behavioural endpoints assessed in the FET test and delayed effects observed during subsequent rearing in clean water.

4.2 Materials and methods

4.2.1 Chemicals

3,3',4,4',5-Pentachlorobiphenyl (PCB126, CAS No. 57465-28-8) was purchased from Dr. Ehrenstorfer GmbH. Dimethyl sulfoxide, 3,4-dichloroaniline, Tricaine methane sulfonate (MS222), n-hexane, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, $NaHCO_3$, KCl, Na_2SO_4 and H_2SO_4 were obtained from Sigma-Aldrich Co. $^{13}C_{12}$ isotope labelled PCB126 was obtained from Cambridge Isotope Laboratories (CIL) and was part of a prepared mixture of 12 $^{13}C_{12}$ isotope labelled PCBs (CIL EC 4937). $^{13}C_{12}$ labelled PCB70 was from the same source (CIL EC 4914).

4.2.2 Zebrafish maintenance and embryo exposure

Zebrafish adults obtained from the University of Zürich were a cross between pet-shop and wild-type WIK strain fish, selected for the high level of genetic variability that is relevant for ecotoxicity tests (Coe et al. 2009). Fish were kept in tanks at the breeding facility of Eawag (Dübendorf, Switzerland) following standard maintenance procedures as described previously

(Groh et al. 2011). Animal test authorization was approved by the Veterinary Office of canton Zurich, Switzerland. Fish were euthanized by prolonged immersion in a solution of 1g/L of Tricaine methane sulfonate (MS222) neutralized with $NaHCO_3$.

Test concentrations were selected based on a literature review (Joensson et al. 2007a, Sisman et al. 2007, Na et al. 2009, Waits and Nebert 2011), and range finding tests with PCB126 concentrations between 1 and 100,000 ng/L (results not shown). The selected concentrations aimed to cover a range that would produce no mortality within the exposure period (0 to 5 dpf), but would cover conditions that caused from none to very evident morphological effects in exposed fish.

In all experiments, exposure of fertilized eggs collected from community mating was carried out from 3 hours post fertilization (hpf) until 5 days post fertilization (dpf) following standard guidelines (ISO 1996, OECD 2013b). All glassware materials for preparation of solutions and exposure were pre-cleaned with n-hexane. Exposure solutions were always freshly prepared in laboratory glass bottles using pre-aerated ISO water (ISO 1996). First, the stock solution (1 mg PCB126 / mL DMSO) was drawn using a Hamilton syringe and pre-diluted to 0.1 µg/mL in water. This solution was then used to prepare the exposure solutions at nominal concentrations of 3, 30, 100, 300 and 3000 ng/L (all containing 0.01% DMSO, the maximum recommended in the OECD 236 FET test guideline (OECD 2013b)). Negative controls included water and solvent (0.01% DMSO) controls. The positive control contained 4 mg/L of 3,4-dichloroaniline. Before the start of a test, glass exposure vessels were pre-soaked with respective test solutions for 24 hours.

At the onset of exposure, 30 embryos per condition were transferred individually to 3 mL of fresh exposure solutions in 5 mL glass beakers, which were randomly distributed in three groups of 10 embryos. Exposure was semi-static, with partial water exchange (2 mL) performed daily with fresh exposure solutions. All experiments were carried out at a temperature of 26 ± 1 °C and 14 h light / 10 h dark cycle. All experiments met the validity criteria (ISO 2007b, OECD 2013b) of minimum rates of fertilization (70 %), hatching (90 %), and survival in water/solvent controls until 5 dpf (90 %); and lethality in positive controls (50 %).

4.2.3 Test setups and endpoints measured

Four test setups were used: (i) the FET test (OECD 2013b), (ii) the prolonged FET (pFET) test, largely resembling the test setup used previously with sole (Foekema et al. 2008), (iii) an

adapted protocol for the test of larvae diet, and (iv) a simplified protocol to obtain samples for chemical analysis. In (i), (ii) and (iv), the chemical exposure was performed as described above, always from 3 hpf until 5 dpf. In (iii), no chemical exposure was applied.

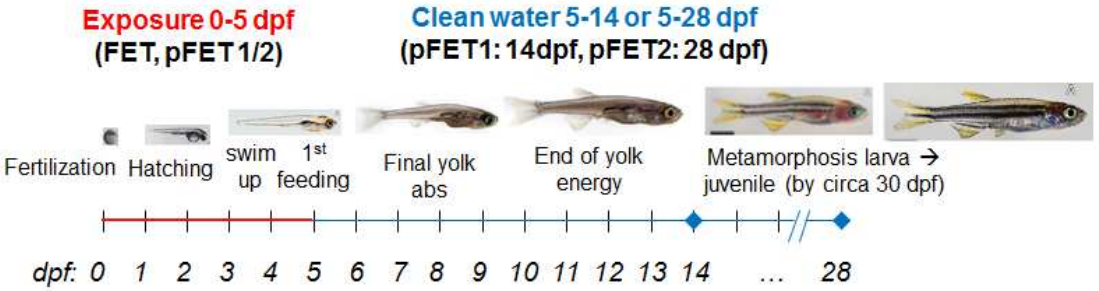

Figure 4.1: Temporal exposure setups of the FET and the pFET tests.

(i) FET test

For each test, 30 fish were used per treatment condition and the test was independently performed three times. Visual scoring of morphology and behaviour was performed daily using a stereomicroscope. Morphological endpoints were assessed as described previously (Lammer et al. 2009). In addition, inflation and pigmentation of the swim-bladder was assessed. Sub-lethal effects are described either for each category, or as by the cumulated occurrence of any of the listed endpoints for sub-lethal toxicity (i.e. any sub-lethal effect). Behavioural responses were qualitatively assessed in a systematic manner and the occurrence of the following categories was registered: spontaneous movement, swim-up behaviour, spontaneous swimming, swimming abnormally with tail up or down, standing position (larva resting in upright position close to well bottom), falling laterally (larva presenting equilibrium loss), and laying laterally. Results of visual observations are presented as average ± standard deviation of number of scores for three biological replicates. At the end of the test (5 dpf) the larvae were euthanized.

(ii) pFET test

At the end of chemical exposure (5 dpf), fish were rinsed with control (ISO) water (ISO 1996) and transferred in groups of 10 to 250 mL of clean ISO water in 500 mL crystallization dishes. The total volume of clean water per dish was gradually increased between 5 and 8 dpf, until the final volume of 500 mL was reached and kept until the end of the test (i.e. 250, 300, 400 and 500 mL on 5, 6, 7 and 8 dpf respectively). Fish were fed with Sera® micron and live artemia. Remaining food and dead fish were removed daily, and partial water exchanges were

performed every other day. Visual scoring of mortality, behavioural and morphological endpoints was performed daily.

Two pFET experiments were carried out. The first experiment (hereafter referred to as pFET-14), with 60 fish per condition distributed in six groups of 10 fish per crystallization dish, continued until 14 dpf and aimed to evaluate if delayed mortality would occur in zebrafish larvae. The second experiment (hereafter referred to as pFET-28), with 30 fish per condition distributed in three groups of 10 fish per crystallization dish, was continued until 28 dpf to also investigate the occurrence of delayed sublethal effects during the metamorphosis from larval to juvenile stages (Parichy et al. 2009).

To measure the fish length at the end of the pFET-28, fish were anesthetized with MS222 and transferred to a Petri dish containing a millimetre paper scale. Images were taken using a camera coupled with a stereomicroscope after which fish were euthanized. Images were used to measure standard length (distance between the snout and the caudal peduncle) and to identify occurrence of developmental milestones, according to the previously described staging by externally visible anatomy for post-embryonic zebrafish development (Parichy et al. 2009).

(iii) Diet test until 18 dpf

Three commercial fish diets were tested: JBL NovoGranoMix mini, TetraMin Junior, and Sera micron. No chemical exposure was performed. At 5 dpf, larvae were transferred to crystallization dishes in the same way as for the pFET, except that each dish contained 40 larvae. From 5 dpf on, fish received one of the commercial diets and in addition live artemia twice a day. The test was conducted until 18 dpf, with 120 fish per condition distributed in three groups of 40 fish per crystallization dish. At the end of the test, the total number of surviving larvae was scored, and fish were submitted to euthanasia.

(iv) Collection of samples for chemical analysis

During the exposure period, exposure solution and fish were sampled at three time points: before the start of exposure (sampling of solutions only); at 2 dpf before the daily partial solution renewal (solutions and fish); and at 5 dpf before the end of the exposure period (solutions and fish). The exposure solution and fish of the 3 ng/L treatment were not analysed because chemical concentrations in these samples would be too close to the limit of detection (LOD). Exposure solution samples consisted of three replicates of ca. 5 mL each (15 mL for

30 ng/L, to ensure that the detection limit of the analytical method was exceeded), collected in 10 mL glass sampling tubes. For each replicate, ca. 0.5 mL of solution from each of ten individual exposure vessels (30 for 30 ng/L) were pooled. Fish samples consisted of three replicates of five fish each (15 fish for 30 ng/L). In addition, we analysed the pre-diluted stock solution in water (nominal concentration of 0.1 µg/mL), and the freshly prepared exposure solutions sampled before pipetting them into exposure chambers.

4.2.4 Chemical analysis

For exposure solution extraction, 100 pg of quantification standard ($^{13}C_{12}$ PCB126) and 5 mL of n-hexane were added per 5 mL of exposure solution and shaken using a vortex mixer. After phase separation by short centrifugation of the emulsion at 1000 rpm, the n-hexane was pipetted out and dried over a pre-cleaned Na_2SO_4 filter. The extraction step was repeated four times, all n-hexane fractions were combined into a flask and evaporated to almost dryness in a rotary evaporator system (45-50 °C, 300 mbar). The remaining residue was transferred to a pre-cleaned GC-vial and the evaporation flask was rinsed several times with n-hexane which was added to the rest of the sample. Next, the extract was evaporated under a gentle nitrogen stream. Afterwards, 100 pg of recovery standard ($^{13}C_{12}$ PCB70) was added before the sample was submitted to chemical analysis. For fish sample extraction, 250 pg of quantification standard ($^{13}C_{12}$ PCB126) were added to five fish in a 4 mL flask. Around 0.5 mL H_2SO_4 95-97% was added to the flask and fish tissues were completely dissolved by ultrasonication for around 1 hour. Subsequently, n-hexane was added to the homogenized samples and thoroughly mixed by hand and by vortex. The n-hexane extract was collected and dried as explained above and transferred to a pre-cleaned vial. This procedure was repeated four times, and the combined extract was evaporated under a gentle nitrogen stream. Afterwards, 250 pg of the recovery standard ($^{13}C_{12}$ PCB70) were added before the sample was submitted to chemical analysis. The extraction efficiencies of both methods were greater than 99%, as tested by additional n-hexane re-extraction of several samples as described above.

The LOD and the limit of quantification were set by definition at signal to noise ratios of greater than three ($s/n \geq 3$) and ten ($s/n \geq 10$) respectively. The quantitative determination of PCB126 in extracts was achieved by gas chromatography/high resolution mass spectrometry (GC/HRMS). Analyses were carried out on a MAT95 high-resolution mass spectrometer (Thermo Finnigan MAT, Bremen Germany) coupled to a Varian 3400 gas chromatograph (Walnut Creek, CA, USA), equipped with an A200S autosampler (CTC Analytics, Zwingen, Switzerland). Samples were injected in splitless mode (splitless time 60s) at an injector

temperature of 260 °C. For the gas chromatographic separation a RTX5 Sil-MS column (30 m x 0.25 mm, film thickness 0.10 µm) was used with helium as carrier gas at a pressure of 100 kPa. The initial column temperature was 100°C. After 1 minute, the temperature was ramped at 10°C/min to 300°C. The ion source was operated at 220 °C, the electron energy was 70 eV, and the mass spectrometer was tuned to a mass resolution of 8000-10000. The two most abundant signals of the molecular ion cluster of the native and $^{13}C_{12}$ labelled pentachlorobiphenyl were recorded in the single ion monitoring mode. Calculation of the PCB126 level in the extracts was based on comparison with the $^{13}C_{12}$ labelled PCB126 used as internal standard.

4.2.5 Statistical analysis

Statistical analysis was performed using the software packages GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) and SigmaPlot. EC_{50} and LC_{50} values and respective confidence intervals (CI) were determined by non-linear fit of the sigmoidal dose-response curve. For statistical analysis of differences between exposure conditions in the FET and pFET tests, the data were checked for normality (Shapiro-Wilk's) and equal variance. Depending on these tests' results, the applied analysis of variance was either parametric (one-way ANOVA followed by Dunnett's Multiple Comparisons Test) or non-parametric (Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's Multiple Comparisons Test). Delayed mortality in pFET experiments was assessed using Kaplan-Meier survival analysis (Jager et al. 2008) applying the log-rank test followed by pairwise Holm-Sidak Multiple Comparisons Test. For construction of survival curves, the data from three technical replicates were pooled for each treatment within each of the tests performed (pFET-14 and pFET-28).

4.3 Results

4.3.1 Survival of larvae receiving the different diets

At 18 dpf, the fish larvae receiving sera micron and live artemia presented the highest survival rate (95 ± 0 %) from the tested conditions, followed by JBL NovoGranoMix (79 ± 3 %) and TetraMin Junior (64 ± 8 %). Although these results should be interpreted with caution, since the experiment was not repeated, the fish diet by Sera was adopted for the following pFET experiments.

4.3.2 External and internal exposure to PCB126

Measured exposure and internal concentrations of PCB126 are shown in Table 1. All samples from DMSO controls showed PCB126 concentrations below the LOD. The concentration of the pre-diluted stock solution in water (0.11 ± 0.001 µg/mL) was in good agreement with the planned nominal value (0.1 µg/mL). Nevertheless, deviations from nominal values were observed in exposure solutions, which reached circa 10% of respective nominal concentrations before the partial solution renewal. Zebrafish embryos accumulated PCB126 over time, and strong induction of *cyp1a* mRNA was observed at the two highest concentrations at 5 dpf (data not shown). The highest tissue concentration was found after five days of exposure at the highest nominal concentration tested, 3000 ng/L (9.2 nM), yielding 2854 ± 258 µg PCB126/kg wet weight. Tissue concentrations of PCB126 increased about 5-fold between 2 and 5 days of exposure in all exposure concentrations (Table 1). However, a steady-state was not likely reached during the 5 days of exposure in the experiments. A bioconcentration factor (BCF) was determined for the 5 dpf larvae, that were assumed to have a wet weight of 0.35 mg (Markovich et al. 2007) including 7.7 µg of total lipids (Petersen and Kristensen 1998). The average logBCF value, obtained from the four different exposure concentrations, was 4.05 ± 0.06 in terms of wet weight, and 5.68 ± 0.05 on a lipid-normalized basis.

Table 4.1: PCB126 concentrations in exposure solutions and in zebrafish.

Nominal PCB126, ng/L	**Exposure Solutions, ng/L**				**Zebrafish [c]**					
	Fresh prep [a]	**In exposure vessels [b]**			**pg/embryo or larva**		**ng/g wet weight [d]**		**ng/g lipid [e]**	
		0 h	**48 h**	**120 h**	**48 h**	**120 h**	**48 h**	**120 h**	**48 h**	**120 h**
0[f]	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
30	27	11.7 ±1.6	3.1 ±0.2	3.7 ±0.2	2.8 ±0.4	12.8 ±0.5	2.8 ±0.4	38.7 ±1.6	426 ±64	1,655 ±69
100	65	54.0 ±3.6	11.8 ±1.9	11.3 ±0.5	8.4 ±1.0	40.8 ±3.5	8.4 ±1.0	124 ±11	1,293 ±161	5,295 ±451
300	242	183 ±17	33.1 ±4.8	28.3 ±3.7	24.3 ±1.8	127 ±4.8	24.3 ±1.8	385 ±15	3,742 ±278	16,497 ±622
3000	2103	1,588 ±145	313 ±17	287 ±10	242 ±24	942 ±85	242 ±24	2854 ±258	37,190 ±3,611	122,332 ±11074

[a] Samples taken from freshly prepared solutions (n=1)

[b] Samples taken from exposure vessels at indicated exposure durations; values shown are average ± standard deviation (n=3)

[c] Zebrafish sampled after indicated exposure durations; values shown are average ± standard deviation (n=3)

[d] Average wet weight of 48 h-old embryo: 1 mg, of 120 h-old larvae: 0.35 mg

[e] Average total lipid weight per 48 h-old embryo: 6.5 µg, per 120h-old larvae: 7.7 µg (Petersen and Kristensen, 1998)

[f] DMSO control condition (0.01% DMSO)

4.3.3 Morphological and behavioural effects observed in the FET tests

No mortality occurred up to 5 dpf, but various sublethal morphological and behavioural effects were observed at 4 and 5 dpf (Fig. 4.2). The most dramatic effect was seen on swim bladder inflation, which at 5 dpf was significantly reduced at nominal aqueous concentrations as low as 30 ng/L (Fig 1B), yielding an EC50 of 169 ng/L (CI: 117 to 244 ng/L) or 0.52 nM (CI: 0.36 to 0.75 nM). If expressed based on internal PCB126 concentrations, the EC50 for no swim bladder inflation translates to 68.85 pg PCB126/larvae (CI: 48.64 to 97.46 pg PCB/larvae) or to 208 ng PCB126/g wet weight (ca. 600 pmol PCB126/g larvae wet weight). Reduction in swim-bladder pigmentation was also observed, but this endpoint was significantly different to controls only at the highest PCB126 concentration (62% and 75% of fish with reduced pigmentation of swim-bladder at 4 and 5 dpf, respectively, $p<0.001$). Thus, its sensitivity was similar to that of other, more traditional, morphological endpoints, such as heart edema and cranial malformations (Fig. 4.2A, 4.2B). Overall, fish exposed to 3000 ng/L were affected most severely, at 5 dpf presenting multiple sublethal effects per individual, including no swim bladder inflation (82 %), edema of heart (98 %) and yolk (93 %), cranial malformations such as short-nose and deformed jaw (83 %), heart malformations such as elongated and/or unlooped heart combined with reduced heart rate (70 %), and malformation of yolk sac (41 %) and spinal column (13 %). Fewer sublethal effects were observed in fish exposed to the 300 ng/L condition, but swim bladder inflation at 5 dpf was affected to a similar extent as in fish exposed to 3000 ng/L (Fig. 4.2B).

The behavioural endpoints examined were relatively insensitive, exhibiting significant difference to controls only at the highest exposure concentration at both 4 dpf (Fig. 4.2C) and 5 dpf (Fig. 4.2D). In particular, at 4 dpf the swim-up behaviour was reduced at 3000 ng/L compared to all other conditions ($p<0.05$), and more larvae were laying laterally in this treatment ($p<0.01$). At 5 dpf, normal swimming activity was observed in all treatments except for 300 ng/L in which swimming activity was slightly reduced and 3000 ng/L where hardly any swimming activity was observed ($p<0.05$). At 3000 ng/L, the prevalent behaviour was laying laterally ($p<0.001$), with remaining larvae exhibiting equilibrium loss while staying close to the vessel bottom (falling laterally) (Fig. 4.2D).

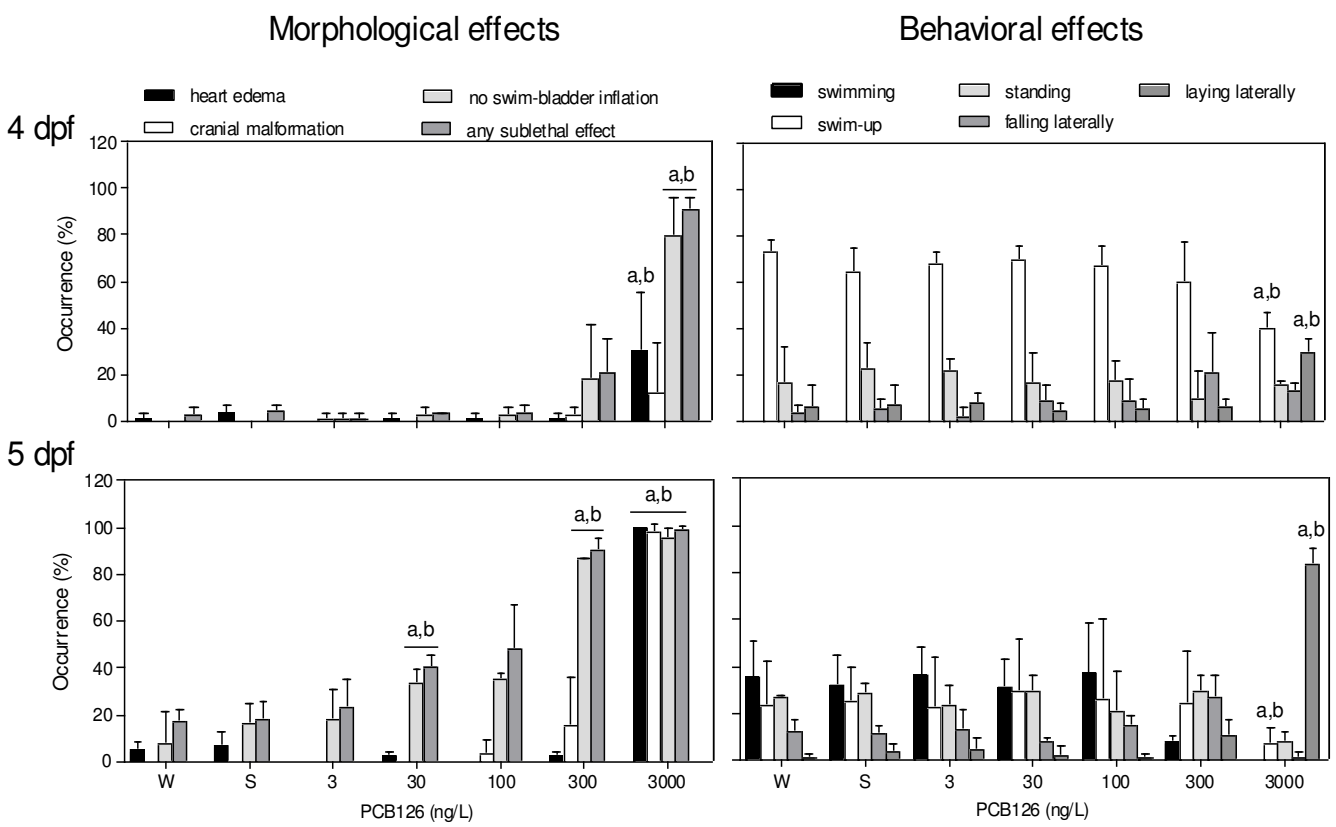

Figure 4.2: Morphological and behavioural effects assessed in 4 and 5 dpf zebrafish exposed to PCB126. Zebrafish embryos were exposed from 3 hpf until 5 dpf. The occurrence (%) of morphological effects at 4 dpf and 5 dpf (left side), and behavioural effects at 4 dpf and 5 dpf (right side), is shown for each respective exposure condition (W - water control, S - solvent control (0.01% DMSO) and different PCB126 concentrations in ng/L). Bars show the average ± standard deviation (n=3). Significant differences ($p<0.05$): one-way ANOVA followed by Dunett's Multiple Comparisons, a - compared to water control, b - compared to solvent control.

4.3.4 Delayed mortality and sublethal effects observed in pFET tests

In both pFET experiments, delayed mortality was observed after larvae were transferred to clean water (Fig. 4.3). A particularly steep increase in mortality occurred by 8 dpf: by then, almost all larvae had died in the two highest exposure concentrations and survival was reduced by up to 20 % in the 30 ng/L PCB126 exposure. Survival at the two highest concentrations (300 ng/L and 3000 ng/L PCB126) was significantly different from both water and solvent controls in both tests. In the 30 ng/L and 100 ng/L groups there was a tendency for lower survival, however a significant difference occurred only in the pFET-14 test when comparing the 30 ng/L condition to the water control (Fig. 4.3). The LC_{50} values at 8 dpf were 188 ng/L (CI: 143 to 247 ng/L) and 202 ng/L (CI: 156 to 262 ng/L) for pFET-14 and pFET-28, respectively. At the end of each experiment, the LC_{50} values were almost one order of

magnitude lower, i.e. 20 ng/L (CI: 10 to 39 ng/L) and 29 ng/L (CI: 11 to 73 ng/L) for pFET-14 and pFET-28 respectively. The similarity of the final LC_{50} values, as well as median survival times for the two highest concentrations (Fig. 4.3), obtained in pFET-14 and pFET-28, indicates that significant delayed mortality primarily occurred within the first three days after the end of exposure and hardly increased after 14 dpf.

At 28 dpf, a higher occurrence of sublethal effects, which included the reduced inflation of swim bladder and spinal malformations, was observed in fish exposed to 100 ng/L (Fig. 4.4A), a concentration which was one third of the one that caused 100% delayed mortality (i.e. 300 ng/L). Fish from the 100 ng/L group also presented decreased standard length (Fig. 4.4B) when compared to the other conditions, as well as generally delayed development (Fig. 4.4C). While circa 60% of the fish from controls, 3 ng/L and 30 ng/L treatments were already in the last stages of larval development, passing the stage of appearance of the pelvic fin ray (PR), only one individual of the 100 ng/L condition reached this developmental stage at 28 dpf. Also, about one third of the individuals exposed to 100 ng/L did not present the appearance of the anal fin ray (AR), expected to occur at circa 12 dpf, and instead were still at the stage of early or late inflation of the anterior swim bladder lobe (aSB+). Furthermore, considering that the onset of pigment pattern metamorphosis occurs in larvae of ca. 6.5 mm length at around 14 dpf (Parichy and Turner 2003, Parichy et al. 2009), it is worth noting that one third of the fish in the 100 ng/L treatment did not reach this length by the end of the pFET-28 experiment.

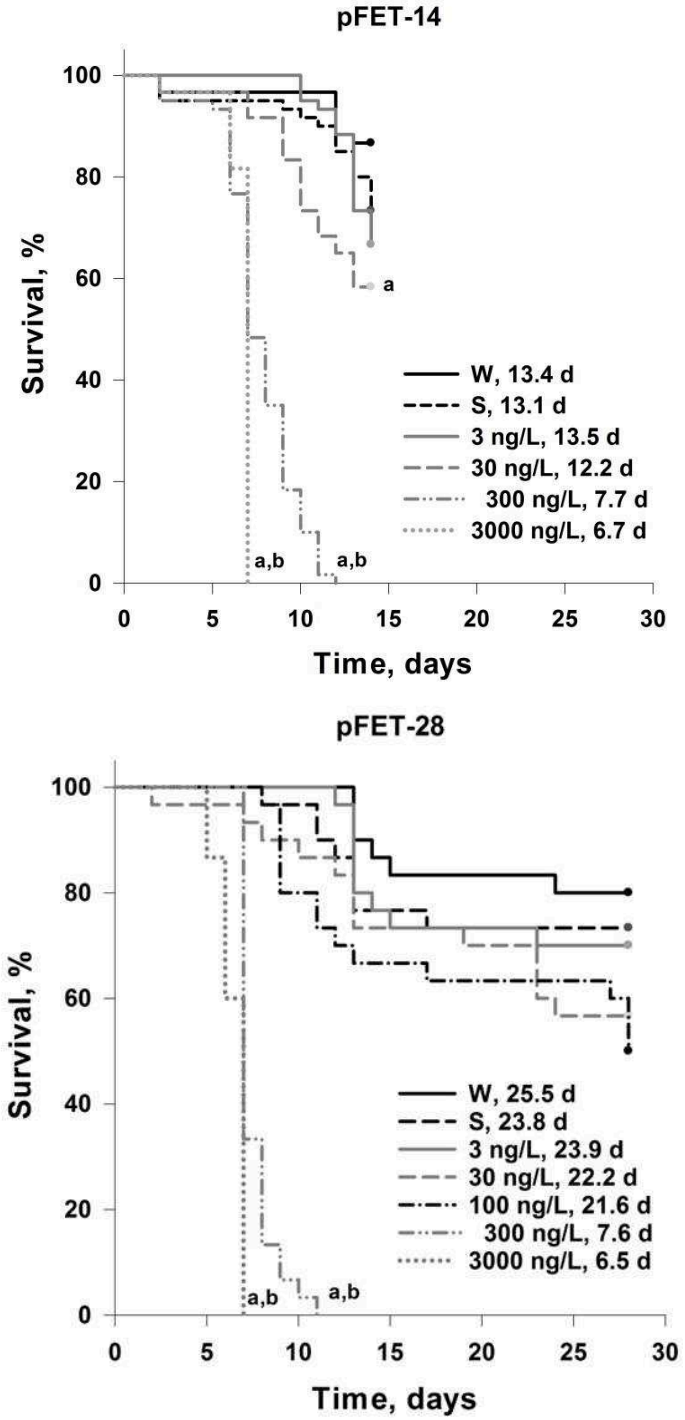

Figure 4.3: Delayed mortality observed in zebrafish maintained in clean water following PCB126 exposure during the first five days of development. Kaplan-Meier survival curves are shown for each exposure condition (W- water control, S - solvent control (DMSO 0.01 %) and different PCB126 concentrations) as observed in pFET-14 (A) and pFET-28 (B) experiments. Mean survival times (in days) are given for each condition on the respective graph legend. Significant differences: log-rank test followed by Holm-Sidak Multiple Comparisons Test, a - compared to water control, b - compared to solvent control.

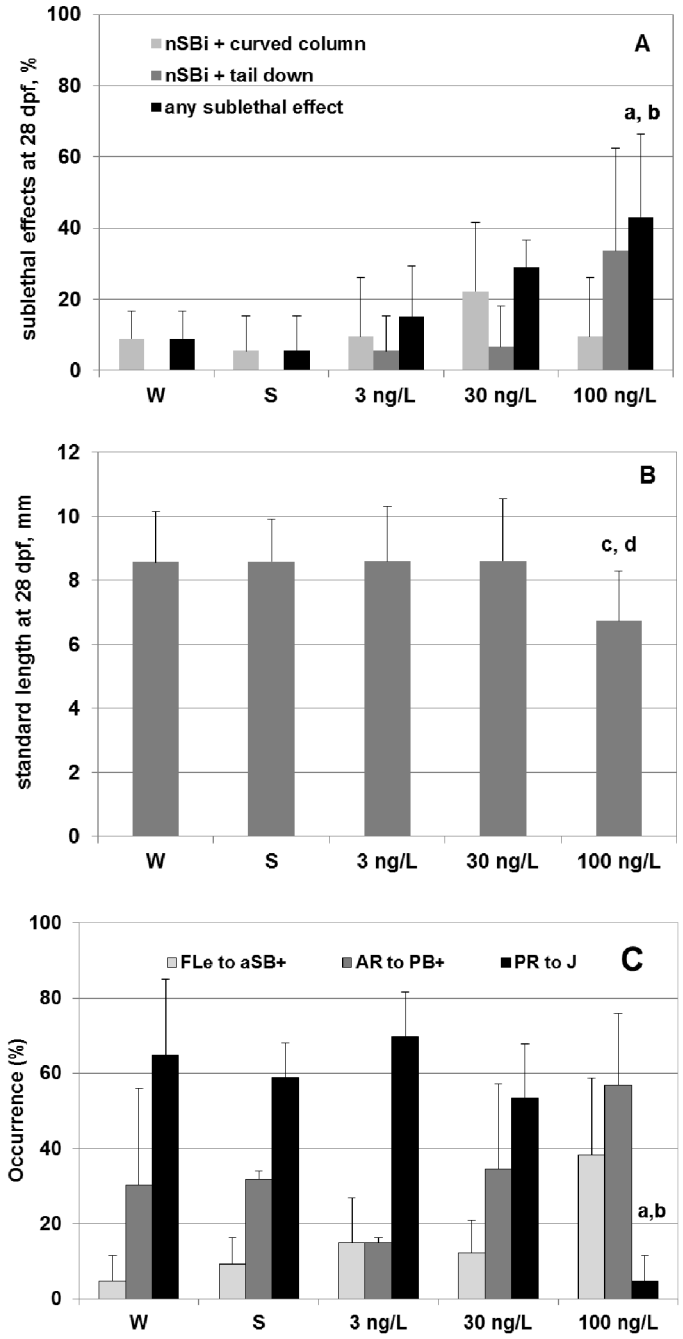

Figure 4.4: Sublethal effects and growth assessed in 28 dpf zebrafish larvae raised in clean water after exposure to PCB126 during the first five days of development. Bars show the average ± standard deviation values (n=3) for the occurrence of sublethal effects (A), standard length (B), and occurrence of developmental milestones (C) in surviving zebrafish in water (W) and solvent (S) controls and different PCB126 concentrations at day 28 of the pFET-28 experiment. Observed sublethal effects included no swim bladder inflation (nSBi), always combined either with lateral curvature of the column or/and with the condition of tail falling down during swimming. Occurrence of developmental milestones is grouped into three developmental ranges: from early flexion to following inflation of anterior swim bladder lobe (FLe to aSB+), from anal fin ray appearance to following pelvic fin bud appearance (AR to PB+), and from pelvic fin ray appearance to juvenile stage (PR to J). Significant differences ($p<0.05$) in (A) and (C): one-way ANOVA followed by Dunnett's (a - to water control, b - to solvent control); and in (B): Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's (c - to water control, d - to solvent control).

4.4 Discussion

The present study used PCB126 as a model compound to examine the occurrence of delayed toxicity in zebrafish exposed during early life stages (from 3 hpf to 5 dpf), to examine the relationship between a variety of endpoints (lethal and sublethal), and to obtain cross-species information by comparing results obtained with zebrafish to those observed in sole under similar exposure conditions.

The zebrafish FET test has recently been accepted and recommended as alternative to acute toxicity tests performed with adult fish (OECD 2013b). The next challenge for the scientific community is the identification of endpoints that can be assessed during the early developmental stages covered by the FET test that can be predictive of effects occurring at later stages (Villeneuve et al. 2014), for both chronic as well as delayed toxicity (Groh et al. 2015). In this study, we show that simply maintaining the zebrafish in clean water following the FET test exposure period can provide relevant information on the delayed mortality and sublethal effects that might occur following a short exposure during early life. In the environment, peak exposures can follow as a result from sediment resuspension (Schneider et al. 2007, Di Paolo et al. 2010) or storm (Rossi et al. 2004, Zgheib et al. 2012) and flood events (Wölz et al. 2008). For instance, concentrations of PCBs in urban storm waters reached more than 400 and 700 ng/L in Switzerland (Rossi et al. 2004) and France (Zgheib et al. 2012) respectively. Also, a major route of exposure of fish early life stages to dioxin-like compounds is via maternal transfer, which can result in increase of internal concentrations through the mobilization of the yolk reserves (Daley et al. 2014, Foekema et al. 2014). Thus, the pFET test covers an exposure scenario of high environmental and biological relevance. The occurrence of relevant delayed toxicity was also identified following exposure of fish to pyrethroid (Floyd et al. 2008) and phenylpyrazole (Beggel et al. 2012) insecticides, indicating that the prospect for delayed toxicity evaluation by the pFET test is expected also for other classes of compounds. Importantly, compared to the observation of toxic effects during the FET test period only, the assessment of delayed mortality and sublethal effects in the pFET pointed to a higher hazard resulting from the early PCB126 exposure.

4.4.1 Accumulation of PCB126 in zebrafish during early life exposure

Unlike in previous PCB126 toxicity studies that reported only nominal concentrations (Joensson et al. 2007b, Sisman et al. 2007, Na et al. 2009), here both aqueous and internal

PCB126 concentrations were measured during the exposure period. Surprisingly, despite the verified stock solution concentration, pre-soaking of vessels and daily solution renewal according to recommended procedures (OECD 2010a), the concentrations measured at the onset of the exposure (0 h) were roughly half of the nominal ones, and after 48h and 120 h they constituted only about 10% of the nominal values (Table 1). These losses were probably caused by the adsorption of PCB126 to glass materials during solution preparation and exposure (Wolska et al. 2005), which could have been further favoured by the relatively high ambient exposure temperatures (Lung et al. 2000) and the high ratio of surface area to volume in the small vessels used for exposures (Tanneberger et al. 2013).

Zebrafish early life stages can bioaccumulate very hydrophobic compounds such as the PCB126. A steep increase in the internal concentrations was observed after 5 days of exposure compared to those measured after 2 days of exposure. This could be due to the presence of chorion that functions as an uptake barrier during the first days of development until hatching (Braunbeck et al. 2005), which occurs between 2 dpf and 3 dpf. Similarly, in studies with zebrafish embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the chorion has been suggested to act as a barrier to chemical uptake (Baker et al. 2013), and a substantial increase in body burden was observed between 1 dpf and 3 dpf, correlating with the hatching period (Lanham et al. 2012). Petersen and Kristensen (1998) determined the bioaccumulation kinetics of the PCBs 31 and 105 in zebrafish embryos and larvae, and found that steady state was not reached up to 10 dpf for the larger PCB105. That supports our finding that steady state concentrations were not reached during PCB126 exposure over the first 5 days of development. Accordingly, our experimentally determined logBCF values are valid for 5 dpf-old fish only, i.e. the latest time point at which zebrafish are accepted as non-protected life stage according EU legislation. However, it probably underestimates internal concentrations that would be achieved over a longer exposure period. According to the regression model for predicting logBCFs for zebrafish larvae at 10 dpf (Petersen and Kristensen 1998), we would expect a wet-weight normalized logBCF of 5.5 and a lipid normalized logBCF of 7.0, assuming a $\log K_{ow}$ for PCB126 of 6.89, (Hawker and Connell 1988). Instead, the BCF values we measured over the shorter exposure period were 1.5 orders of magnitude lower. Thus, both toxicokinetic processes and the temporal pattern for the accumulation of a lipophilic chemical like PCB126 need to be considered when attempting to use zebrafish embryos to predict long-term bioaccumulation and toxicological effects in fish (Kühnert et al. 2013, Sanz-Landaluze et al. 2015).

4.4.2 Behavioral and morphological effects of early life PCB126 exposure and their correlation to delayed toxicity

Although diverse behavioural endpoints are increasingly included in zebrafish studies (Melvin and Wilson 2013, Selderslaghs et al. 2013), only a few toxicity test guidelines so far have addressed behavioural effects to some extent (ASTM 2012, OECD 2013a). The swim-up behaviour of early larvae is usually not considered in the toxicological studies, despite its relevance for the inflation of the swim bladder and consequently swimming capacity (Lindsey et al. 2010). We observed that exposure to PCB126 affects the timing and prevalence of this particular behaviour. In control larvae, swim-up was prevalent in 4 dpf larvae and was succeeded by swimming in 5 dpf larvae. In 3000 ng/L PCB126-exposed larvae, swim-up behaviour occurrence was severely reduced, with increased incidence of fish laying laterally. The 300 ng/L PCB126-exposed larvae showed equilibrium loss and a prolonged swim-up period that continued into 5 dpf, when normal swimming should already occur. The observed behavioural effects of PCB126 are in agreement with those reported for TCDD, which caused loss of equilibrium and lethargy in different fish species (Elonen et al. 1998). However, the behavioural effects we monitored turned out to be relatively insensitive, with statistically significant differences noted only in the highest exposure concentration. More sophisticated functional analyses as well as application of automated prolonged monitoring of swimming characteristics and activity patterns (Moser 2011, Sloman and McNeil 2012, Di Paolo et al. 2015c) might enable detection of more subtle significant differences in behaviour in future studies.

Morphological effects observed in the FET tests were similar to those reported in previous investigations of PCB126 toxicity in zebrafish (Joensson et al. 2007a, Sisman et al. 2007, Na et al. 2009, Waits and Nebert 2011) and reflected typical AhR-mediated dioxin-like effects, such as disruption of osmoregulation and induction of malformations in cardiovascular system and skeleton (Carney et al. 2006, Xiong et al. 2008, King-Heiden et al. 2009). Indeed, in addition to induction of *cyp1a*, previous studies have shown that other genes with relevance to skeletal malformation (e.g *sox9b* (Xiong et al. 2008) and *col11a2* (Yokoi et al. 2009)) and swim bladder inflation (e.g. *myca* (Henry et al. 1997)) are modulated following the exposure to TCDD. Also, the disruption of retinoid homeostasis, which regulates tissue formation and epithelial integrity, is a well-known mechanism of developmental toxicity caused by dioxin-like compounds (Nilsson and Håkansson 2002) and its occurrence was related to developmental malformations in early life stages of amphibians exposed to PCB126 (Gutleb

et al. 1999). Among the observed effects in our study, impaired swim bladder inflation, craniofacial and skeletal malformations are considered to be particularly relevant predictors for chronic toxicity. These effects are likely to have a strong impact on feeding and swimming abilities, thus directly affecting the larval development and survival.

Reduced occurrence of swim bladder inflation, recently recommended for inclusion as standard morphological endpoint in FELS tests (Li et al. 2011, Villeneuve et al. 2014), was the most sensitive endpoint among the various morphological and behavioural effects analysed in the FET tests. This is in accordance with previous reports of swim bladder inflation being one of the most sensitive targets for toxicity mediated by AhR agonists (Sisman et al. 2007, King-Heiden et al. 2009, Joensson et al. 2012). It has been suggested that impairment of swim bladder inflation could be due either to disruption of normal development during organ formation or to disruption of normal functioning after formation (Villeneuve et al. 2014). For AhR ligands, both alternatives are likely. Histopathological assessment of non-inflated swim bladder in PCB126-exposed zebrafish demonstrated impairment of swim bladder development, as the organ membranes and pneumatic duct were seen to form a compact structure with necrosis points, instead of an open organ with a thin epithelia observed in inflated bladders (Joensson et al. 2012). In TCDD-exposed zebrafish and medaka, both non-inflation as well as inflation followed by deflation were observed (Henry et al. 1997). In our study, no recovery of swim bladder inflation was observed during the clean water period following the early life exposure to PCB126. Interestingly, the EC50 value for occurrence of no swim bladder inflation by 5 dpf (169 ng/L) was in the same range as the LC_{50} value in 8 dpf fish following the first mortality wave (188 ng/L and 202 ng/L for pFET-14 and pFET-28, respectively). Detrimental effects on swim bladder inflation occurring in early life during the organ formation period may be indicative of other severe AhR-mediated effects, including mortality, at later stages (Villeneuve et al. 2014).

Also, disruption of cartilaginous and bone tissue development can have adverse effects in later life stages, affecting prey handling (jaw and cranial malformations) and swimming (column malformations). Although increased incidence of craniofacial malformations during the exposure period was visually observed only in the two highest concentrations, skeletal malformations developed into prevalent delayed sub-lethal effects at later life stages in surviving zebrafish. The observed column malformations were similar to those described for TCDD, with early exposure causing axial skeletal malformations analogous to scoliosis at later stages (Baker et al. 2013). Jaw malformations in response to PCB126 observed in this

study were similar to those reported for TCDD previously (Xiong et al. 2008), indicating the likely involvement of common AhR-mediated pathways (Carney et al. 2006, King-Heiden et al. 2012). Interestingly, the lack of a functional swim bladder and the appearance of lordosis seem to be correlated, also described for juvenile *Dicentrarchus labras* and *Sparus aurata* reared in captivity. Both effects became more prevalent when fish were forced to swim against a current compared to individuals that were kept in static water (Chatain 1994). In our study, fish exposed to 30 ng/L PCB126 and higher concentrations presented impaired swim bladder inflation, which indicates that the animals needed to spend more energy for swimming and feeding. This greater energy expenditure could have contributed to the growth impairment observed in fish exposed to 100 ng/L PCB126, the condition at which also significant column malformations and impaired swimming activity occurred. Furthermore, as discussed in the next section, bioamplification might have been promoted in fish from this condition due to insufficient growth dilution (Daley et al. 2013).

4.4.3 Comparison of delayed PCB126 toxicity in zebrafish and sole at similar exposure scenarios

The critical developmental periods for the occurrence of delayed mortality in zebrafish corresponded well to those previously reported for sole (Foekema et al. 2008, Foekema et al. 2014). In both species, exposure during the non-feeding stage was sufficient for induction of delayed effects during the clean water period. The most pronounced wave of delayed mortality ensued after the yolk was fully absorbed, in a few days after the fish become free-feeding, i.e. at around 7 dpf in zebrafish and 12 dpf in sole. This critical period of transition to entire dependence on exogenous food sources, as opposed to strict reliance on endogenous yolk resources, has already been identified as prone to mortality (Flynn et al. 2009). When very hydrophobic chemicals such as PCB126 accumulate in the lipid-rich yolk, the progression of yolk absorption will lead to chemical mobilization into the embryo tissues, until the toxic residue concentrations are reached in target organs. Compounds having a $\log K_{ow}>5$ are not easily eliminated, presenting highest internal concentration when lipid reserves become depleted (Foekema et al. 2012, Daley et al. 2013). Our study shows that in zebrafish, similarly to sole, the transition into the free-feeding stage following yolk resorption represents the most critical window for toxicity elicited by lipophilic chemicals. It is the moment at which the internal lipid-normalized concentration might be higher than in any other life stage of the fish (Foekema et al. 2012).

The second critical time point in zebrafish occurs at ca. 12 dpf, when the energy obtained from the yolk-sac is completely depleted. In fact, if zebrafish larvae are not fed they are still able to survive on energy of maternal origin until 10-12 dpf (Rombough 2002) after which mortality ensues in the non-fed fish (Kienle et al. 2009, Imrie and Sadler 2010). Additionally, the 12-14 dpf time point in zebrafish larval development marks the onset of metamorphosis for pigment pattern and development of photoreceptors (Parichy and Turner 2003, Budi et al. 2008). Consequently, only the fish able to properly eat and transform the ingested food into energy will be able to survive after 12 dpf and acquire the extra energy required to proceed into later stages and undergo proper metamorphosis. Indeed, in the second highest concentration group (300 ng/L) the last fish died at 12 dpf, while surviving individuals from the next lower exposure group (100 ng/L) presented reduced growth and delayed development by the end of the test. In addition, one third of these individuals did not reach the developmental stage usually occurring at ca. 12 dpf, which indicates that the fish did not obtain enough energy to properly support further development and metamorphosis. Delayed metamorphosis following exposure to PCBs has already been observed in sole (Foekema et al. 2008, Foekema et al. 2014) and flounder (Soffientino et al. 2010), as well as in amphibians (Gutleb et al. 1999). Such developmental delay may be related to reduction in food ingestion or assimilation due to reduced prey capture, as observed in *Fundulus heteroclitus* exposed to PCB126 (Couillard et al. 2011) and zebrafish exposed to TCDD (Chollett et al. 2014). Also, more specific toxicity mechanisms might be involved, such as interferences with thyroid hormones. In rats, PCB126 has been demonstrated to produce TCDD-induced hypothyroidism by glucuronidation of thyroxine (Martin and Klaassen 2010). Thyroidal hormones are of crucial importance for the normal growth and development of fish and tadpole embryos and larvae (Power et al. 2001). Also for zebrafish their involvement in the transition from embryo to larvae phase has been demonstrated (Liu and Chan 2002).

Similarly to sole (Foekema et al. 2008), in zebrafish the LC_{50} values decreased with longer observation duration. Around the period of the first wave of delayed mortality, zebrafish were 2-times less sensitive than sole, difference which increased to up to an order of magnitude at later time points. The LC50 for delayed mortality in zebrafish exposed during 0-5 dpf and observed at 8 dpf was 188 ng/L, while for sole an LC_{50} of 82 ng/L was reported at the corresponding developmental time point (12 dpf) after exposure during 0-4 dpf (Foekema et al. 2008). Interestingly, in experiments with sole, the extension of the exposure period up to 10 dpf (to cover the entire non-feeding yolk-sac stage) did not significantly change the 12 dpf

LC_{50}, indicating that the first 4 days of exposure were sufficient in acquiring the critical body burdens causing the observed effects (Foekema et al. 2008). Indeed, it has been previously shown that, despite the lower uptake rate in embryos protected by chorion when compared to larvae, the also lower embryonic elimination rate may result in BCFs at comparable levels (Petersen and Kristensen 1998). At later time points, LC_{50} values dropped down to 20 ng/L in zebrafish and 1.7-3.7 ng/L in sole, the range in latter depending on the initial exposure duration (Foekema et al. 2014). A later study from the same group reported the internal LC_{50} concentration for PCB126 in sole exposed at 0-6 dpf to be 1.3 µg/g lipid which roughly corresponded to the levels obtained the 3 ng/L exposure condition (Foekema et al. 2014). In zebrafish after 0 to 5 dpf exposure, a comparable PCB126 internal concentration (1.7 µg/g lipid) was reached at the nominal exposure concentration of 30 ng/L, which is an order of magnitude higher (Table 1). It has been shown that higher ambient temperatures result in lower accumulation of PCBs during waterborne exposures of embryos and larvae, possibly due to increased elimination rates (Petersen and Kristensen 1998). Thus, the observed differences in sensitivity can at least partially be explained by different water temperatures used in zebrafish (26ºC) and sole (12 to 13 ºC) experiments, which could result in lower PCB126 accumulation in zebrafish compared to sole at similar exposure levels. Furthermore, the lipid fraction of fertilized eggs of sole (1.6%) (Foekema et al. 2012) is around three times higher than in zebrafish (0.5%) (Nyholm et al. 2008), contributing to relatively higher bioamplification and tissue concentrations of PCB126.

Importantly, when attempting to perform inter-species comparison studies with laboratory models or with wild fish, it is of relevance to consider that they might vary in their capacity to adapt to chemical exposure (Whitehead et al. 2011). For example, different *Fundulus heteroclitus* populations presented high variability in sensitivity to PCB126 exposures, with tolerance being directly correlated with the PCB levels at their respective residence sites (Nacci et al. 2010). However, the adaptive tolerance to specific stressors, such as high concentrations of certain classes of pollutants, might reduce genetic diversity in fish (Paris et al. 2015) and impair their resistance to additional stressors. For instance, pollution-resistant *Platichthys flesus* individuals were indicated to present lower tolerance to thermal stress when compared to individuals from moderately contaminated site (Lavergne et al. 2015). Since the definition of adequate protection goals depends on the identification of the most vulnerable life stages, populations and species, there is benefit from the combined interpretation of

laboratory studies together with the information on the capacity of different wild fish to adapt or not to environmental stressors (Hamilton et al. 2015).

Despite the slight differences in zebrafish and sole LC_{50} values, the effects of early life stage exposure to lipophilic compounds on the later survival and occurrence of sublethal effects are quite similar in zebrafish and sole. In both species, the period marking the end of the yolk-sac stage and complete reliance on free-feeding turned out to be most critical for survival. Also, a similar reduction in metamorphosis rates was observed in exposure groups exhibiting sublethal effects. This indicates the similarity of the effects induced by early life exposure and the comparability of critical developmental stages across divergent species, emphasizing the interspecies relevance of experiments with zebrafish.

4.5 Conclusions

We investigated an experimental setup for observing delayed toxic effects in zebrafish, and assessed the suitability of diverse endpoints measured during the early life exposure period for prediction of delayed lethal or sublethal effects. Among the evaluated endpoints, the effects on swim bladder inflation and on cartilaginous tissues appeared to be the most promising for prediction of delayed mortality. In particular, the EC_{50} value for swim bladder inflation in the FET test was very similar to the LC_{50} value obtained in the pFET test at 12 dpf. The patterns of delayed mortality and delayed sublethal effects on growth and development were similar between sole and zebrafish based on measured internal concentrations. Our study reveals that zebrafish early life stages present an easily accessible, well described and relevant experimental model for evaluation of the delayed toxicity potential of lipophilic chemicals in fish. Since the prolonged observation period enhances the predictive value of the test and involves a more effective use of experimental animals, the zebrafish pFET test should be applied more broadly for the evaluation of lipophilic chemicals or environmental samples suspect of causing delayed mortality or sublethal effects.

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Chapter 5: Antiandrogenicity and estrogenicity assessment of surface waters

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Part of this chapter is being prepared for submission to the peer review journal Water Research as:

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Screening for cytotoxic, anti-androgenic and estrogenic effects of surface water of the Saale-Mulde Catchment by in vitro assays

Abstract

Endocrine-disrupting aquatic pollutants belong to varied classes of chemicals, such as pesticides, herbicides, fungicides, biocides, chemical catalysts, plastics and plasticizers, pharmaceuticals and phytoestrogens. Wastewater treatment plant effluents are important sources of these compounds to aquatic environments, leading to the exposure of aquatic organisms and humans. Although antiandrogenic compounds started being investigated relatively recently, they have already been determined in different environments, effluents and wastes. That motivates monitoring efforts to determine the occurrence and biological effects of antiandrogenic chemicals in aquatic systems. The present study investigated more than 30 surface water sample extracts of the Saale-Mulde catchment in Germany for antiandrogenicity utilizing an androgen reporter gene cell-based assay, complemented by cytotoxicity evaluation by three different tests. Target chemical analysis allowed performing mass-balance analysis to estimate how much of the bioactivity could be explained by the measured compounds. Cytotoxicity results indicate good agreement between the three applied methods, indicating that the selection of exposure concentrations with basis on the MTT test results is an adequate approach. Therefore highest test concentrations in the antiandrogenicity assay presented at least 80 % cell viability, being for most samples at the concentrations factor of 25. Bioassay flutamide equivalents ranged from 28.8 ng/L up to 6.1 µg/L water, and no bioactivity was found in the procedure blank. Despite the wide range of measured activities, antiandrogenicity was identified in most of the water samples, indicating a potential threat to aquatic organisms and humans. The highest value was obtained for a sample collected at the Holtemme River near a health facility, which presented reduction of magnitude of AR response in a dose-response manner. The mass-balance analysis indicated bioassay flutamide equivalents always higher than respective chemical equivalents, with ratios between 4 and 4,000. That indicates that the chemical analysis of a selected list of compounds was of limited value to estimate the antiandrogenic potential of surface waters, and the complementary assessment through bioassays is recommended. The dose-response antiandrogenic effect identified at the River Holtemme sample was very poorly explained by the target chemical analysis, even if it presented the highest concentration of the antiandrogenic pharmaceutical bicalutamide. As an outcome, an effect-directed analysis case study was set to investigate the antiandrogenic activity of the Holtemme river surface water sample, presented in Chapter 6.

Keywords: antiandrogenic activity, reporter gene bioassay, surface waters, wastewater treatment plant effluents.

5.1 Introduction

Endocrine-disrupting chemicals (EDCs) can be defined as natural or synthetic compounds that can alter the organism hormonal and homeostatic system through diverse mechanisms that converge upon endocrine and reproductive systems. Those include interactions with nuclear hormone receptors (e.g. estrogen, androgen, thyroid), but also with nonnuclear steroid hormone (e.g., membrane) and nonsteroid (e.g., neurotransmitter) receptors; and enzymatic pathways involved in steroid biosynthesis or metabolism (Diamanti-Kandarakis et al. 2009). Compounds that can act as EDCs belong to varied classes of contaminants, such as pesticides, herbicides, fungicides, biocides, chemical catalysts, plastics and plasticizers, pharmaceuticals and phytoestrogens (Diamanti-Kandarakis et al. 2009, Frye et al. 2012).

Effluents from wastewater treatment plants (WWTP) are important sources of EDCs to aquatic environments, leading to exposure of aquatic organisms and humans to complex mixtures of these chemicals (Petrovic et al. 2002, Kasprzyk-Hordern et al. 2009, Kusk et al. 2011, Maletz et al. 2013). Estrogenic compounds are the most studied EDCs in aquatic systems, and the recognition of their threats lead to the inclusion of estrogenic pharmaceuticals in the Water Framework Directive chemical monitoring watch list (EC 2013, Kunz et al. 2015). Antiandrogenic compounds, on the on the other hand, started being investigated more recently. Nevertheless they have been already determined in different aquatic environments (Urbatzka et al. 2007, Weiss et al. 2009, Liscio et al. 2014, Alvarez-Munoz et al. 2015), effluents and wastes (Van der Linden et al. 2008, Thomas et al. 2009a, He et al. 2011). That widespread occurrence results in monitoring efforts to determine the occurrence and bioeffects of EDCs in aquatic systems.

Monitoring studies have been developed by researchers from the Helmholtz Centre for Environmental Research (Germany) in the catchment of the Saale and Mulde Rivers (Saxonia, Saxonia-Anhalt and Thuringia), which are among the most polluted tributaries of the Elbe River (Weigold and Baborowski 2009). This chapter presents the outcomes of an investigation developed in collaboration with the Department of Effect-Directed Analysis in the context of one of these monitoring studies, which evaluated surface waters utilizing chemical analysis and bioassays for different mechanisms of toxicity. Selection of sampling sites was designed to evaluate locations in the vicinity to and downstream of industrial, municipal or hospital WWTPs. Also, reference locations considered as unpolluted or low polluted were included.

The present study investigated surface water samples of the Saale-Mulde catchment for antiandrogenic activities utilizing an androgen reporter (AR) gene cell-based bioassay. Cytotoxic effects of samples were previously investigated using the MTT test in order to exclude concentrations that would reduce cell viability, which is an important step in order to avoid false positives in antagonistic assays. Further, two other cytotoxicity methods were integrated in the antiandrogenicity assay. Chemical analysis evaluated the occurrence of a list of target compounds in the water samples, and mass-balance analysis estimated how much of the identified bioactivity could be explained by the measured compounds. As an outcome, a particularly highly antiandrogenic sample was identified and selected for further EDA investigation.

5.2 Materials and Methods

5.2.1 Chemicals

Dimethyl sulfoxide (DMSO) and the bioassay chemicals flutamide (CAS 13311-84-7), 17β-Estradiol (CAS 50-28-2) and dihydrotestosterone (CAS 521-18-6) were purchased from Sigma Aldrich (Sigma Aldrich Chemie GmbH, Steinheim, Germany).

5.2.2 Sample collection and extraction

Surface water samples were collected and extracted by UFZ researchers as previously described (Bloch 2012). Briefly, 31 surface water samples were collected in diverse locations in the Saxony, Saxony-Anhalt and Thuringia regions in Germany (Table 5.1, Fig. 5.1). Sampling sites were either located near of downstream to WWTP belonging to three general categories (urban area, industry, health facility); or were selected as reference sites. Sample preparation followed the procedure by Singer et al. (2009). After adjusting the pH to 6.5, samples were filtered and stored at 4 °C until extraction by solid-phase extraction using mix-mode cartridges, with 2.5 L of water being concentrated in 1 mL of extract (2,500 times concentrated). A process blank was prepared by submission of the method solvents through the same procedures as sample. For the evaluation in bioassays, the extract was evaporated to dryness and the same volume of DMSO was added.

Table 5.1: Sampling site codes, rivers, locations and state, and general classification. Sampling sites were either located near of downstream to WWTP belonging to three general categories (urban area, industry, health facility); or were selected as reference sites. Adapted from Bloch (2012).

Code	River	Location	State	Classification
B1	Bode	Thale	Saxony-Anhalt	Reference
B2	Bode	Staßfurt	Saxony-Anhalt	Urban area
C1	Chemnitz	Chemnitz	Saxony	Urban area
C2	Cheminitz	Göritzhain	Saxony	Industry
Eu	Eula	Bad Lausick	Saxony	Health facility
Gö	Göltzsch	Rodewisch	Saxony	Health facility
H1	Holtemme	Wernigerode/Silstedt	Saxony-Anhalt	Reference
H2	Holtemme	Wernigerode/Silstedt	Saxony-Anhalt	Health facility
Ilm	Ilm	Bad Sulza	Thuringia	Health facility
LA	Alte Luppe	Leipzig	Saxony	Industry
LN	Neue Luppe	Leipzig	Saxony	Health facility
M1	Zwickauer Mulde	Rautenkranz	Saxony	Reference
M2	Zwickauer Mulde	Zwickau	Saxony	Health facility
M3	Dorfbach	Niederschingmass	Saxony	Industry
M4	Mulde	Trebsen	Saxony	Industry
M5	Mulde	Bad Düben	Saxony	Health facility
M6	Mulde	Bitterfeld	Saxony-Anhalt	Industry
M7	Spittelwasser	Bitterfeld	Saxony-Anhalt	Industry
P1	Pleiße	Ebersbrunn	Saxony	Reference
P2	Pleiße	Böhlen	Saxony	Industry
S1	Saale	Eichicht	Thuringia	Reference
S2	Saale	Rudolstadt	Thuringia	Industry
S3	Saale	Jena	Thuringia	Urban area
S4	Saale	Weißenfels	Saxony-Anhalt	Urban area
S5	Saale	Leuna	Saxony-Anhalt	Industry
S6	Saale	Schkopau	Saxony-Anhalt	Industry
S7	Saale	Halle	Saxony-Anhalt	Urban area
S8	Saale	Calbe	Saxony-Anhalt	Industry
Sol	Sole Channel	Schönebeck	Saxony-Anhalt	Health facility/Industry
WE	Weiße Elster	Ostrau	Saxony-Anhalt	Industry
Z	Ziethe	Köthen	Saxony-Anhalt	Urban area

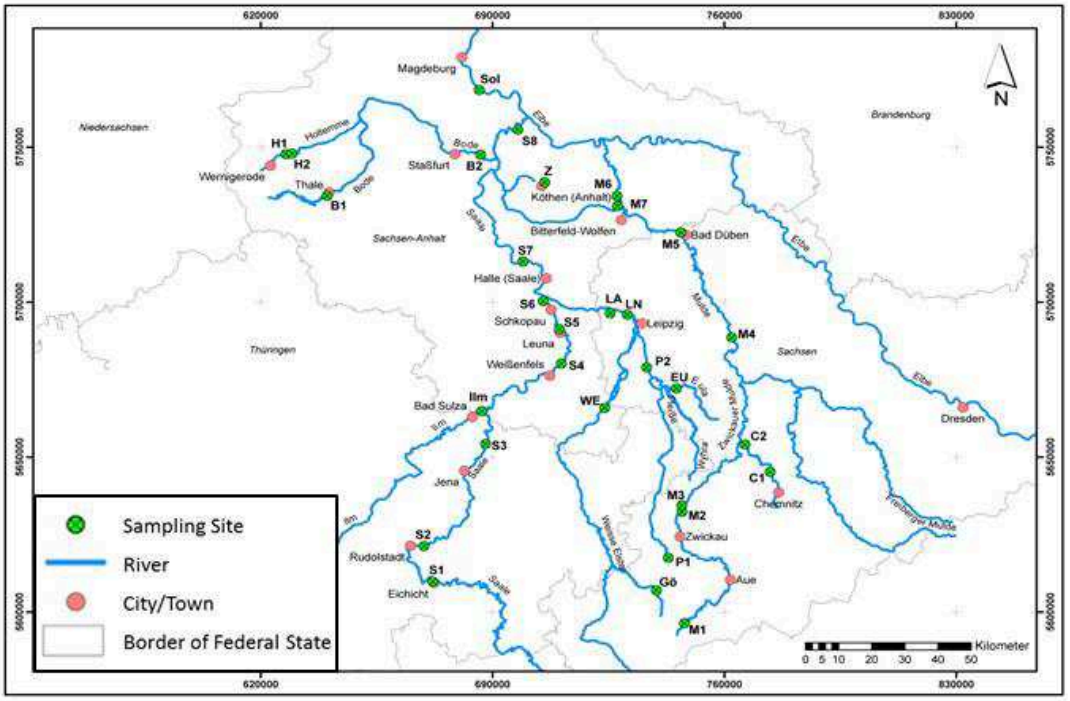

Figure 5.1: Sampling sites (in green) locations in the catchment of the Saale and Mule Rivers in the Saxony, Saxony-Anhalt and Thuringia regions in Germany. Adapted from Bloch (2012).

5.2.3 Testing strategy

A testing strategy was followed for the present study (Fig. 5.2). The water sample extracts were first tested regarding cytotoxic effects in the MTT assay. According to results, five non-cytotoxic concentrations were selected for testing regarding antiandrogenic effects. In addition, some of the water samples were also tested utilizing two fluorometric cytotoxicity assays, i.e. Alamar Blue (AB) and CFDA-AM (Bopp and Lettieri 2008), which were integrated into the antiandrogenic assay. The verification of cytotoxicity was emphasized in order to avoid false positive results.

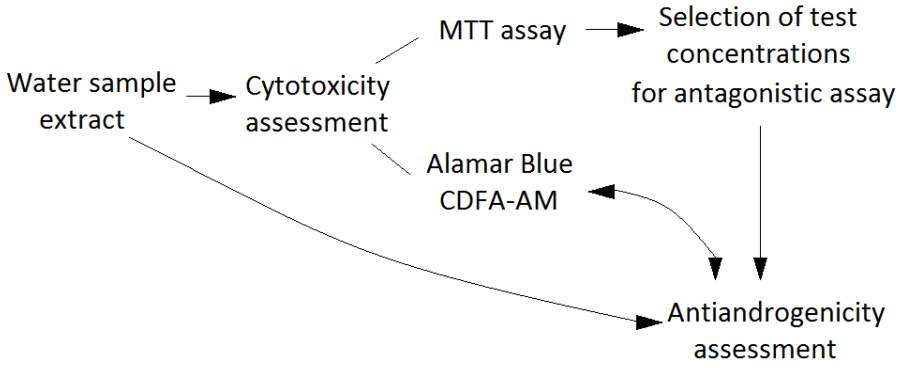

Figure 5.2: Testing strategy applied to evaluate the water sample extracts regarding antiandrogenicity. Cytotoxic effects were also carefully evaluated in order to avoid false positive results.

5.2.4 Cell culture

The human osteoblastic osteosarcoma U2OS cell line stably co-transfected with an expression construct for the human AR and a respective reporter construct was obtained from BioDetection Systems BV (BDS, Amsterdam, the Netherlands) and cultured as previously described (van der Burg et al. 2010). Briefly, cell cultures were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (Invitrogen Life Technologies, Darmstadt, Germany) with phenol red supplemented with 7.5 % fetal calf serum (FCS) (Th. Geyer GmbH, Renningen, Germany), 0.2 % penicillin/streptomycin solution (Invitrogen), 1 % non-essential amino acids (Invitrogen) and G418 antibiotic (0.20 mg/ml) for the selective survival of transfected cells only. Cultures were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C until reaching around 90 % confluence, when cells were used for tests or sub-cultures. Cells were passaged twice a week and medium change was performed every two to three days.

5.2.5 AR reporter gene assay performance

Cell cultures were treated with trypsin, cells were counted and re-suspended in assay medium (phenol red-free 1:1 mixture of DMEM/F12 supplemented with 5 % of stripped FCS, 0.2 % penicillin/streptomycin solution and 1 % non-essential amino acids) to a final concentration of 10^5 cells/ml. The cell suspension was transferred to 96-well plates, with each well receiving 100 µl (10^4 cells). External wells of each plate were filled instead with 200 µl of PBS in order to minimize medium evaporation. After 24 h, the medium was refreshed and cells were exposed to serial dilutions of the water samples water sample extracts in exposure medium. Water extract test concentrations were selected with basis on the MTT test results. To evaluate antagonistic activity, there was co-exposure with a non-saturating DHT concentration (4.2×10^{-10} M). Each plate contained also a serial dilution of the standard chemical flutamide (10^{-9} to 10^{-5} M), solvent (1% DMSO), and DHT (4.2×10^{-10} M DHT) controls. Conditions were performed in triplicate wells in each test and contained final DMSO 1%. After 24 h exposure at 37 °C and 5 % CO_2, the medium was removed, and PBS (50 µL) and Steady Glo® (50 µL) solution were added to each well. The luciferase activity was measured using a luminometer (GloMax®- 96 Microplate Luminometer Promega, USA).

5.2.6 Cytotoxicity assays

Cell viability of was evaluated through three complementary methods. The MTT test was applied to evaluate all the samples. The Alamar-Blue and CFDA/AM assays were additionally

integrated into the investigation of 20 samples (H1, H2, P1, P2, C1, C2, Ilm, Gö, M4, M7, S3, S4, S5, S6, LA, LN, Eu, Sol, WE, Z) to further elucidate potential cytotoxicity.

(i) MTT test cell viability assessment

Cell viability of samples was evaluated though a colorimetric microplate assay with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT test), which is reduced to formazan by viable cells (Mosmann 1983, Berridge et al. 2005). Briefly, cells were exposed following the described downscaling procedures as for the AR binding analysis to water extracts, with the highest concentration factor (CF) being of 12.5 (i.e. 12.5 mL of water equivalent per mL of medium). In addition, for every assay run a positive control plate performing exposure to serial dilutions of 3,5-DCP (2.5 to 40 mg/L) and Triton X100 (0.0625 to 1 %) were performed. At the end of the 24 h incubation period, there was visual verification of no contamination evidence, medium was removed, and each well received 100 µl of freshly prepared 0.5 mg/mL MTT in FCS-free assay medium. In each plate, 6 wells containing no cells received the MTT solution for the measurement of background signal. After 30 min of incubation at 37 °C and 5 % CO_2, the occurrence of formazan crystals was confirmed under microscope observation. The medium was discharged, 200 µl of DMSO was added per well, and plates were shaken for 15 min for crystal solubilisation. The amount of formed formazan was measured with a microplate spectrophotometer (Tecan Infinite® M200, Tecan, Switzerland) at an absorbance wavelength of 492 nm.

(ii) Alamar-Blue and CFDA/AM Assay (AB-CFDA/AM)

The fluorometric AB and CFDA/AM assays (AB-CFDA/AM) were integrated into the antiandrogenicicy assay for the test of selected samples. For that, the AB-CFDA/AM-working solution (5 % v/v AB and 4 µM CFDA/AM) was freshly prepared at the day of the assay and stored at room temperature and in the dark. After exposure following the anti-AR CALUX® procedure (see below), the exposure media was removed. Cells were rinsed with PBS, 125 µL of the working solution was added to each well, and incubation was done in the dark at room temperature for 30 min. Afterwards, the fluorescence of AB (at 530 - 595 nm) and of CFDA-AM (at 493 - 541 nm) were measured in an infinite M200 Tecan reader (Tecan, Switzerland).

5.2.5 Chemical analysis

Chemical analysis of the water sample extracts by quantitative target screening was performed by UFZ through liquid chromatography / high resolution mass spectrometry using an ion trap-Orbitrap hybrid instrument as previously described (Hug et al. 2015). For the

target analysis of endocrine compounds an Agilent 1260 LC system coupled to an ABSciex QTrap 6500 mass spectrometer was used. For the analysis of compounds in ESI- mode (bisphenol A, bisphenol F, 17α-ethinylestradiol, 17β-estradiol, estriol, estrone, 2-phenylphenol, 4-nonylphenol) a Kinetex C18 column (100 x 3.0 mm, 2.6 µm particle size, Phenomenex) was used with elution done using 1 mM ammonium fluoride and methanol. For the analysis of compounds in ESI+ mode (4-androstenedione, anastrozole, androsterone, canrenone, cyproterone, DHT, drospirenone, epiandrosterone, exemestane, finasteride, gestoden, levonorgestrel, medroxyprogesterone, norethindrone, norgestimate, progesterone, raloxifene, tamoxifen, 4-hydroxytamoxifen, testosterone, trenbolone, triamcinolone) the same column was used with 0.1 % formic acid and methanol with 0.1 % formic acid. For quantification, internal standard calibration was used with deuterium-labelled compounds.

5.2.6 Bioassay data analysis

Cell viability results are expressed as fold changes (transformed to % values) of measurements obtained from treated cells in comparison to cells exposed to DMSO only, after subtraction of average background signal from all conditions. A threshold values of 80 % was applied to evaluate the occurrence of reduced cell viability (OECD 2012d). Results from the antagonistic AR assay are expressed as magnitude of AR response, obtained by normalizing the fold change relative to DMSO controls from co-exposed cells to the fold change of cells exposed to DHT only. Significance of differences versus the DHT control were evaluated through one-way ANOVA followed by Dunnett's multiple comparison test. Further, the bioassay flutamide equivalents (Bio-FEQ) were calculated utilizing point estimation approach (BDS 2011, Besselink 2015).

5.2.7 Mass balance approach

Mass balance was applied to comparatively evaluate the obtained results from bioassays and chemical analysis. For that, the chemical analysis results for the different samples were utilized to calculate respective flutamide equivalents (Chem-FEQ) with basis on bioassay literature data. The literature was reviewed for bioassays reporting IC_{50} values for the antiandrogenic chemicals evaluated through chemical analysis and for flutamide. Flutamide toxicity equivalent factors (TEF_{Flu}) were obtained by the ratio $IC_{50\text{-flutamide}}/IC_{50\text{-chemical}}$ for the following chemicals: bicalutamide, bisphenol A, diuron, fipronil, propiconazole, tebuconazole and triclosan. Measured chemical concentrations were then multiplied by respective TEF_{Flu} to estimate Chem-FEQ values.

5.3 Results and Discussion

5.3.1 Cytotoxic activity of samples

(i) MTT-based selection of concentration factors for the antiandrogenicity assay

Following the 80 % cell viability threshold, the highest CF values for the test of water sample extracts in the antiandrogenicity assay were selected (Table 5.2). For most of the samples (23 out of 31), the CF of 25 was applied as highest test concentration, while for the remaining samples lower CFs (from 6.5 down to 2.5) were applied.

Table 5.2: Highest test concentration factors (CF) selected for testing in the androgen receptor bioassay, based on respective cell viability (%) identified in the MTT test.

Sample	**Selected highest test CF**	**Cell viability in the MTT test (%)** Average and standard deviation (3 experiments)
S1	12.5	82 ± 11
S2	12.5	70 ± 17
S3	12.5	86 ± 14
S4	12.5	92 ± 2
S5	12.5	80 ± 7
S6	12.5	89 ± 2
S7	12.5	87 ± 3
S8	12.5	89 ± 1
WE	12.5	92 ± 3
M1	12.5	79 ± 10
M2	12.5	90 ± 2.3
M3	12.5	108 ± 8.9
M4	12.5	79 ± 6.3
M5	12.5	87 ± 0.2
M6	12.5	101 ± 4.3
M7	12.5	84 ± 7.3
B1	12.5	80 ± 6.8
B2	12.5	91 ± 2.7
P1	2.5	81 ± 5.5
P2	3.125	77 ± 2.8
H1	6.25	90 ± 1.9

H2	12.5	88 ± 4.9
C1	6.25	80 ± 3.4
C2	12.5	91 ± 7.6
LA	6.25	90 ± 4.9
LN	6.25	92 ± 8.0

(ii) Comparability between the MTT and the AB-CFDA/AM results

For the great majority of the samples evaluated in the three assays, there was good agreement between the different cytotoxic assays, as shown for the method blank (Fig. 5.3A) and for the sample H2 collected at the Holtemme River (Fig. 5.3B). Therefore the selection of exposure concentrations with basis on results from the MTT test was considered to be an adequate procedure for antagonistic assays.

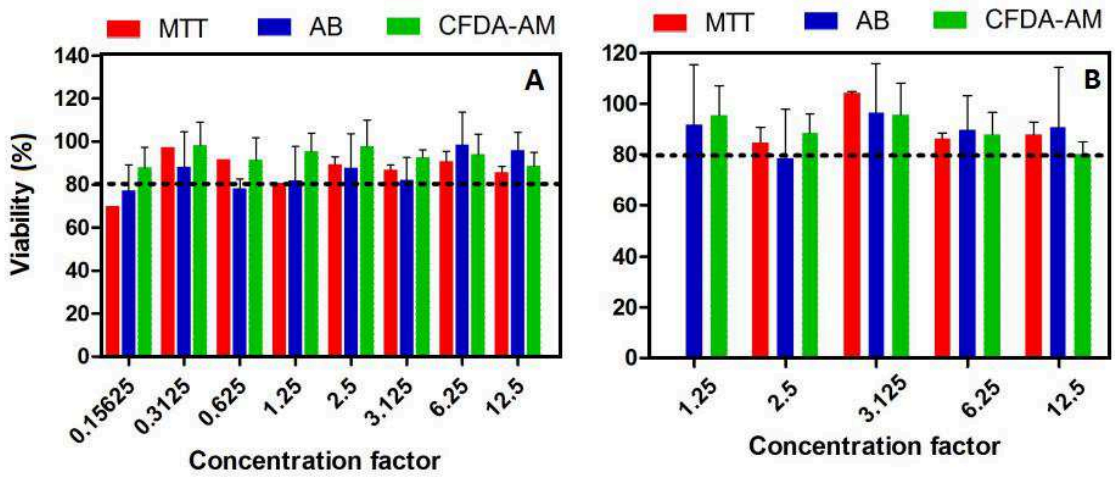

Figure 5.3: Cell viability obtained though the different cytotoxicity assays for the method blank (A) and the sample collected and the Holtemme River (B).

5.3.2 Flutamide toxicity equivalent factors for the measured antiandrogenic chemicals

Calculated TEF_{Flu} and respective references are presented in Table 5.3.

Table 5.3: TEF_{Flu} obtained for the antiandrogenic compounds based on bioassay data utilizing different cell lines.

Detected compounds	TEF_{Flu}	Cell line	Reference
Bicalutamide	2.10	MDA-kb2	(Ma et al. 2003)
Bisphenol A	0.40	U2OS	(Rostkowski et al. 2011)
Diuron	0.075	MDA-kb2	(Ait-Aissa et al. 2010)
Fipronil	0.075	MDA-kb2	(Ait-Aissa et al. 2010)
Propiconazole	0.066	MDA-kb2	(Ait-Aissa et al. 2010)
Tebuconazole	0.22	T47D-ARE	(Roelofs et al. 2014)
Triclosan	0.611	U2OS	(Rostkowski et al. 2011)

5.3.3 Antiandrogenicity bioassay

Obtained Bio-FEQ values ranged between 28.8 ng/L up to 6.1 µg/L water (Table 5.4). When the Bio-FEQ for one sample was detected only in one (or none) experiment, it was classified as not detected (n.d.) in the table. The procedure blank did not cause antiandrogenic effect. The highest Bio-FEQ was obtained for the sample H2 collected at the Holtemme River near a health facility, which presented reduction of magnitude of AR response in a dose-response manner (Fig. 5.3). These results confirm that WWTPs can be important sources of contamination to the aquatic environment, in agreement with previous studies that identified antiandrogenic activity in surface waters and WWTP effluents (Christiaens et al. 2005, Urbatzka et al. 2007, Hill et al. 2010). Although partial removal of EDCs can be achieved through conventional WWTP treatment (Kirk et al. 2002), advanced treatments might be needed to properly remove antiandrogenic activity from effluents, similarly to processes developed for estrogenicity (Maletz et al. 2013). Surprisingly, the reference sites also presented occurrence of antiandrogenic activity, indicating that in contrast to what was expected they in fact presented some level of contamination with antiandrogenic compounds.

Table 5.4: Bio-FEQ values (ng/L water) obtained for the different samples. Average and standard deviation values for 3 to 5 experiments. When the Bio-FEQ for one sample was detected only in one (or none) experiment, it was classified as not detected (n.d.).

Sample	Classification	Bio-FEQ (ng/L)	Average and standard deviation
B1	Reference	5.77E+02	1.35E+02
B2	Urban area	5.61E+02	3.91E+02
C1	Urban area	6.12E+01	8.44E+00
C2	Industry	2.06E+02	1.38E+02
Eu	Health facility	n.d.	
Gö	Health facility	2.85E+02	1.28E+02
H1	Reference	9.10E+01	7.73E+01
H2	Health facility	6.10E+03	1.04E+03
Ilm	Health facility	2.27E+02	1.84E+02
La	Industry	1.95E+02	2.17E+02
LN	Health facility	n.d.	
M1	Reference	3.21E+02	1.65E+02
M2	Health facility	1.01E+03	8.45E+02
M3	Industry	1.01E+03	6.02E+02
M4	Industry	4.87E+02	2.92E+02
M5	Health facility	3.26E+02	2.73E+01
M6	Industry	1.02E+03	6.81E+02
M7	Industry	7.45E+02	6.50E+02
P1	Reference	2.88E+01	2.13E+01
P2	Indistry	n.d.	n.d.

S1	Reference	5.15E+02	2.49E+02
S2	Industry	5.70E+02	2.54E+02
S3	Urban area	6.63E+02	4.16E+02
S4	Urban area	3.66E+02	2.87E+02
S5	Industry	2.38E+02	2.36E+02
S6	Industry	3.65E+02	2.52E+02
S7	Urban area	3.28E+02	2.35E+02
S8	Industry	6.28E+02	1.40E+02
Sol	Health facility/Industry	6.43E+02	1.96E+02
WE	Industry	6.17E+02	1.23E+02
Z	Urban area	2.64E+02	3.08E+02

n.d.: not detected.

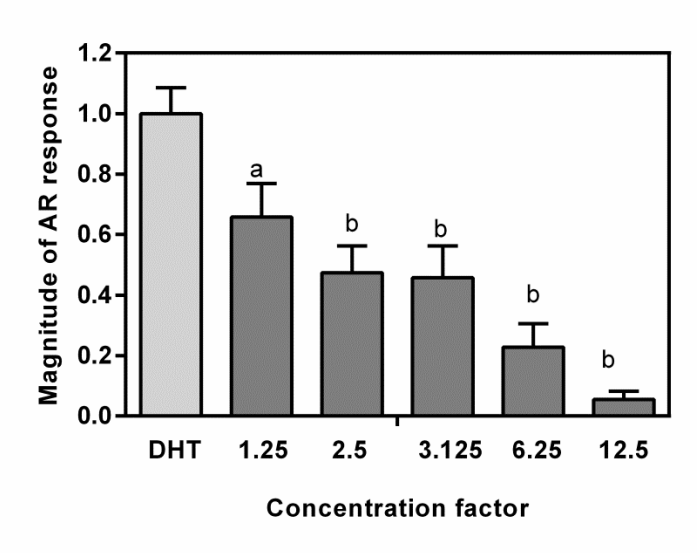

Figure 5 4: Magnitude of AR response versus different concentration factors of the sample H2 collected at the Holtemme River. Averages and standard deviations for 3 experiments. Significant differences versus the DHT control evaluated through one-way ANOVA followed by Dunnett's multiple comparison test (a: $p<0.01$, b: $p<0.001$).

5.3.4 Comparison between Bio- and Chem-FEQ values

The Bio-FEQ values obtained with the antiandrogenicity where always higher than the respective Chem-FEQ value for the same sample (Fig. 5.4A). Ratios between Bio- and Chem-FEQ values (Fig. 5.4B) ranged between circa 4 (P1) and 4,000 (M2). That indicates that, in general, the chemical analysis of a selected list of compounds was of limited values to estimate the antiandrogenic potential of water samples. For the sample H2 collected at the Holtemme River, the Bio-TEQ was circa 130 times higher than estimated Chem-FEQ, with the chemical analysis data explaining less than 1 % of the identified bioactivity. The Holtemme receives the effluents from the WWTP of the town Wernigerode, where a health facility is located, and has previously been identified as highly active in a microbial live cell

array (Hug et al. 2015). Indeed, the sample presented the highest concentration among all water samples of the antiandrogenic pharmaceutical bicalutamide (3.6 ng/L). However the chemical content was not able to explain the Bio-FEQ of 6.1 µg/L. These results, together with the fact that this sample presented the highest antiandrogenic activity of all samples (Table 5.4) and dose-response reduction of the magnitude of AR response (Fig. 5.3), led to the decision to establish an effect-directed analysis case study to investigate it. This follow-up study is presented in Chapter 6.

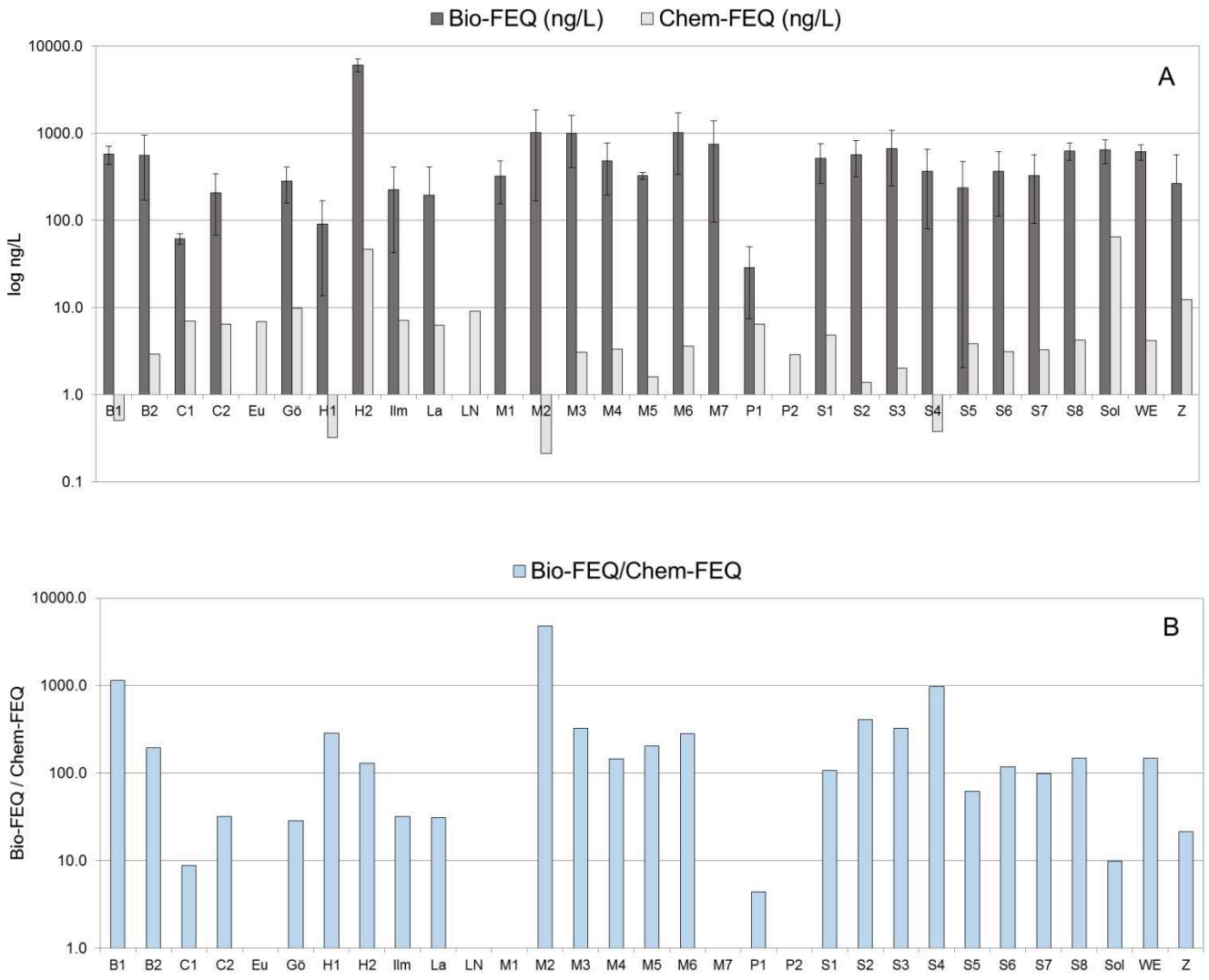

Figure 5 5: Bio- and Chem-FEQ (ng/L) values (A) and ratios Bio-FEQ/Chem/FEQ (B) for the different water samples.

5.4 Conclusions

The biotesting strategy applying the MTT test to evaluate cell viability showed to be an adequate approach, as confirmed by the results obtained with the integrated AB and CFDA-AM assays. In future studies, either the pre-test with MTT or the integrated CFDA-AM assays can be applied to verify cell viability. Despite the wide range of measured activities, antiandrogenicity was identified in most of the water samples collected in the vicinity of WWTP but also in sites selected as reference locations, indicating that antiandrogenic compounds present in surface waters present a potential threat to aquatic organisms and humans. Therefore the assessment of surface waters through bioassays to complement chemical assessment is fully supported. The highest antiandrogenic bioactivity was identified at the sample collected at the River Holtemme, which presented dose-response antiandrogenic effect that was very poorly explained by the target chemical analysis. The outcomes of this study led to the decision to establish an effect-directed analysis case study to investigate the antiandrogenic activity in the Holtemme river surface water. This follow-up study is presented in Chapter 6.

Acknowledgements

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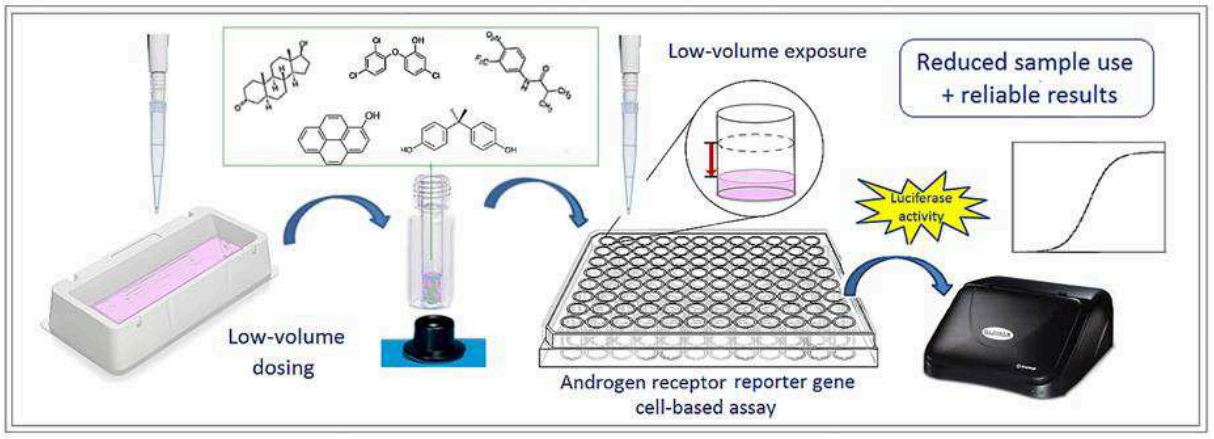
Chapter 6: Miniaturization of a reporter gene assay for antiandrogenicity assessment and application in an EDA case study



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Effect-directed analysis of antiandrogenic compounds in river water through *in vitro* and *in vivo* bioassays and a novel fractionation approach.

Abstract

Bioactivity screening studies often face sample amount limitation with respect to the need for reliable, reproducible and quantitative bioassay results. That was the situation in the present study, since for the effect-directed analysis of the antiandrogenic surface water sample identified in Chapter 5 limited volume was available. Therefore approaches that minimize sample use are needed. Low-volume exposure and chemical dilution procedures were applied in an androgen receptor reporter gene human cell line assay to evaluate the agonist 5α-dihydrotestosterone (DHT); and the antagonists flutamide, bisphenol A, 1-hydroxypyrene and triclosan. Cells were exposed in around 1/3 of the medium volume recommended by the protocol (70 µL/well). Further, chemical losses during pipetting steps were minimized by applying a low-volume method for compound dilution in medium inside microvolume glass inserts. Simultaneously, compounds were evaluated following conventional procedures for comparison of results. Afterwards, the downscaling procedures were applied in the effect-directed analysis study, to evaluate cytotoxicity or antiandrogenicity of method blanks and water fractions obtained by high performance liquid chromatography parallel fractionation with four stationary phase columns. Low-volume exposure tests produced DHT EC_{50} (3.4-3.7 x 10^{-10} M) and flutamide IC_{50} (2.2-3.3 x 10^{-7} M) values very similar to those from regular assays and previous studies. Results were within assay acceptance criteria, supporting the relevance of the downscaling setup for both agonistic and antagonistic tests. The low-volume exposure was also successful in determining IC_{50} values for 1-hydroxypyrene (2.1-2.8 x 10^{-6} M), bisphenol A (2.6-3.3 x 10^{-6} M), and triclosan (1.2-1.9 x 10^{-6} M) in agreement with values obtained through high-volume exposure. Experiments following both low-volume dosing and exposure methods produced similar flutamide and triclosan IC_{50} values to those obtained with regular tests; and within acceptance criteria. In the effect-directed analysis investigation, no cytotoxicity or antiandrogenicity were caused by the method blanks. For the water fractions, reduced magnitudes of androgenic response were identified only for one fraction per column, which occurred in a dose-dependent manner. The low-volume experimental procedures provide a simple and effective solution for studies that need to minimize sample use while maintaining assay reliability. Additionally, the procedures efficiently evaluated water sample fractions and method blanks regarding antiandrogenicity or cytotoxicity. The downscaling methods can be applied for the evaluation of samples, fractions or chemicals which require minimal losses in the steps of pipetting, transference to medium and exposure in bioassays.

Keywords: downscaling, miniaturization, low-volume, androgen receptor binders, reporter gene bioassay, (anti)androgens.

6.1 Introduction

The modulation of the androgen receptor (AR) signaling by androgens is of crucial importance for the male phenotype development and maintenance in humans and vertebrates, and to a lesser extent also for female development and physiological regulation (Staub and De Beer 1997, Fang et al. 2003, Ogino et al. 2009). At the molecular level, these processes are modulated by the binding of endogenous androgens to the AR, which then translocates into the nucleus and activates the transcription of target genes (Tan et al. 2015). In addition to testosterone, its metabolite 5α-dihydrotestosterone (DHT) is a highly active androgen in mammals, while teleost fish present also 11-ketotestosterone (Ogino et al. 2009).

AR antagonists such as the synthetic pharmaceuticals flutamide and bicalutamide inhibit androgenic actions by competing for AR binding sites (Tan et al. 2015). In addition to these drugs, different classes of pollutants act as AR antagonists (Gray et al. 2001), such as the plastic component bisphenol A (Teng et al. 2013), the biocide triclosan (Gee et al. 2008), the pesticides linuron and vinclozolin (Lambright et al. 2000, Melnick et al. 2002, Roy et al. 2004), and PAH contaminants like the metabolite 1-hydroxypyrene and diesel exhaust particle extracts (Kizu et al. 2003, Rostkowski et al. 2011). Not surprisingly, antiandrogenic activity has been identified in various effluents and wastes (Van der Linden et al. 2008, Thomas et al. 2009a, He et al. 2011), diverse natural freshwater and marine environments (Urbatzka et al. 2007, Weiss et al. 2009, Liscio et al. 2014, Alvarez-Munoz et al. 2015), and represents a concern for drinking water quality (Wenzel 2003, Hu et al. 2013).

Reporter gene bioassays are often applied in screening studies that aim to identify new antiandrogens due to their therapeutic potential (Tan et al. 2015), toxic effects (Roy et al. 2004) or environmental occurrence (Vinggaard et al. 1999, Kojima et al. 2004). Since AR antagonism in environmental samples is often caused by unknown compounds, effect-directed analysis (EDA) has been applied to investigate diverse matrices, including water and sediments (Urbatzka et al. 2007, Thomas et al. 2009a, Weiss et al. 2009, Liscio et al. 2014), and exposed fish and clams (Hill et al. 2010, Rostkowski et al. 2011, Alvarez-Munoz et al. 2015). Several bioassays with stably transfected AR reporter gene cell lines (Blankvoort et al. 2001, Roy et al. 2004, Sonneveld et al. 2005, van der Burg et al. 2010, Bartonkova et al. 2015) or recombinant yeast (Urbatzka et al. 2007, Liscio et al. 2014) are available. These methods, recommended for application as effect-based tools for water quality monitoring in Europe (Wernersson et al. 2015), have been integrated in EDA and monitoring studies in European river basins (Brack et al. 2013, Altenburger et al. 2015, Brack et al. 2015).

A common issue often faced by bioassay screening studies is sample amount limitation with respect to the need for reliable, reproducible and quantitative results. The performance of tests in microtiter plates can considerably reduce the needed volumes of assay medium and consequently of samples. For instance, assays performed in 96-well plates use around 200-250 µL of medium per well (Roy et al. 2004, Sonneveld et al. 2005, van der Burg et al. 2010, He et al. 2011). Still, sample use can amount to significant volumes, due to serial dilutions required to obtain quantitative results, and replicate wells and experiments to overcome intra- and inter-assay variabilities (Brack et al. 2016). Further sample losses can also occur during pipetting steps for dosing of samples in bioassay medium. Therefore there is need to improve procedures that can minimize sample use in bioassays, while maintaining method reliability.

The objectives of this study were to: (i) develop low-volume procedures for the steps of exposure and chemical dilution in medium in an AR reporter gene human cell line assay (van der Burg et al. 2010); (ii) evaluate the performance of the downscaling procedures in the agonistic setup though the testing of DHT; and in the antagonistic form though the testing of flutamide, bisphenol A, 1-hydroxypyrene and triclosan; and (iii) utilize the downscaling assay as the guiding tool in the EDA investigation of the antiandrogenic water sample identified in Chapter 5 of this thesis. After method development and preliminary tests, the low-volume procedures were applied in the agonistic setup to evaluate DHT; and in the antagonistic form to evaluate flutamide, bisphenol A, 1-hydroxypyrene and triclosan. The cells were exposed in 96-well plates in medium volume of around 1/3 of common procedures. Further, a low-volume dosing method was applied to dilute the chemicals in medium inside microvolume glass inserts, minimizing sample losses during pipetting steps. The low-volume exposure procedure was applied to evaluate all the compounds, and the low-volume dilution method was applied to evaluate flutamide and triclosan. Evaluation of the chemicals following regular procedures was simultaneously performed for comparison of results. Afterwards, the downscaling procedures were applied in the EDA case study to evaluate the water fractions regarding antiandrogenic activity.

6.2 Material and Methods

6.2.1 Chemicals

Dimethyl sulfoxide (DMSO) and the test chemicals (Table 6.1) dihydrotestosterone (CAS 521-18-6), flutamide (CAS 13311-84-7), triclosan (CAS 3380-34-5), 1-hydroxypyrene (CAS 5315-79-7) and bisphenol A (CAS 80-05-7) were purchased from Sigma Aldrich (Sigma

Aldrich Chemie GmbH, Steinheim, Germany) with a purity of at least 97 %. Stock and serial dilution solutions were prepared by dilution of chemicals in DMSO and were stored at -20°C.

Table 6.1: Test chemicals evaluated in bioassays, plus respective CAS numbers, formulas and molecular weights.

Test chemicals		CAS	Formula	Molecular weight
Dihydrotestosterone	Endogenous steroid	521-18-6	$C_{19}H_{30}O_2$	290.44
Flutamide	Synthetic pharmaceutical	13311-84-7	$C_{11}H_{11}F_3N_2O_3$	276.21
Triclosan	Biocide	3380-34-5	$C_{12}H_7Cl_3O_2$	289.54
Bisphenol A	Plastic component	80-05-7	$C_{15}H_{16}O_2$	228.28
1-Hydroxypyrene	Pyrene metabolite	5315-79-7	$C_{16}H_{10}O$	218.25

6.2.2 Cell culture

The human osteoblastic osteosarcoma U2OS cell line stably co-transfected with an expression construct for the human AR and a respective reporter construct was obtained from BioDetection Systems BV (BDS, Amsterdam, the Netherlands) and cultured as previously described (van der Burg et al. 2010). Briefly, cell cultures were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (Invitrogen Life Technologies, Darmstadt, Germany) with phenol red supplemented with 7.5% fetal calf serum (FCS) (Th. Geyer GmbH, Renningen, Germany), 0.2 % penicillin/streptomycin solution (Invitrogen), 1 % non-essential amino acids (Invitrogen) and G418 antibiotic (0.20 mg/mL) for the selective survival of transfected cells only. Cultures were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C until reaching around 90 % confluence, when cells were used for tests or sub-cultures. Cells were passaged twice a week and medium change was performed every two to three days.

6.2.3 AR reporter gene assay performance

Cell cultures were treated with trypsin, cells were counted and re-suspended in assay medium (phenol red-free 1:1 mixture of DMEM/F12 supplemented with 5 % of stripped FCS, 0.2 % penicillin/streptomycin solution and 1 % non-essential amino acids) to a final concentration of 10^5 cells/mL. The cell suspension was transferred to 96-well plates, with each well receiving 100 µL (10^4 cells). External wells of each plate were filled instead with 200 µL of PBS in order to minimize medium evaporation. After 24 h, the medium was refreshed and cells were exposed to serial dilutions of the test chemicals in exposure medium, which were previously prepared in 24-well plates (1 mL of exposure medium for each test

condition). In the antagonistic assay, there was co-exposure with a non-saturating DHT concentration (4.2×10^{-10} M). Each plate contained also a serial dilution of the standard chemical, which was either DHT (10^{-12} to 10^{-7} M) in the agonistic assays, or flutamide (10^{-9} - 10^{-5} M) in the antagonistic assays. The antiandrogens bisphenol A, 1-hydroxypyrene and triclosan were evaluated in serial dilutions ranging from 10^{-9} to 10^{-5} M, which does not cause cytotoxicity according to previous studies (van der Burg et al. 2010, Rostkowski et al. 2011). Each plate contained solvent controls (1 % DMSO), and also DHT controls (4.2 x 10^{-10} M DHT) in the antagonistic assays. All conditions were performed in triplicate wells in each test and contained final DMSO concentration of 1 %. After 24 hours of exposure at 37 °C and 5 % CO_2, the cells were visually inspected for cell viability. The medium was removed and cells were lysed in 1 % Triton X-100 lysis buffer (BDS). Cell lysates were transferred to opaque white plates (30 µL/well), and after addition of 60 µL/well of D-Luciferin solution (BDS illuminate mix) the luciferase activity was measured using a luminometer (GloMax® 96 Microplate Luminometer, Promega GmbH, Germany).

6.2.4 Miniaturized volume procedures

Miniaturized volume procedures were evaluated for the dosing of chemicals and for the exposure incubation. All other procedures were performed following the regular protocol.

(i) Low-volume exposure setup

After dilution of test chemicals in exposure medium as described above, exposure was conducted in medium volume of 70 µL/well. This setup was applied to evaluate DHT and the four antiandrogens in the agonistic and antagonistic assays, respectively. Each plate contained also the serial dilution of the respective standard chemical plus controls following 70 µL/well exposure. Experiments following 200 µL/well during exposure were simultaneously performed in order to compare results obtained with the two setups. Experiments were repeated four to five times for each chemical.

(ii) Low-volume dosing procedure

A low-volume dosing system was applied to evaluate flutamide and triclosan in the antagonistic assay. Test chemicals were diluted in exposure medium inside 300 µL microvolume glass inserts, to simulate the procedure that can be done with sample extracts or fractions, often collected and stored in such vials. First, 0.25 µL of each stock solution of the serial dilution were transferred into the glass inserts placed in 2 mL vials. Medium containing

DHT and DMSO was prepared in 24-well plates and added to each insert to a final volume of 250 µL. Vials were shaken by vortex for about 10 seconds, and the obtained exposure mediums were applied in the low-volume (70 µL/well) exposure setup. Tests following the regular dosing (in 24-well plates) and exposure volume (200 µL/well) were simultaneously conducted. Experiments were performed one time for triclosan and three times for flutamide.

6.2.5 MTT test cell viability assessment

Cell viability of single chemicals and mixtures was evaluated though a colorimetric microplate assay with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT test), which is reduced to formazan by viable cells (Mosmann 1983, Berridge et al. 2005). Briefly, cells were exposed following the described downscaling procedures as for the AR binding analysis. At the end of the 24 h incubation period, there was visual verification of no contamination evidence, medium was removed, and each well received 100 µL of freshly prepared 0.5 mg/mL MTT in FCS-free assay medium. In each plate, 6 wells containing no cells received the MTT solution for the measurement of background signal. After 30 min of incubation at 37 °C and 5 % CO_2, the occurrence of formazan crystals was confirmed under microscope observation. The medium was discharged, 200 µL of DMSO was added per well, and plates were shaken for 15 min for crystal solubilisation. The amount of formed formazan was measured with a microplate spectrophotometer (Tecan Infinite® M200, Tecan, Switzerland) at an absorbance wavelength of 492 nm.

6.2.6 Evaluation of water sample fractions in the EDA case study

For the EDA case study, the water sample was submitted to high performance liquid chromatography parallel fractionation approach utilizing four types of stationary phase columns: aminopropyl (NH_2), octadecyl (C18), pyrenyl ethyl (PYE) and pentafluorophenyl (PFP). Method blanks, which were produced by submitting only respective solvents to column procedures, were evaluated for cytotoxic and antiandrogenic activity. Water sample fractions were identified according to the elution time in minutes. Fractions were tested individually and also as re-combined (pooled) fractions per column. In order to obtain specific concentration factors in bioassay when utilizing the low-volume dosing system, needed volumes from obtained fractions were transferred to glass inserts contained in amber vials and evaporated to dryness. Exposure concentrations corresponded to concentration factors of 25 (i.e. 25 mL_{water}/mL_{medium}), 12.5, 6.25 or 3.125.

6.2.7 Acceptance criteria, data analysis and statistical analysis

AR bioassay acceptance criteria were based on EC_{50} or IC_{50} values for the respective standard chemical, and on minimum luciferase fold inductions for: (i) the highest DHT

concentration relatively to the DMSO control in the agonistic assay; and (ii) the DHT control with respect to the highest flutamide concentration in the antagonistic assay. In the agonistic assay, DHT should produce EC_{50} between 1.97-6.53 × 10^{-10} M and 20-fold induction; while in the antagonistic assay a flutamide IC_{50} of 1.08-10.74 × 10^{-7} M and 10-fold induction was expected. Agonistic assay results are expressed as induction values, obtained by converting the response of each concentration to a percentage of the maximum DHT response, after subtracting the solvent response from both. Results from the antagonistic assay are expressed as magnitude of AR response, obtained by normalizing the fold change relative to DMSO controls from co-exposed cells to the fold change of cells exposed to DHT only. Results were fitted through non-linear regression with three-parameter logistic function by constraining bottom to zero using GraphPad Prism software version 6.02 (GraphPad Software, San Diego, CA, USA). The $logEC_{50}$ / $logIC_{50}$ for individual experiments were compared by two-way ANOVA followed by Tukey's multiple comparisons test, and averages were calculated. After verifying that for each setup there was overlap between the 95 % confidence intervals (C.I.) obtained in individual experiments, this data was pooled and fitted by non-linear regression. For cell viability, results are expressed as fold changes of measurements obtained from treated cells in comparison to cells exposed to DMSO only, after subtraction of average background signal from all conditions. Threshold values of 0.8 were applied to estimate occurrence of reduction of both cell viability and magnitude of AR response in the evaluation of water sample fractions and respective method blanks.

6.3 Results and Discussion

6.3.1 Low-volume exposure agonistic assays

DHT fold induction always exceeded the minimum of 20-fold, with average and standard deviation values of 134 ± 44 (70 µL/well) and 113 ± 36 (200 µL/well). EC_{50} values from individual experiments (Table 6.2) did not differ significantly, and exposure volume or experimental repetitions were not sources of variation. Also, both regression curves (Fig. 6.1) presented very similar shape. DHT EC_{50} values for single tests of 3.4 to 3.7 (70 µL) and 3.1 to 4.2 (200 µL) × 10^{-10} M were within the bioassay acceptance criteria, and in the same range as average values (3.1 and 4.5 × 10^{-10} M) determined in the assay pre-validation study (van der Burg et al. 2010). That supports the relevance of the miniaturized exposure setup for the agonistic assay performance, and the maintenance of 4.2 × 10^{-10} M DHT for co-exposure in the low-volume antagonistic assay.

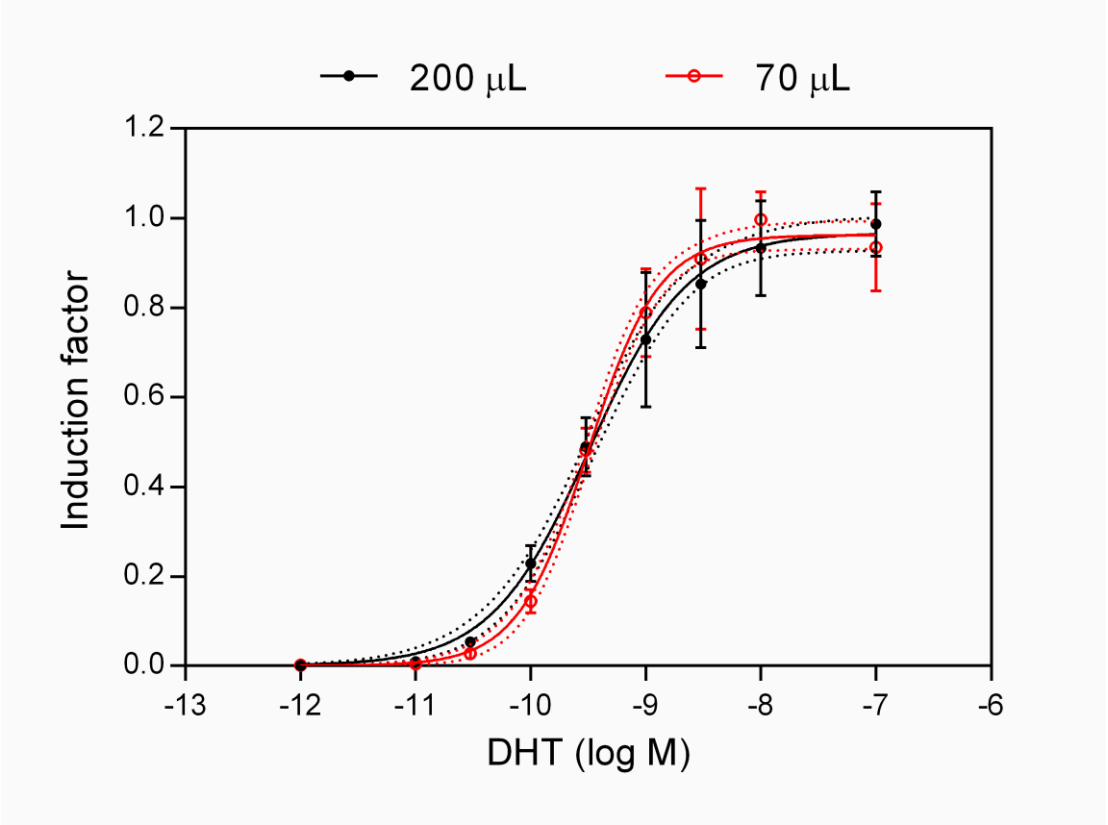

Figure 6.1: Induction factors versus DHT concentrations (log M) evaluated in the agonist AR assay following exposure in 70 µL/well (red, -○-) or 200 µL/well (black, -●-). Mean and standard deviation values plus 95% confidence bands for 3 experiments in each exposure setup.

Table 6.2: EC_{50} or IC_{50} values and respective 95% confidence intervals and curve top/slope values for the androgen (EC_{50}) DHT and for the antiandrogens (IC_{50}) flutamide, bisphenol A, hydroxypyrene and triclosan evaluated in the antagonistic AR assay following exposure volumes of 70 or 200 µL of medium per well. Values are presented for each experiment and averages, and for pooled data.

		70 µL/well exposure		**200 µL/well exposure**	
		IC_{50} / EC_{50} and 95 % C.I. (M)		**IC_{50} / EC_{50} and 95 % C.I. (M)**	
Agonistic assay (EC_{50})					
DHT					
EC_{50} Single tests (n)	1	3.50	(3.00 to 4.08) × 10^{-10}	2.54	(1.92 to 3.36) × 10^{-10}
	2	3.37	(2.47 to 4.61) × 10^{-10}	3.90	(2.27 to 6.69) × 10^{-10}
	3	3.00	(2.30 to 3.85) × 10^{-10}	2.84	(1.73 to 4.68) × 10^{-10}
	4	3.06	(2.34 to 4.00) × 10^{-10}	3.25	(2.53 to 4.18) × 10^{-10}
	av	3.09	× 10^{-10}	3.13	× 10^{-10}
EC_{50} Pooled data		3.23	(2.68 to 3.89) × 10^{-10}	3.16	(2.80 to 3.56) × 10^{-10}
Top		0.96	(0.93 to 0.99)	0.97	(0.93 to 1.01)
Slope		1.41	(1.20 to 1.61)	1.02	(0.87 to 1.17)
Antagonistic assay (IC_{50})					
Flutamide					
IC_{50} Single tests (n)	1	2.66	(2.03 to 3.49) × 10^{-7}	2.54	(1.84 to 3.49) × 10^{-7}
	2	2.20	(1.81 to 2.68) × 10^{-7}	2.34	(1.82 to 3.02) × 10^{-7}
	3	2.23	(1.87 to 2.65) × 10^{-7}	2.09	(1.78 to 2.46) × 10^{-7}
	4	3.32	(2.61 to 4.23) × 10^{-7}	3.22	(2.61 to 3.98) × 10^{-7}
	5	3.00	(2.16 to 4.16) × 10^{-7}	3.30	(2.44 to 4.48) × 10^{-7}
	av	2.65	× 10^{-7}	2.66	× 10^{-7}
IC_{50} Pooled data		2.64	(2.36 to 2.95) × 10^{-7}	2.64	(2.27 to 3.08) × 10^{-7}
Top		0.96	(0.93 to 0.99)	0.99	(0.95 to 1.03)
Slope		-1.54	(-1.78 to -1.31)	-1.27	(-1.48 to -1.06)
Bisphenol A					
IC_{50} Single tests (n)	1	2.99	(2.24 to 3.99) × 10^{-6}	3.07	(2.42 to 3.89) × 10^{-6}
	2	2.56	(1.89 to 3.45) × 10^{-6}	3.36	(2.22 to 5.07) × 10^{-6}
	3	3.09	(2.22 to 4.30) × 10^{-6}	2.96	(2.48 to 3.53) × 10^{-6}
	4	3.33	(2.46 to 4.51) × 10^{-6}	2.53	(1.84 to 3.48) × 10^{-6}
	av	2.54	× 10^{-6}	2.65	× 10^{-6}
IC_{50} Pooled data		2.99	(2.57 to 3.48) × 10^{-6}	2.92	(2.48 to 3.45) × 10^{-6}
Top		1.09	(1.06 to 1.13)	1.14	(1.11 to 1.18)
Slope		-1.26	(-1.50 to -1.02)	-1.24	(-1.50 to -0.98)
Hydroxypyrene					
IC_{50} Single tests (n)	1	2.55	(2.02 to 3.23) × 10^{-6}	2.66	(2.19 to 3.24) × 10^{-6}
	2	2.77	(2.38 to 3.24) × 10^{-6}	2.77	(2.38 to 3.24) × 10^{-6}
	3	2.14	(1.47 to 3.11) × 10^{-6}	2.40	(1.95 to 2.94) × 10^{-6}
	4	2.51	(1.97 to 3.19) × 10^{-6}	2.31	(1.82 to 2.93) × 10^{-6}
	av	2.48	× 10^{-6}	2.53	× 10^{-6}
IC_{50} Pooled data		2.50	(2.19 to 2.85) × 10^{-6}	2.55	(2.29 to 2.83) × 10^{-6}
Top		1.07	(1.04 to 1.11)	1.04	(1.01 to 1.06)
Slope		-2.09	(-2.68 to -1.51)	-1.97	(-2.38 to -1.56)
Triclosan					
IC_{50} Single tests (n)	1	1.24	(0.96 to 1.61) × 10^{-6}	1.11	(0.86 to 1.44) × 10^{-6}

	2	1.41	(1.07 to 1.84) × 10^{-6}	1.15	(0.96 to 1.39) × 10^{-6}
	3	1.56	(1.09 to 2.21) × 10^{-6}	1.01	(0.84 to 1.22) × 10^{-6}
	4	1.89	(1.51 to 2.36) × 10^{-6}	1.29	(1.06 to 1.57) × 10^{-6}
	av	1.51	× 10^{-6}	1.14	× 10^{-6}
IC_{50} Pooled data		1.58	(1.33 to 1.87) × 10^{-6}	1.12	(1.01 to 1.25) × 10^{-6}
Top		1.04	(1.00 to 1.08)	1.13	(1.10 to 1.16)
Slope		-1.29	(-1.55 to 1.03)	-1.54	(-1.77 to -1.31)

6.3.2 Low volume exposure antagonistic assays

The low-volume antagonistic tests also produced IC_{50} values (Table 6.2) and shape of regression curves (Fig. 6.2) similar to those from regular exposure setup experiments. Flutamide fold induction in individual experiments always exceeded the minimum of 10-fold, with average and standard deviation values of 31 ± 21 and 32 ± 22 in the 70 and 200 µL/well, respectively. For the model antiandrogen flutamide, the experimental repetitions were a significant variation source (96% of total variation, $p<0.01$) but not the different exposure setups. Also, inter-experimental differences occurred independently of exposure procedures (Fig. 3), and no significant differences occurred for simultaneously performed low- and regular volume experiments. This observation is in favor of variation being related to intrinsic bioassay variability, which tends to be higher in the antagonistic format when compared to the agonistic test (van der Burg et al. 2010). Flutamide IC_{50} values of 2.2 to 3.3 (70 µL) and 2.1 to 3.3 (200 µL) x 10^{-7} M were in similar range to previous studies (1.0 - 5.2 × 10^{-7} M) that applied the regular assay with the same cell model (van der Burg et al. 2010, Rostkowski et al. 2011, Mertl et al. 2014, Wang et al. 2014). All results were within the method acceptance criteria, which further supports the application of the miniaturized exposure volume procedure also for the antagonistic setup.

The results confirmed the AR antagonistic activity of 1-hydroxypyrene, a metabolite of the abundant PAH pyrene. IC_{50} values for individual experiments (2.1 to 2.8 and 2.3 to 2.8 × 10^{-6} M in 70 and 200 µL/well) did not significantly differ considering exposure volume or experimental repetitions. Current values were in high agreement with previous study (2.0 × 10^{-6} M) that identified hydroxypyrene in antiandrogenic bile fractions of fish exposed to wastewater effluents (Rostkowski et al. 2011). Also, mononitrated 1-hydroxypyrene isomers were previously identified as AR antagonists, and as agonists and antagonists of the estrogen receptor (Kameda et al. 2011). In fact, 1-hydroxypyrene was also identified as an antiestrogen present in diesel exhaust particle fraction by Noguchi *et al.* (2007). Considering the potential for future EDA and screening studies of 1-hydroxypyrene and related compounds, the miniaturized setup showed to consistently assess its AR antagonistic activity.

For triclosan, the different exposure volumes were identified as a source of variation (56% of total variation, $p<0.05$), however no significant differences were identified when comparing individual experiments through the multiple comparison test (Fig. 6.3). In contrary for bisphenol A no significant differences were found. Both compounds presented IC_{50} values (Table 6.2) slightly higher than previous studies. Present bisphenol A IC_{50} values of 2.6 to 3.3 (70 µL) and 2.5 to 3.4 (200 µL) $\times 10^{-6}$ M were around the double of previous studies (1.1 and 1.5 $\times 10^{-6}$ M) (Rostkowski et al. 2011, Wang et al. 2014), similarly to triclosan IC_{50} values (1.2 to 1.9 and 1.0 to 1.3 $\times 10^{-6}$ M in 70 and 200 µL/well) when compared to the literature (0.7 $\times 10^{-6}$ M) (Rostkowski et al. 2011). These differences could be related to the different DHT concentration applied for co-exposure, which was of 4.2 $\times 10^{-10}$ M and of 2.0 $\times 10^{-10}$ M in the present and previous studies, respectively. Also, different positive control conditions were used for normalization of data, since we exposed cells to the same non-saturating DHT concentration used for co-exposure, while previously that was done by applying a concentration three orders of magnitude higher (10^{-7} M of DHT) (van der Burg et al. 2010, Rostkowski et al. 2011). Finally, experiments were performed with final DMSO concentration of 1%, instead of the most often performed 0.1% DMSO. It is relevant to mention that triclosan has been shown to potentiate the response of MDA-kb2 cells to DHT when in co-exposure in concentrations down to 1 $\times 10^{-9}$ M (Christen et al. 2010), which was our lower exposure concentration. This enhancement of the DHT AR agonism by low triclosan concentrations could be related to a slight tendency for a bell-shaped dose response curve observed in our study (Fig. 6.2 D).

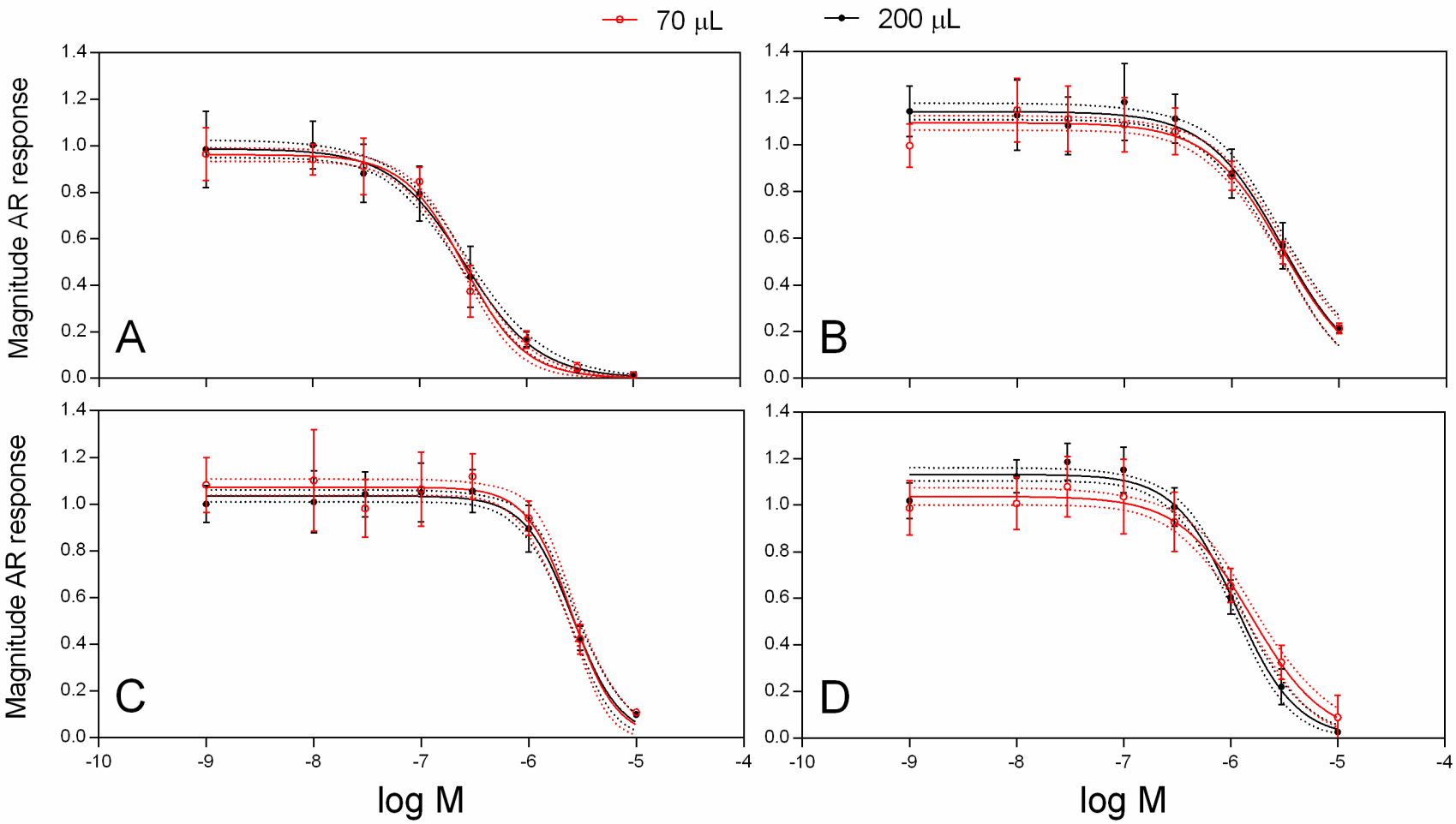

Figure 6.2: Magnitudes of AR response versus concentrations (log M) of (A) flutamide (5 tests), (B) bisphenol A (4 tests), (C) 1-hydroxypyrene (4 tests) and (D) triclosan (4 tests) following exposure volumes of 70 µL/well (red, -○-) or 200 µL/well (back, -●-) in the antagonistic assay. Mean and standard deviation values plus 95% confidence bands for 4 to 5 independent experiments in each exposure setup.

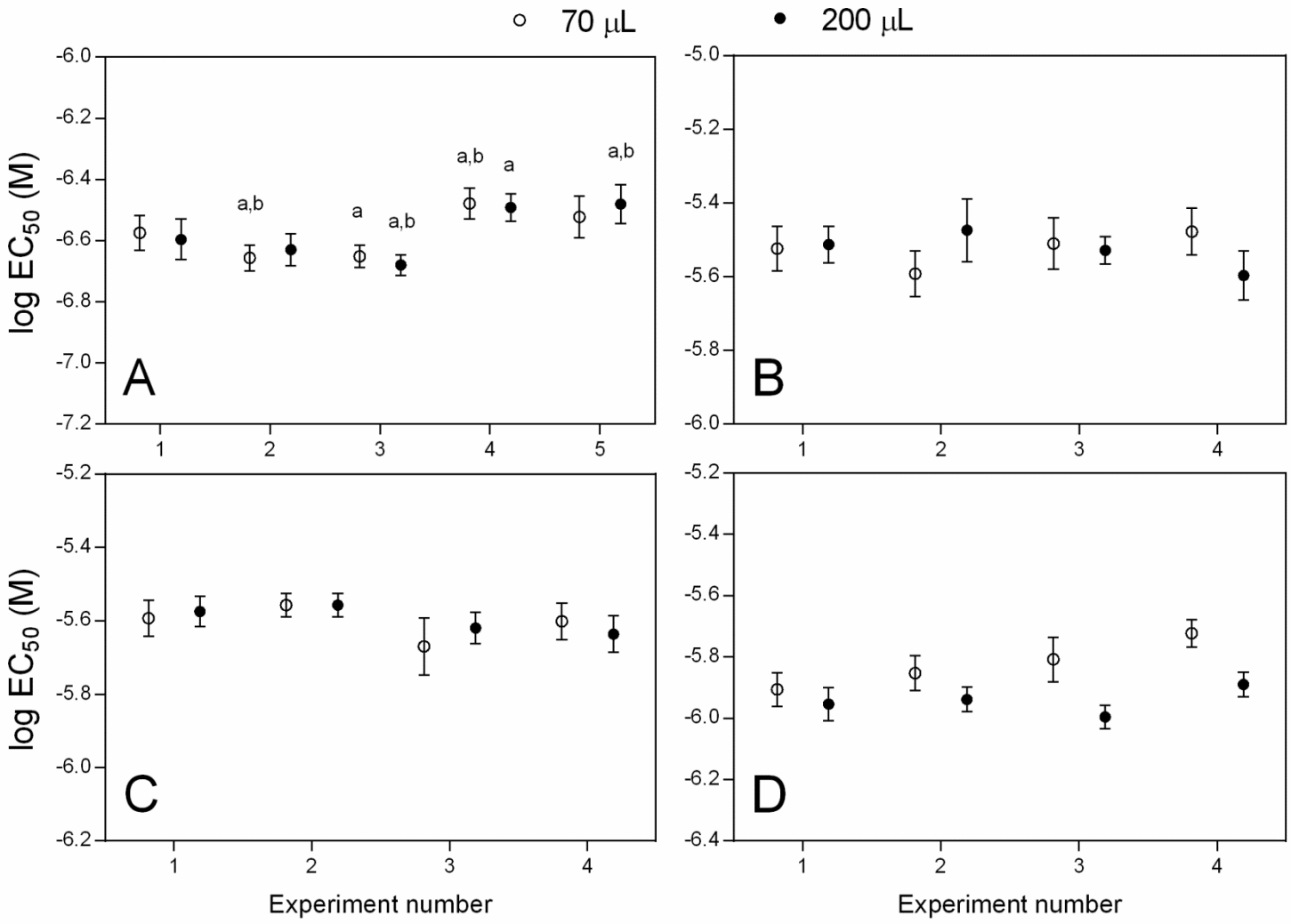

Figure 6.3: Obtained log IC_{50} and standard error (log M) values for individual experiments evaluating (A) flutamide (5 tests), (B) bisphenol A (4 tests), (C) 1-hydroxypyrene (4 tests) and (D) triclosan (4 tests) following exposure volumes of 70 µL/well (○) or 200 µL/well (●) in the antagonistic assay. Significant differences from at least one experiment following (a) the same or (b) different exposure volume, but never from simultaneously performed tests.

6.3.3 Low volume dosing and exposure antagonistic assays

Antagonistic bioassays following the low-volume dosing followed by exposure in 70 µL/well produced IC_{50} values (Table 6.3) and regression curves (Fig. 6.4) highly similar to concurrent tests following regular procedures. No differences were identified when comparing flutamide IC_{50} for different individual experiments. Again, flutamide results were within the bioassay acceptance criteria regarding IC_{50} (Table 6.3) and also fold induction values (23 ± 11 and 18 ± 3 in the miniaturized and regular assays, respectively). These results demonstrate that the low-volume dosing system promoted adequate chemical dilution in medium. The combined application of the miniaturized dosing and exposure procedures resulted in minimal losses of test chemicals during the steps of pipetting, transference to medium and exposure, while providing results within assay acceptance criteria.

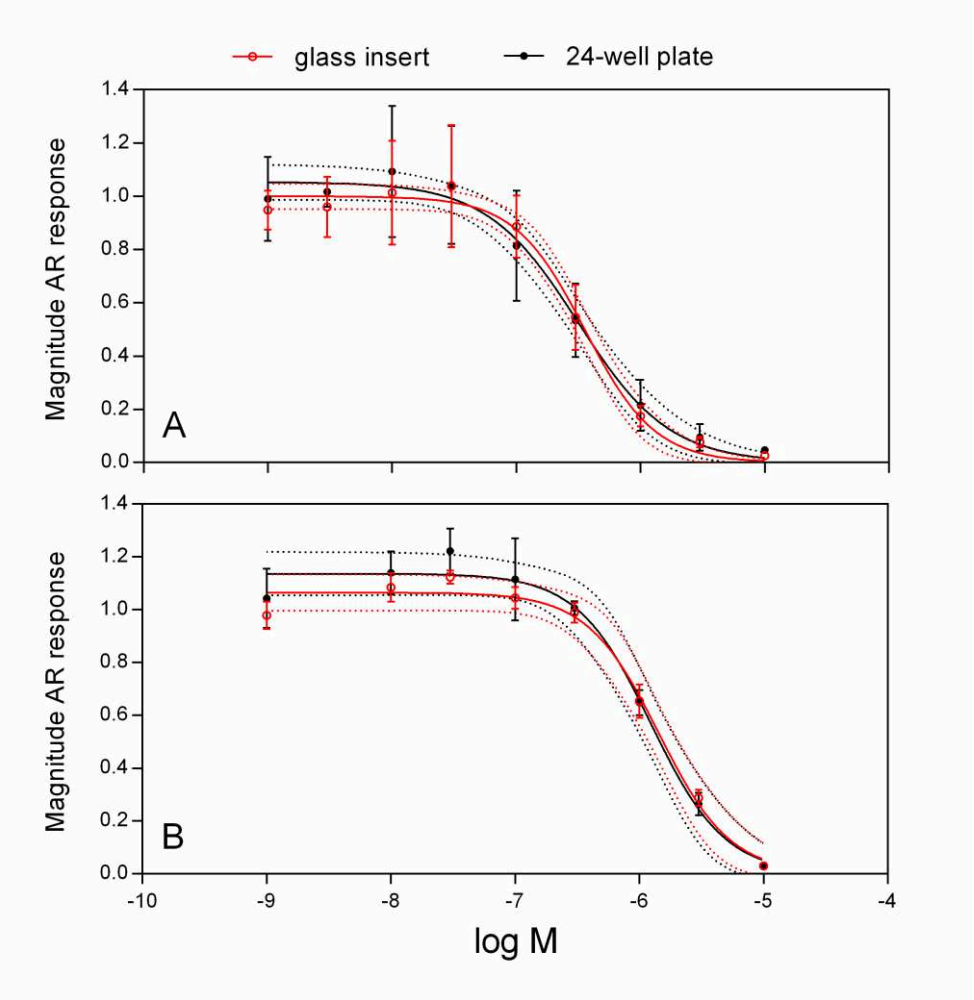

Figure 6.4: Magnitudes of AR response versus concentrations (log M) of (A) flutamide (3 tests) or (B) triclosan (1 test) following dilution in glass inserts and exposure in 70 µL/well (red, -○-), or dilution in 24-well plates and exposure in 200 µL/well (black, -●-). Mean and standard deviation values plus 95% confidence bands for 1 or 3 independent experiments in each setup.

Table 6.3: IC_{50} values and 95 % confidence intervals obtained for flutamide and triclosan evaluated in the antagonistic AR assay following dilution in exposure medium in glass inserts and exposure in 70 µL/well; or dilution in 24-well plates and exposure in 200 µL/well.

		Glass insert dilution and 70 µL/well exposure IC_{50} and 95 % C.I. (M)		**24-well plate dilution and 200 µL/well exposure IC_{50} and 95 % C.I. (M)**	
Flutamide					
IC_{50} Single tests (n)	1	4.03	(3.00 to 5.42) × 10^{-7}	3.73	(2.88 to 4.85) × 10^{-7}
	2	3.31	(2.57 to 4.25) × 10^{-7}	2.09	(1.48 to 2.93) × 10^{-7}
	3	3.02	(2.49 to 3.66) × 10^{-7}	2.88	(2.49 to 3.34) × 10^{-7}
	av	3.45	× 10^{-7}	2.90	× 10^{-7}
IC_{50} Pooled data		3.55	(2.89 to 4.36) × 10^{-7}	3.14	(2.34 to 4.21) × 10^{-7}
Top		1.00	(0.95 to 1.05)	1.05	(0.99 to 1.12)
Slope		-1.54	(-1.96 to -1.12)	-1.18	(-1.53 to -0.83)
Triclosan					
IC_{50} Single test (n)	1	1.44	(1.07 to 1.93) × 10^{-6}	1.24	(0.90 to 1.72) × 10^{-6}
Top		1.07	(1.00 to 1.13)	1.14	(1.05 to 1.22)
Slope		-1.51	(-2.10 to -0.91)	-1.46	(-2.09 to -0.84)

6.3.4 Evaluation of the water sample fractions utilizing the low volume dosing and exposure procedures

Results from bioassay performed following the downscaling procedures for dosing and exposure indicated no bioactivity of method blanks in the tested concentrations; and identified only one antiandrogenic fraction for each column.

(i) Cytotoxic and antiandrogenic activity of the method blanks of the different columns

Values of cell viability (Fig. 6.5A) and magnitude of AR response (Fig 6.5B) were always above the threshold of 0.8 (dashed lines in graphs), indicating that no significant bioactivities were caused by the method blanks of the different columns in concentration factors between 3.125 and 25. For the further test of water extract fractions, the concentration factors of 6.25 or 12.5 were utilized.

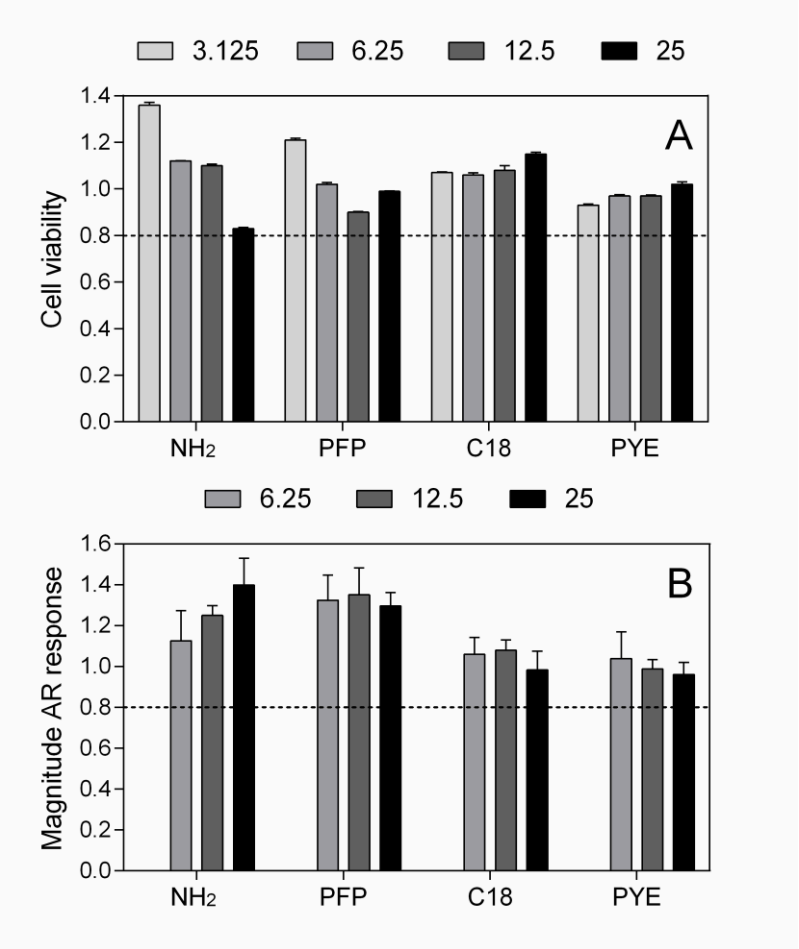

Figure 6.5: Cell vitality (A) and magnitude of AR response (B) versus different concentration factors of the method blanks of the NH_2, PFP, C18 and PYE columns. The dashed lines indicate the 0.8 threshold value for the bioactivities. Average and standard deviation values for one experiment per column.

(ii) Antiandrogenic activity of the fractions obtained with the different columns

For the test of individual fractions (identified by respective elution times in Fig. 6.6), reduced magnitudes of AR response lower than the threshold of 0.8 was clearly identified only for one fraction per column (green columns Fig 6.6). Tested concentration factors were of 12.5, except for the C18 column (C) which was of 6.25. The numbers of active fractions and magnitudes of AR response were: 19 (0.65) for NH2; 29 (0.37) for PFP; 25 (0.57) for C18; and 37 (0.45) for the PYE column. The antiandrogenic activity, which occurred in a dose-dependent manner, was confirmed by the results from tests of serial dilution of the active fractions (Fig. 6.7); and of the recombined fractions per column (Fig. 6.8).

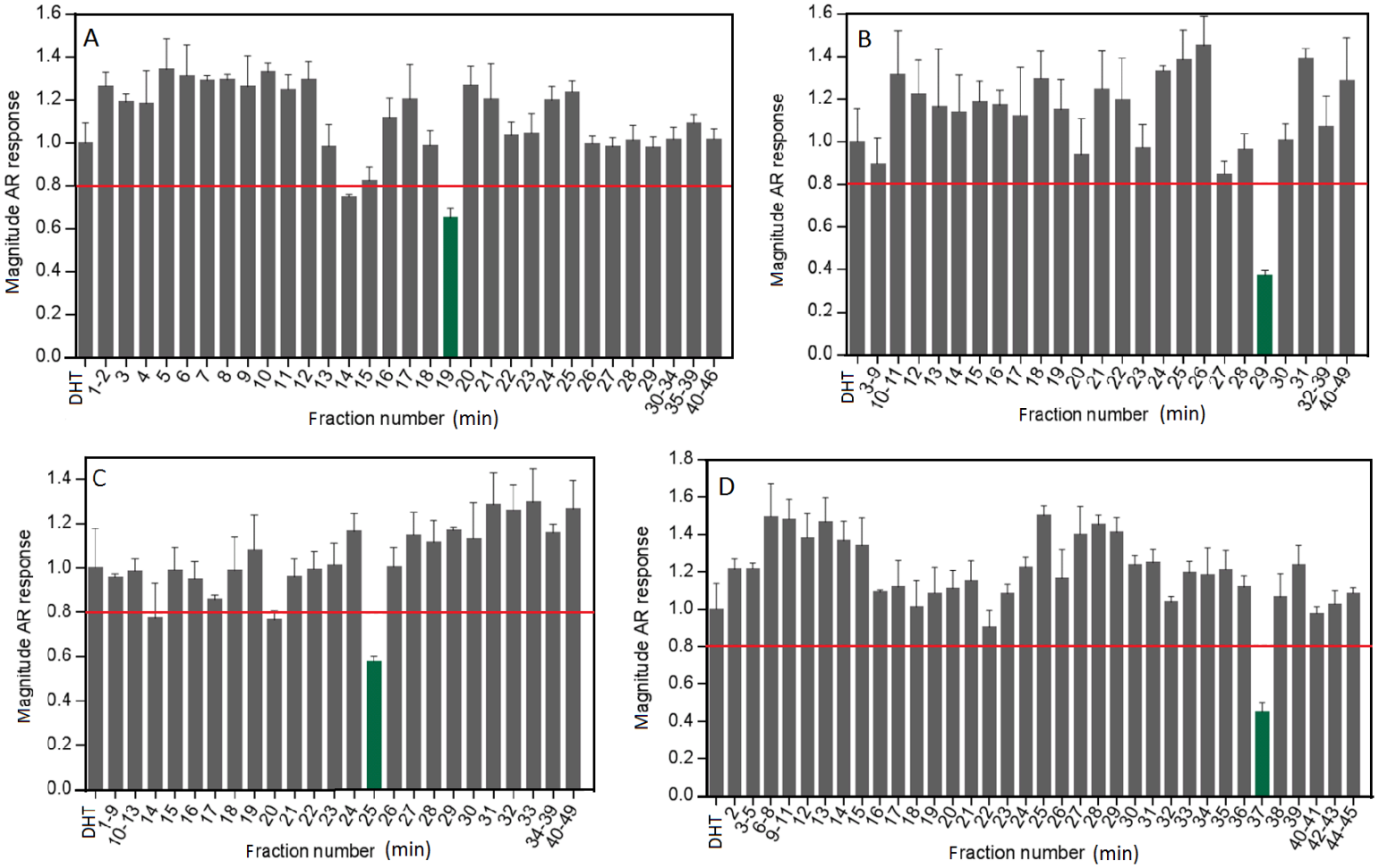

Figure 6.6: Magnitude of AR response versus water extract fractions (min) obtained from the NH2 (A), PFP (B), C18 (C) and PYE (D) columns. The red line indicates the threshold value of 0.8. Tested concentration factors were of 12.5, except for the C18 column (C) which was of 6.25. Fractions that presented clear antiandrogenic activity are identified in green. Average and standard deviation values for one experiment.

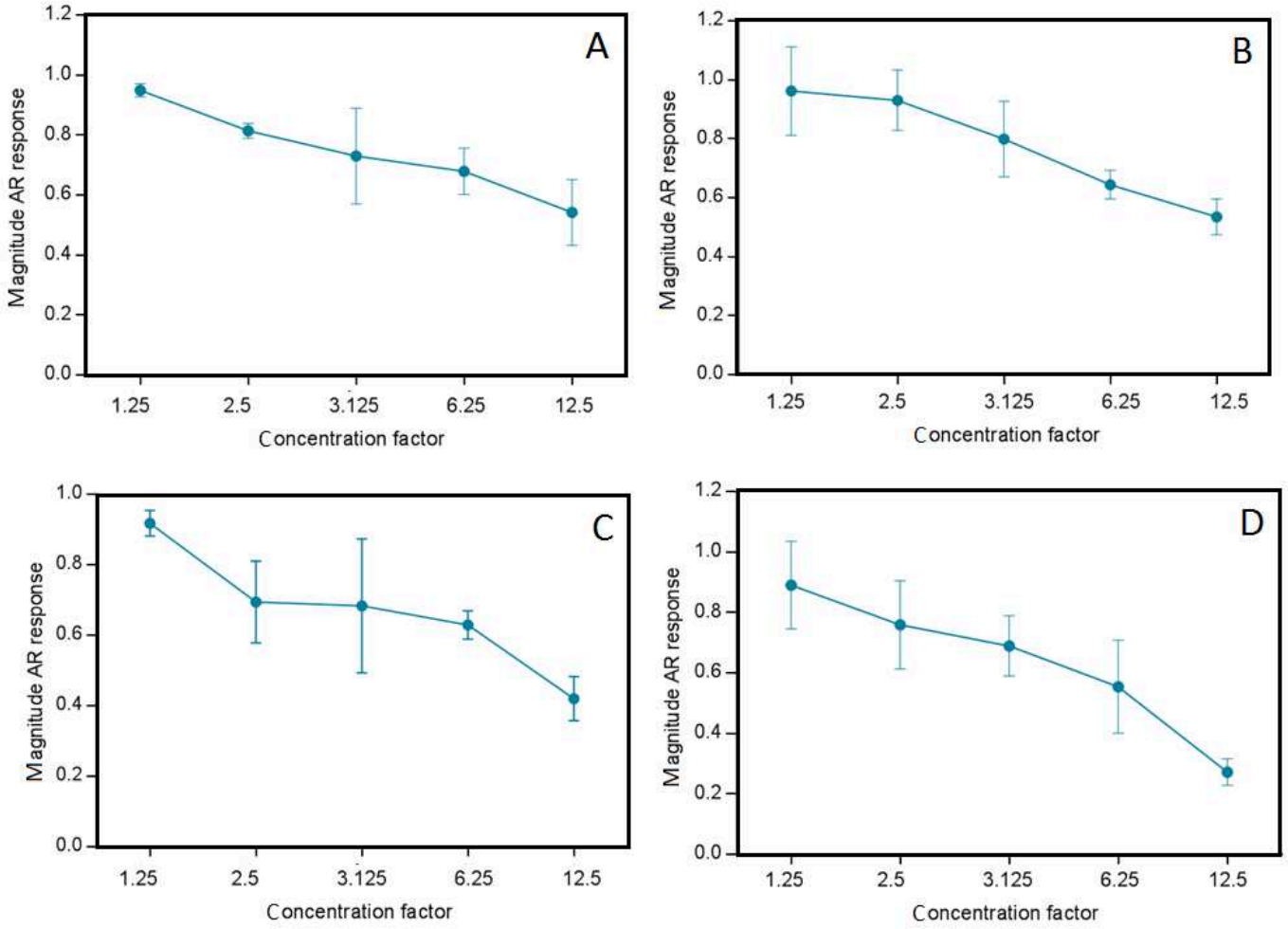

Figure 6.7: Magnitude of AR response versus different concentration factors of the active fractions obtained from the different columns, being fraction 19 for NH_2 (A), fraction 29 for PFP (B), fraction 25 from C18 (C) and fraction 37 from PYE (D). Average and standard deviation values for three replicates in one experiment.

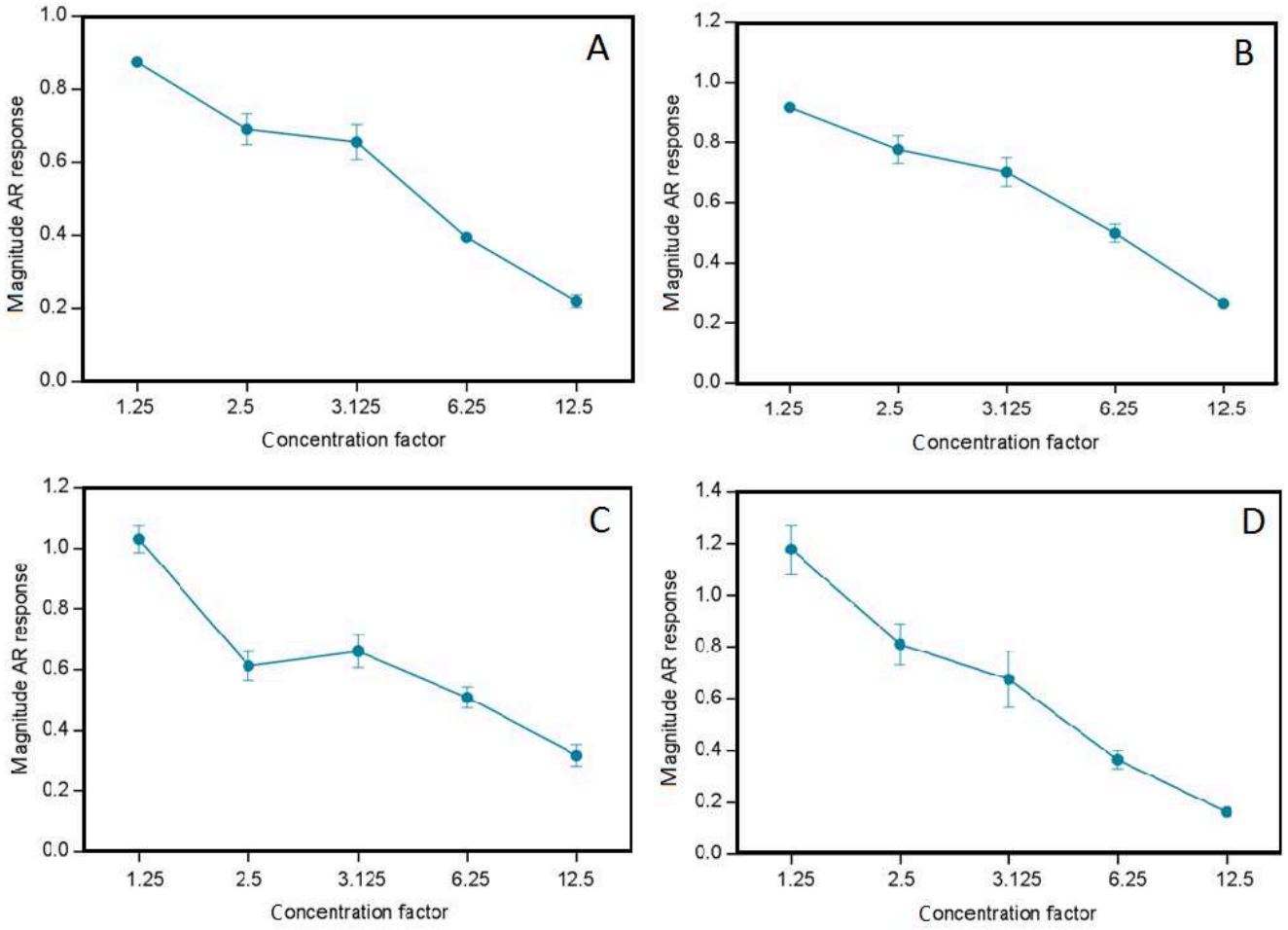

Figure 6 8: Magnitude of AR response versus different concentration factors of recombined fractions obtained from the NH_2 (A), PFP (B), C18 (C) and PYE (D) columns. Average and standard deviation values for three replicates in one experiment.

6.4. Conclusions

The newly developed low-volume procedures for dosing and exposure in the AR reporter gene cell-based assay produced results comparable to those from regular tests when evaluating different compounds. Also, the acceptance criteria of agonistic and antagonistic assays were met. Additionally, the low-volume procedures efficiently evaluated water sample fractions and method blanks regarding antiandrogenicity or cytotoxicity. The downscaling procedures provide a simple and effective solution for studies that need to minimize sample use while maintaining reliable, reproducible and quantitative bioassay results. In the future, the miniaturized methods can be adapted to additional bioassays for application in screening and EDA investigations.

Acknowledgements

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Chapter 7: Induction of the p53 pathway and modulation of cell viability following exposure to genotoxic compounds as single chemicals and mixtures

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Part of this chapter has been submitted to the peer reviewed journal Environmental Science and Pollution Research as:

Di Paolo, C., Müller, M., Thalmann, T., Hollert, H., Seiler, T.B.
p53 induction and cell viability modulation by genotoxic single chemicals and mixtures.

Abstract

The p53 tumour suppression protein activation has a central role in transcriptional regulation of a network of genes in response to stressors that interfere with DNA integrity and cell proliferation. Different in vitro bioassays, such as reporter gene cell lines that allow the high-throughput quantification of p53 binding to DNA response elements can reveal activation of the p53 pathway as an endpoint for DNA damage. Still, knowledge gaps can hamper the application of such assays to evaluate environmental samples containing a multitude of contaminants. Limited information is available on p53 induction following exposure to chemical mixtures, and the interference of cytotoxicity has been discussed to be an issue for p53 induction analysis. In this study, the effects of genotoxic chemicals as single exposures or mixtures on the induction of the p53 pathway were investigated using a cell-based reporter gene assay. Evaluated chemicals included the model compounds or environmental contaminants actinomycin D (ActD), cyclophosphamide (CPP), 4-nitroquinoline 1-oxide (NQO) and 3-nitrobenzanthrone (3-NBA). Single exposures were conducted in the absence or presence of S9 metabolic activation, while binary and tertiary mixtures were tested in the absence of S9 only. All conditions were also analysed regarding effects on cell viability through the MTT test. Cell viability reduction tended to present direct correlation with p53 induction values. ActD and CPP caused the strongest p53 induction for exposures without and with S9, respectively. NQO and 3-NBA presented intermediate and the lowest p53 inductions, respectively. Except for 3-NBA, the peak of p53 induction occurred at chemical concentrations that caused cell viability below 80 %, which is often set as minimum viability accepted in cell-based assays. For the mixtures, there was in general good agreement between predicted and measured p53 induction factors for the lowest concentration ranges, while higher concentrations gave lower values than expected. Similarly to single chemical exposures, p53 peaks often occurred concurrently with viability lower than 80 %. Cytotoxicity evaluation supported not only the selection of concentration ranges for the p53 assay, but also the interpretation of its results. The often used 80 % cell viability threshold as a basis to select the maximum test concentration for cell-based assays is not considered to be the most suited option for the p53 assay. Instead, future studies applying the p53 assay are recommended to include in evaluated test range also concentrations that cause up to 50 % cell viability reduction. The identification of the LOEC and peak concentration will thus unlikely be missed for the compounds that are capable of causing meaningful p53 induction.

Keywords: p53 tumour suppression protein, p53 pathway, DNA damage, genotoxicity, cytotoxicity, reporter gene cell line bioassay.

7.1 Introduction

Activation of the p53 tumour suppression protein has a central role in transcriptional regulation of a network of genes in response to stressors that interfere with DNA integrity and cell proliferation. Under undisturbed conditions, the p53 tumour suppressor protein is maintained at low levels by negative feedback loops, such as by the well-studied mouse double minute 2 homolog protein (MDM2). However, a variety of DNA-damaging agents can interfere with this equilibrium, triggering p53 activation and stabilization, and leading to its accumulation. The binding of p53 to DNA response elements (p53RE) will then result in transcriptional modulation of target genes and activation of the p53 regulatory network. Several responses can follow, including DNA damage, cell cycle arrest or apoptosis, ultimately promoting tumour protecting mechanisms (Harris and Levine 2005, Lavin and Gueven 2006, Beckerman and Prives 2010, Kumari et al. 2014).

Different *in vitro* bioassays have evaluated the activation of the p53 pathway as an endpoint for DNA damage in human health assessment. Levels of p53 protein can be measured by flow cytometry, ELISA or Western blot analysis (Salazar et al. 1997, Yang and Duerksen-Hughes 1998, Clewell et al. 2014). Also, the activation of p53RE by p53 protein binding can be quantified through high-throughput assays with p53RE luciferase (p53RE-Luc) or p53RE β-lactamase reporter gene cell lines (Sohn et al. 2002, Kester et al. 2003, Briat and Vassaux 2008, Knight et al. 2009, van der Linden et al. 2014). Since p53 isoforms are active in non-mammal vertebrates (e.g. fish, amphibians) and invertebrates (e.g. clams, nematodes), the p53 pathway has also relevance for environmental risk assessment (Bhaskaran et al. 1999, Bensaad et al. 2001, Rutkowski et al. 2010, Storer and Zon 2010, Wernersson et al. 2015).

Still, knowledge gaps can hamper the application of such assays to evaluate environmental samples containing different contaminants. Limited information is available on p53 induction following exposure to chemical mixtures, since most of investigations have focused on single compound exposure (Duerksen-Hughes et al. 1999, Knight et al. 2009, Salazar et al. 2009, van der Linden et al. 2014). Although p53 modulation after co-treatment with different drugs has been investigated by some studies, their focus was mainly of therapeutic concern (Choong et al. 2009, Chen et al. 2014, Zajkowicz et al. 2015). Also, the interference of cytotoxicity has been discussed to be an issue for p53 induction analysis in samples of water and animal tissue (Yeh et al. 2014, Jin et al. 2015a). Consequently, for the integration of p53 induction as an

endpoint in the analysis of environmental samples, further knowledge is required on the correlation with cell viability and on the effects of mixtures.

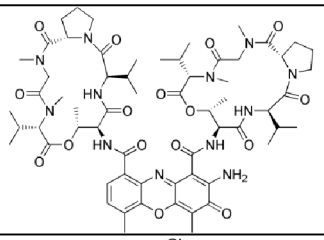
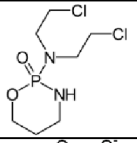
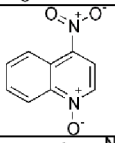
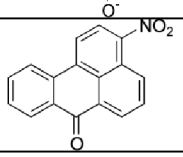
In this study, the correlation between cell viability modulation and the induction of the p53 pathway was investigated using a p53RE-Luc cell line assay (van der Linden et al. 2014) following the exposure to genotoxic chemicals as single exposure or mixtures. Tested compounds included the DNA-interacting drug actinomycin D (ActD) and the DNA-damaging prodrug cyclophosphamide (CPP) as model p53-inducers in the absence and presence of metabolic activation, respectively (Strauss et al. 2007, Choong et al. 2009). In addition, there was evaluation of two nitroaromatic compounds, i.e. 4-nitroquinoline 1-oxide (NQO), a quinolone derivative and UV-mimetic DNA-damaging chemical (Han et al. 2007); and of the diesel exhaust component 3-nitrobenzanthrone (3-NBA) (Landvik et al. 2010). Single exposures tested compounds in the absence (ActD, NQO, 3-NBA) or presence (CPP, NQO, 3-NBA) of metabolic activation. Binary and tertiary mixtures of ActD, NQO and 3-NBA were tested in the absence of the metabolic system only. All conditions were also analysed regarding effects on cell viability by means of a colorimetric method using the tetrazolium bromide salt (Mosmann 1983). This and similar methods are routinely applied in combination with diverse cell-based assays to evaluate chemicals and samples regarding cytotoxicity, with the minimum accepted cell viability being often set at 80 % (Brinkmann et al. 2014a, Xiao et al. 2016). Results are discussed considering different mechanisms of toxicity of chemicals, and regarding the potential application of methods and the testing strategy for the evaluation of environmental contaminants and samples.

7.2 Materials and Methods

7.2.1 Chemicals

The test chemicals (Table 7.1) ActD, CPP and NQO were provided by Sigma Aldrich (Sigma Aldrich Chemie GmbH, Steinheim, Germany), and 3-NBA was provided by Chiron (Chiron AS, Trondheim, Norway). Stock and serial dilution solutions were prepared by chemical dilution in Dimethyl sulfoxide (DMSO, Sigma Aldrich Chemie GmbH, Steinheim, Germany) and stored at 4°C, except for the CPP solutions which were stored at -20°C.

Table 7.1: Test chemicals and respective CAS numbers, formulas and molecular weights.

Test chemicals	CAS number	Formula	Molecular weight	
Actinomycin D (ActD)	50-76-0	$C_{62}H_{86}N_{12}O_{16}$	1255.42	
Cyclophosphamide (CPP)	50-18-0	$C_7H_{15}Cl_2N_2O_2P$. H_2O	279.10	
4-Nitroquinoline 1-oxide (4-NQO)	56-57-5	$C_9H_6N_2O_3$	190.16	
3-Nitrobenzanthrone (3-NBA)	17117-34-9	$C_{17}H_9NO_3$	275.30	

7.2.2 Cell culture

The human osteoblastic osteosarcoma U2OS cell line stably transfected with a p53 reporter construct was obtained from BioDetection Systems BV (BDS, Amsterdam, the Netherlands) and cultured as previously described (van der Linden et al. 2014). Briefly, cell cultures were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) (Invitrogen Life Technologies, Darmstadt, Germany) with phenol red supplemented with 7.5 % fetal calf serum (FCS) (Th. Geyer GmbH, Renningen, Germany), 0.2 % penicillin/streptomycin solution (Invitrogen), 1 % non-essential amino acids (Invitrogen) and G418 antibiotic (0.20 mg/mL) for the selective survival of transfected cells only. Cultures were maintained in a humidified atmosphere with 5 % CO_2 at 37 °C until reaching around 90 % confluence, when cells were used for tests or sub-cultures. Cells were passaged twice a week and medium change was performed every two to three days.

7.2.3 p53 induction assessment

Cell cultures were trypsinized, cells were counted and re-suspended in assay medium (phenol red-free 1:1 mixture of DMEM/F12 supplemented with 5 % of stripped FCS, 0.2 % penicillin/streptomycin solution and 1 % non-essential amino acids) to a final concentration of 10^5 cells/ml. The cell suspension was transferred to 96-well plates (100 µl or 10^4 cells per well), and external wells were filled instead with 200 µl of PBS. After 16 to 20 h, the medium was refreshed and cells were incubated for 24 h with serial dilutions of the test chemicals (200

µl/well) at 37 °C and 5 % CO_2. When cells were co-exposed with rat liver homogenate S9 (supernatant 9000), each well received also 20 µl of freshly prepared S9-mix (10 % S9, 200 µM NADPH, 3 mM glucose-6-phosphate, 0.3 U/ml glucose-6-phosphate dehydrogenase, and 5 mM magnesium chloride in assay medium). After 3 h, the medium was removed, cells were washed with PBS and received fresh assay medium (200 µl/well) for another 21 h until a total of 24 h incubation. Each plate contained also solvent controls (1 % DMSO). All conditions were performed in triplicate wells in each test and contained final DMSO concentration of 1 %. After the incubation period, the medium was removed and cells were lysed in 1 % Triton X-100 lysis buffer (BDS). D-Luciferin solution (BDS illuminate mix) was added and the luciferase activity was measured using a luminometer (GloMax® 96 Microplate Luminometer, Promega GmbH, Germany).

7.2.4 MTT test cell viability assessment

Cell viability of single chemicals and mixtures was evaluated though a colorimetric microplate assay with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT test), which is reduced to formazan by viable cells (Mosmann 1983, Berridge et al. 2005). Briefly, cells were exposed following the same procedures as for p53 induction analysis. At the end of the 24 h incubation period, there was visual verification of no contamination evidence, medium was removed, and each well received 100 µL of freshly prepared 0.5 mg/mL MTT in FCS-free assay medium. In each plate, 6 wells containing no cells received the MTT solution for the measurement of background signal. After 30 min of incubation at 37 °C and 5 % CO_2, the occurrence of formazan crystals was confirmed under microscope observation. The medium was discharged, 200 µl of DMSO was added per well, and plates were shaken for 15 min for crystal solubilisation. The amount of formed formazan was measured with a microplate spectrophotometer (Tecan Infinite® M200, Tecan, Switzerland) at an absorbance wavelength of 492 nm.

7.2.5 Exposure to single substances and mixture

Incubation in the presence of S9-mix was applied to evaluate NQO, 3-NBA, and CPP following single exposures. Incubation in the absence of S9-mix was applied to evaluate ActD, NQO and 3-NBA following single exposures; and also binary (ActD/3-NBA, NQO/3-NBA, ActD/NQO) and a tertiary mixture (ActD/NQO/3-NBA). Single and mixture exposure ranges (Table 7.2) evaluated chemicals following two-fold serial dilutions based on log10 M concentrations. For single exposure, the highest test concentrations of ActD and CPP were adopted from the protocol provided by BDS, aiming to cover from none up to peak induction;

for NQO +/-S9 they were selected with respect to preliminary genotoxicity and cytotoxicity tests; and for 3-NBA the highest test concentration was based on its solubility in aqueous solutions (PubChem) and according to previous studies. For the mixtures, ActD and NQO were evaluated following the same dilution series as for the single exposure, except that the highest concentration was excluded. This procedure aimed to avoid the occurrence of reduced cell viability in the test of mixtures. In the mixtures, the exposure concentration of 3-NBA was kept the same in all dilution steps, being also the second highest concentration of the single exposure testing.

Table 7.2: Exposure concentrations of single chemicals and mixtures for bioassays performed in the presence (+S9) or absence (-S9) of metabolic activation systems.

	Bioassays +S9	**Bioassays -S9**	
Test chemicals	**Single exposure (M)**	**Single exposure (M)**	**Binary and tertiary mixtures (M)**
ActD		1×10^{-6} - 1×10^{-10}	1×10^{-7} - 3×10^{-11}
NQO	1×10^{-4} - 1×10^{-8}	1×10^{-9} - 1×10^{-5}	3×10^{-6} - 3×10^{-10}
3-NBA	9×10^{-5} - 1×10^{-8}	9×10^{-5} - 1×10^{-8}	Constant at 3×10^{-5}
CPP	1×10^{-3} - 1×10^{-7}		

7.2.6 Data analysis

The p53 induction results are expressed as induction factor (IF) values, obtained by normalizing the response of each concentration to the DMSO response. IF threshold values for occurrence of p53 induction activity are set at 1.7 and 2.0 for incubations in the absence and presence of S9-mix, respectively (van der Linden et al. 2014). The lowest concentration value reaching the threshold value is identified as the lowest observed effect concentration (LOEC), and the concentration causing highest IF value is described as the peak concentration. For cell viability, results are expressed as fold changes of measurements obtained from treated cells in comparison to cells exposed to DMSO only, after subtraction of average background signal from all conditions. Fold changes are then converted to cell viability percentage (%) values, considering the solvent control to present 100 %. Cell viability IC_{50} and IC_{20} values were obtained through three parameter non-linear regression (bottom constrained to 0) using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA). Ratios between MTT IC_{50} or IC_{20} values and p53 LOEC and peak values were

also calculated. For mixtures, predicted responses were estimated by the sum of the respective single chemical responses in the MTT and p53 assays.

7.3 Results and Discussion

7.3.1 Single exposures

Considering results for the different compounds, profiles of cell viability IC_{50} (M) values (Table 7.2: ActD -S9 < NQO +S9 < NQO -S9 < 3-NBA +/- S9 < CPP -S9) tended to present direct correlation with p53 induction LOECs (M) (Table 7.3: ActD -S9 < NQO -S9 < NQO +S9 < 3-NBA +/-S9 < CPP +S9), as can be observed in Figure 7.1. The ratios between MTT IC_{50} or IC_{20} and p53 LOEC or peak are presented in Figure 7.2.

Table 7.3: Cell viability IC_{50} and IC_{20} (M) values plus respective 95 % confidence intervals (C.I.) obtained in the MTT test following single exposures in the absence (-S9) and presence (+S9) of metabolic activation.

	Cell viability IC_{50} (M)		**Cell viability IC_{20} (M)**	
	IC_{50} and 95 % C.I.		IC_{20} and 95 % C.I.	
MTT -S9				
ActD	4.6×10^{-7}	1.6×10^{-7} to 1.3×10^{-6}	2.4×10^{-9}	1.2×10^{-10} to 4.8×10^{-8}
NQO	1.6×10^{-6}	1.1×10^{-6} to 2.3×10^{-6}	2.5×10^{-7}	1.2×10^{-7} to 5.0×10^{-7}
3-NBA	n.a.		7.6×10^{-5}	5.0×10^{-5} to 1.2×10^{-4}
MTT +S9				
CPP	n.a.		6.3×10^{-4}	3.0×10^{-4} to 1.3×10^{-3}
NQO	1.9×10^{-5}	1.1×10^{-5} to 3.2×10^{-5}	5.1×10^{-6}	2.4×10^{-6} to 1.1×10^{-5}
3-NBA	n.a.		7.3×10^{-5}	7.6×10^{-6} to 7.1×10^{-4}

n.a: not available.

Table 7.4: LOEC and peak concentration (M) values for p53 induction factor for the single compounds following exposure in the absence (-S9) or presence (+S9) of metabolic activation; and for the mixtures in the absence of S9-mix. Obtained LOEC average, minimum and maximum values; and IF averages and standard deviations are provided.

	LOEC (M)		**Peak concentration (M)**		**Peak IF**
	average	**min / max**	**average**	**min / max**	**average ± SD**
Single chemicals –S9					
ActD	1×10^{-9}	3×10^{-10} / 1×10^{-9}	1×10^{-8}	1×10^{-8} / 3×10^{-8}	11.5 ± 3.6
NQO	3×10^{-7}	3×10^{-7} / 3×10^{-7}	3×10^{-7}	3×10^{-7} / 1×10^{-6}	3.6 ± 0.7
3-NBA	3×10^{-6}	3×10^{-6} / 1×10^{-5}	3×10^{-5}	3×10^{-5} / 1×10^{-4}	2.8 ± 0.5
Single chemicals +S9					
CPP	3×10^{-5}	3×10^{-7} / 1×10^{-4}	1×10^{-3}	3×10^{-4} / 1×10^{-3}	8.2 ± 3
NQO	1×10^{-6}	1×10^{-8} / 3×10^{-6}	1×10^{-5}	1×10^{-5} / 1×10^{-5}	6.9 ± 0.9

3-NBA	1×10^{-5}	1×10^{-5} / 1×10^{-5}	3×10^{-5}	3×10^{-5} / 1×10^{-4}	4.1 ± 1.3
Binary mixtures –S9					
ActD / 3-NBA					
ActD	3×10^{-11}	3×10^{-11} / 3×10^{-11}	1×10^{-8}	1×10^{-8} / 1×10^{-8}	11.1 ± 0.6
NQO / 3-NBA					
NQO	3×10^{-10}	3×10^{-10} / 1×10^{-8}	3×10^{-7}	1×10^{-7} / 3×10^{-7}	4.8 ± 0.9
ActD / NQO					
ActD	1×10^{-9}	1×10^{-9} / 1×10^{-9}	1×10^{-8}	1×10^{-8} / 1×10^{-8}	7.7 ± 1.4
NQO	1×10^{-8}	1×10^{-8} / 1×10^{-8}	1×10^{-7}	1×10^{-7} / 1×10^{-7}	
Tertiary mixture –S9					
ActD / NQO / 3-NBA					
ActD	3×10^{-11}	3×10^{-11} / 3×10^{-11}	1×10^{-8}	1×10^{-8} / 1×10^{-8}	8.6 ± 2.8
NQO	3×10^{-10}	3×10^{-10} / 3×10^{-10}	1×10^{-7}	1×10^{-7} / 1×10^{-7}	

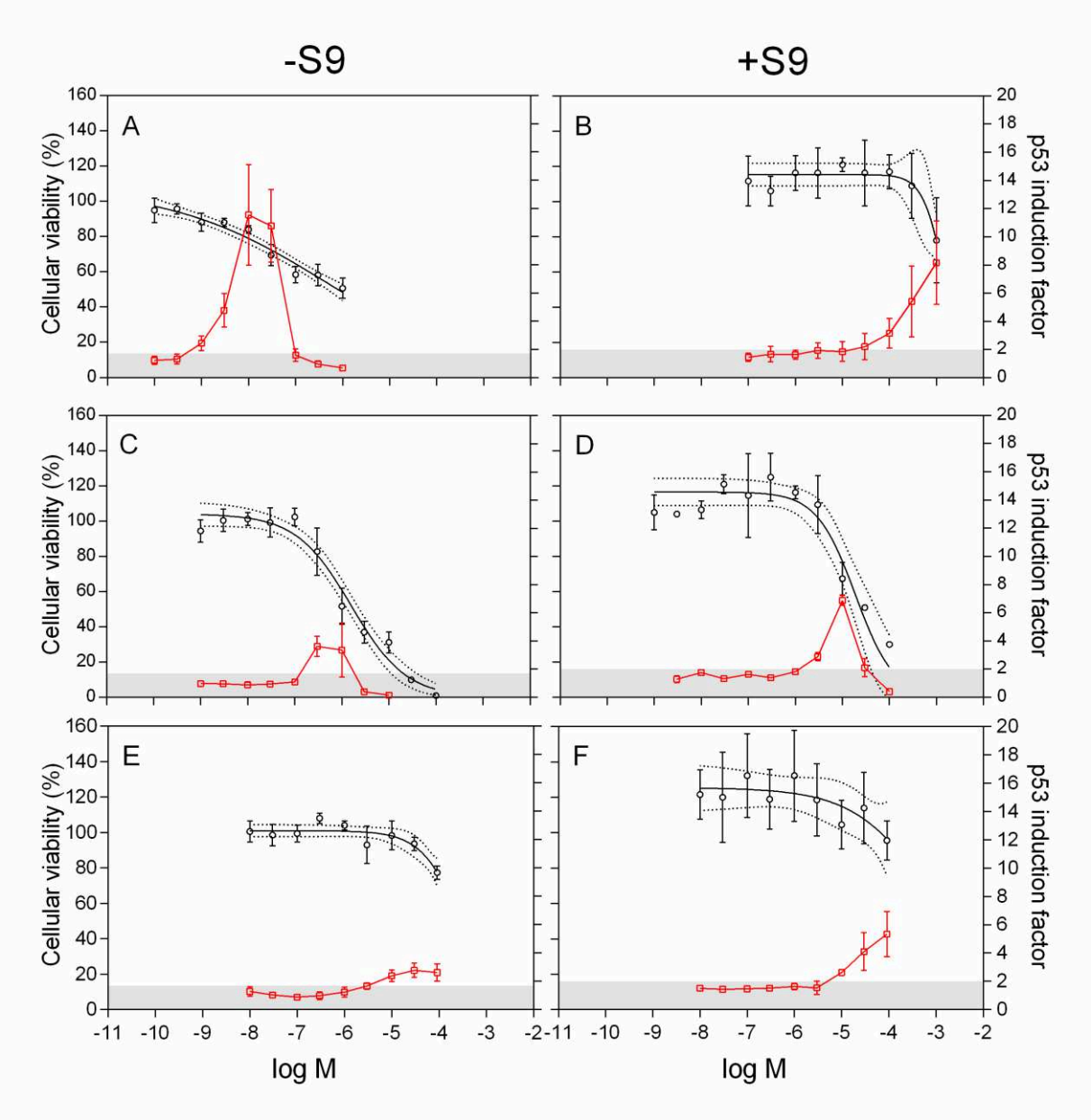


Figure 7.1: Cell viability (%, left Y-axis) and p53 induction factor (right Y-axis) plotted versus concentrations (log M) of cells exposed in the absence (-S9) or presence (+S9) of S9-mix to ActD (A), CPP (B), NQO (C, D), 3-NBA (E, F). Average values and standard errors for cell viability (black, -○-) or p53 induction factors (red, -□-), plus 95% confidence bands for viability. The shadowed areas indicate the threshold values for p53 induction activity in -S9 (1.7) and +S9 (2.0) tests. Number of MTT and p53 induction expertiments, respectively: ActD (4, 14), CPP (3, 7), NQO –S9 (4, 3) and +S9 (3, 3), 3-NBA –S9 (3, 3) and +S9 (3, 2).

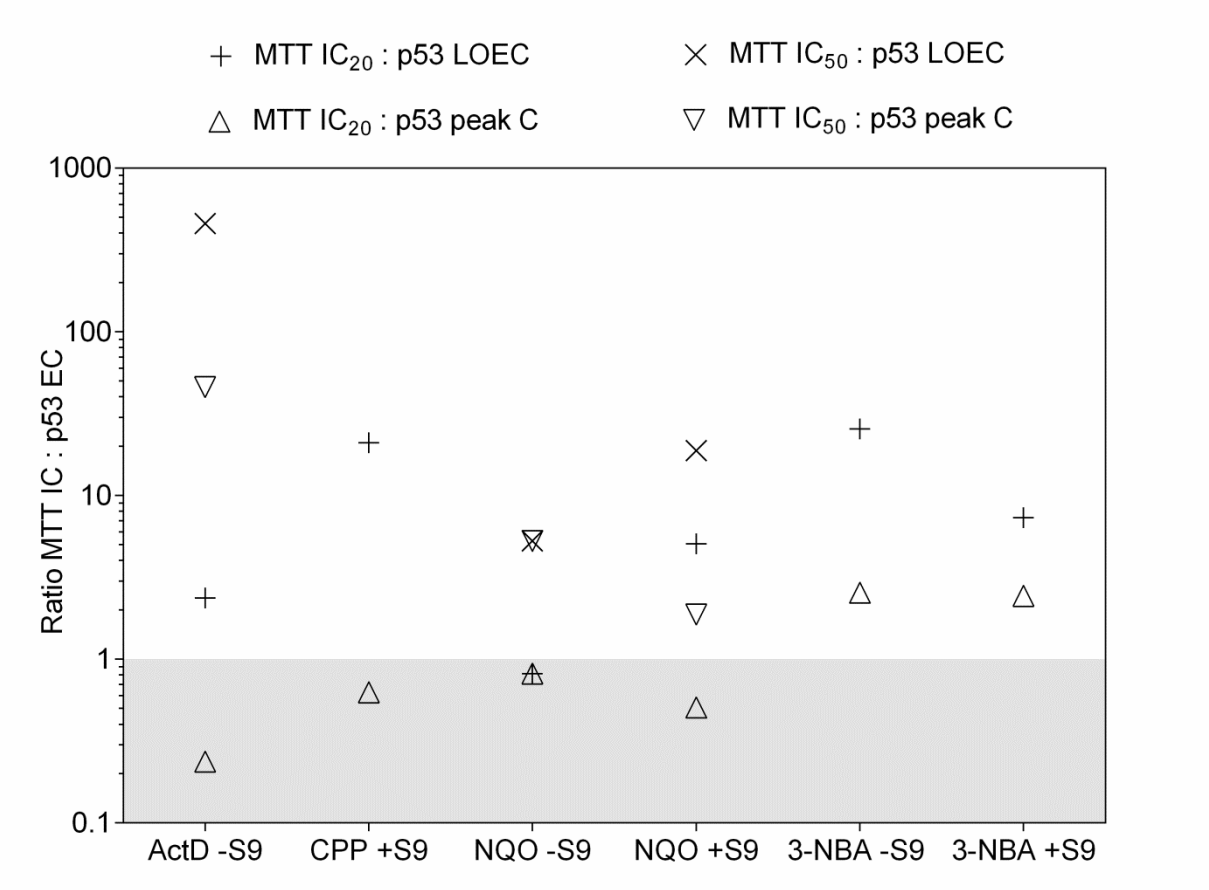


Figure 7.2: Ratios between MTT IC_{50} and IC_{20} cell viability values and respective p53 effect concentration (EC), i.e. LOEC or peak concentration (peak C) for the different single exposures (X-axis). The shadowed area highlights ratio values equal to or lower than 1.

(i) Actinomycin D -S9

ActD -S9 caused the highest values of p53 induction factor among all chemicals (Table 7.4), with an IF peak of 11.5 ± 3.6. Obtained p53 induction LOEC (1 nM) and peak concentration (10 nM) were the same as those found in the method pre-validation study (van der Linden et al. 2014), and both occurred at cell viabilities above 80 % (Table 7.3, Fig. 7.1A). For ActD 30 nM, the high IF was maintained but viability was slightly reduced (70 ± 7 %); followed by further viability reduction and p53 IF drop below the threshold value (Fig. 7.1A) at concentrations ≥100 nM. Ratios of MTT to p53 effect concentrations were always higher than 1 except for MTT_{IC20}:$p53_{peak}$ (around 0.25), which indicates that the concentration causing peak of p53 induction was circa four times higher than the cell viability IC_{20}.

Act-D is an antineoplastic compound often applied for the treatment of tumours, being studied in different cell types. Similar p53 induction and cytotoxicity profiles were described for experiments with diverse cell types, with 1 to 30 nM ActD causing increased p53 activity,

expression and phosphorylation; followed by decrease at 100 nM (Choong et al. 2009, Chen et al. 2014). While at the cytostatic low nanomolar range, ActD was described to cause ribosomal stress that leads to decrease in MDM2 levels and consequently p53 stabilization and activation, at higher cytotoxic concentrations the compound acts as a transcription blocker (Chen et al. 2014). Although the molecular mechanisms are not yet fully understood, it is already known that ActD inhibits RNA synthesis by binding to guanine residues and inhibiting DNA-dependent RNA polymerase (Choong et al. 2009) leading to the accumulation of free ribosomal proteins that can decrease the MDM2 function (van Leeuwen et al. 2011). Importantly, the compound was described to induce genotoxicity relevant endpoints such as the formation of histone gamma-H2AX Foci (Mischo et al. 2005), which is a biomarker of DNA strand breaks and of micronucleus formation (Kuo and Yang 2008, Ivashkevich et al. 2012). Therefore the strong p53 induction identified in our study presents correlation with DNA damage markers.

(ii) Cyclophosphamide +S9

CPP +S9 caused the highest values of p53 induction factor for +S9 exposures (Table 7.4), with an IF peak of 8.2 ± 3.0. The determined CPP p53 induction LOEC (0.3 µM) was lower than the obtained in the pre-validation study (van der Linden et al. 2014), which in fact agreed with our peak concentration (1 mM). This difference might be related to the higher variability recognized to occur in assays containing metabolic system (van der Linden et al. 2014). Cytotoxic effects were not so evident in the tested concentration range, being possible to calculate only the IC_{20} value (63 mM). The ratio MTT_{IC20}:$p53_{peak}$ was lower than 1 (around 0.6), with the p53 peak occurring at a CPP concentration circa 1.6 times the cell viability IC_{20}.

CPP is a widely used antineoplastic pharmaceutical often investigated in *in vivo* studies with rodents. CPP was demonstrated to cause p53 induction in diverse *in vitro* and *in vivo* models. Treatment of primary human cytotoxic T cells with circa 10 µM of a CPP activated analogue led to strong p53 induction, which was correlated with induced ROS production and nuclear relocation of mitochondrial apoptogenic factors (Strauss et al. 2007). In this way the present results support the capability of the p53 assay to identify p53 induction intermediated by different molecular mechanisms. CPP also induced cardiotoxicity and increased mRNA expression of the p53 gene in rats (Asiri 2010), indicating potential organism-level relevance of present results. Due to its use as pharmaceutical, CPP contamination of aquatic environments can be of concern. While advanced treatment with ozonation can achieve its complete removal (Ferre-Aracil et al. 2016), conventional wastewater treatment achieves only

partial reduction, with CPP concentrations up to 0.06 nM occurring in effluents (Steger-Hartmann et al. 1997). of CPP metabolites and transformation products might present a risk to aquatic environments since these compounds can occur in hospital wastewater (Česen et al., 2016).

(iii) 4-Nitroquinoline 1-oxide +/- S9

NQO caused intermediate values of p53 induction factor for exposures both with and without S9-mix (Table 7.4). For NQO –S9, the p53 LOEC and peak occurred at the same concentration (0.3 µM); being very close to the cell viability IC_{20} (0.25 µM) and circa five times lower than the IC_{50} (1.6 µM). Following metabolic activation, there was increase in the p53 LOEC (1 µM) and peak concentrations (10 µM); and cell viability IC_{20} (5.1 µM) and IC_{50} (19 µM) were circa one order of magnitude higher. Still, the IF peak in +S9 (6.9) was nearly the double of the one in -S9 (3.6), which can be attributed to higher p53 induction by the metabolites when compared to the patent compound (Brüsehafer et al. 2016). In both –S9 and +S9 exposures, the ratios MTT_{IC20}:$p53_{peak}$ was lower than 1 (around 0.6), indicating that the p53 peak was achieved in concentrations higher than respective cell viability IC_{20}. Therefore for NQO the assay –/+S9 was able to adequately identify p53 induction despite of concurrent cytotoxic effects.

NQO is a compound often applied in positive control conditions of methods investigating genotoxicity. In previous studies, NQO –S9 caused circa 50% of cell viability at 1-2 µM after 24 h exposure to epidermoid carcinoma cells, which presented increased p53 expression already after 2 h of treatment (Han et al. 2007). In addition to cytotoxicity, NQO –S9 caused micronucleus formation in human lymphoblastoid cells, which was more evident in p53 dysfunctional lines (Brüsehafer et al. 2016). *In vivo* studies following treatment of rats with the NQO metabolite 4-hydroxyaminoquinoline 1-oxide identified that p53 expression, apoptosis and cell proliferation occurred sequentially in pancreas, processes which are considered to be closely related with the compound carcinogenesis following DNA adduct formation (Imazawa et al. 2003). Again, the measured p53 induction can be correlated with other genotoxicity endpoints and demonstrates relevance to support in vivo investigations.

(iv) 3-Nitrobenzanthrone +/- S9

3-NBA caused the lowest values of p53 induction factor for exposures both with and without S9-mix (Table 7.4). For 3-NBA -S9, the p53 LOEC (3 µM) and peak (30 µM) were 25 and 2.5 lower than cell viability IC_{20} (76 µM). Following metabolic activation, there was increase in the LOEC (10 µM) but the peak (30 µM) and cell viability IC_{20} (73 µM) were

maintained. Such profile could indicate that the sensitivity of the assay was affected by the co-incubation with the metabolic activation system. Still, the IF peak in the +S9 experiments (4.1) was around 1.5 times higher than the one in -S9 (2.8), indicating that the assay +S9 is able to identify relevant p53 induction. Additionally, similarly to NQO this profile can be attributed to higher p53 induction by 3-NBA metabolites when compared to the parent compound (IARC 2014). Cytotoxic effects were not so evident in the tested concentration range, being possible to calculate only the IC20 value in both -/+S9 (Table 7.3). Differently than for the other chemicals, the ratios between MTT and p53 effect concentration values were always higher than 1. Therefore, for 3-NBA -/+S9 the peak and LOEC was achieved at concentrations lower than the cell viability inhibition IC20. That indicates that for 3-NBA the p53 induction activity would be identified even if exposure concentrations were limited to those occurring with minimum 80 % of cell viability.

3-NBA is a diesel exhaust component considered as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC 2014). It causes DNA adduct formation and oxidative stress both *in vitro* and *in vivo* (Nagy et al. 2007), and DNA strand breaks identified through the comet assay (Arlt et al. 2004). In experiments with human bronchial epithelial cells, 3-NBA in the low μM range caused increased p53 phosphorylation and nuclear translocation, formation of the DNA strand break marker gamma-H2AX, and apoptosis (Øya et al. 2011). However, our results are contrasting with an investigation on the effects of 3-NBA and its metabolite 3-aminobenzanthrone on mouse hepatoma cells, which described that although both compounds increased p53 phosphorylation, its translocation to the nucleus occurred only after 3-NBA exposure (Landvik et al. 2010). Such differences can be related to cell sensitivity variations, or to the formation of other active 3-NBA metabolites in our study. 3-NBA results also support the relevance of the p53 assay to identify induction intermediated by diverse mechanisms and its correlation with diverse genotoxicity endpoints.

7.3.2 Binary and tertiary mixtures p53 induction and cell viability

Predicted responses in the binary and tertiary mixtures were estimated by the sum of the respective single chemical responses in the MTT and p53 assays; and compared to the measured values in bioassays. In general, there was good agreement between predicted and measured IF values for the lowest concentration ranges, while higher chemical concentrations caused instead lower than expected IF values (Fig. 7.3 and 7.4). For the four lower concentrations of the binary mixtures the predicted and measured values were very similar, confirming additivity (Fig. 7.3B and C) or indicating slight synergism (7.3A) for p53

induction. For the tertiary mixtures instead there was infra-additivity tendency (Fig. 7.3D). As an outcome, predicted LOECs were always in agreement with measured values (Fig. 7.3). That suggests sensitivity of the assay to identify the activity even when mixtures of components are present. IF peaks instead tended to occur at concentrations lower than predicted, except for ActD/3-NBA for which it was maintained (Fig. 7.3A). Further, IF values for the peak and higher test concentrations tended to be lower than respective predicted values, particularly for the binary ActD/NQO (Fig. 7.3C) and the tertiary (Fig. 7.3D) mixture. Such results indicate that the evaluation of mixtures might require careful selection of investigated concentration range in order to properly identify the peak of induction and estimate the respective magnitude of response.

Additivity of the p53 induction response is already explored for therapeutic use of pharmaceuticals. For ActD, combined drug administration applies low doses of the drug aiming to achieve increased p53 activation while avoiding undesired cytotoxic or DNA-damaging effects (Rao et al. 2010, Chen et al. 2014). Synergism has also been described for ActD in the low nM range combined with anti-tumorigenic drugs (Choong et al. 2009). Similarly, our results indicate that at low concentrations additivity of even synergism occurred, while higher concentrations presented reduced measured p53 IF when compared to predicted values. That is in agreement with lower cell viability values obtained for the mixtures (Fig. 7.3) when compared to respective single compounds (Fig. 7.1). Surprisingly, cell viability reduction at highest test concentrations was not as intensive as predicted for the binary mixture containing ActD/NQO (Fig. 7.3C) and for the tertiary (Fig. 7.3D) mixtures. Still, viability values reached circa 40% at highest test concentrations for both mixtures. Although p53 is discussed to present a protective role against DNA damage-induced cell death (Garner and Raj, 2008), other factors might have interfered with exposures at higher concentrations such as chemical interactions between tested compounds.

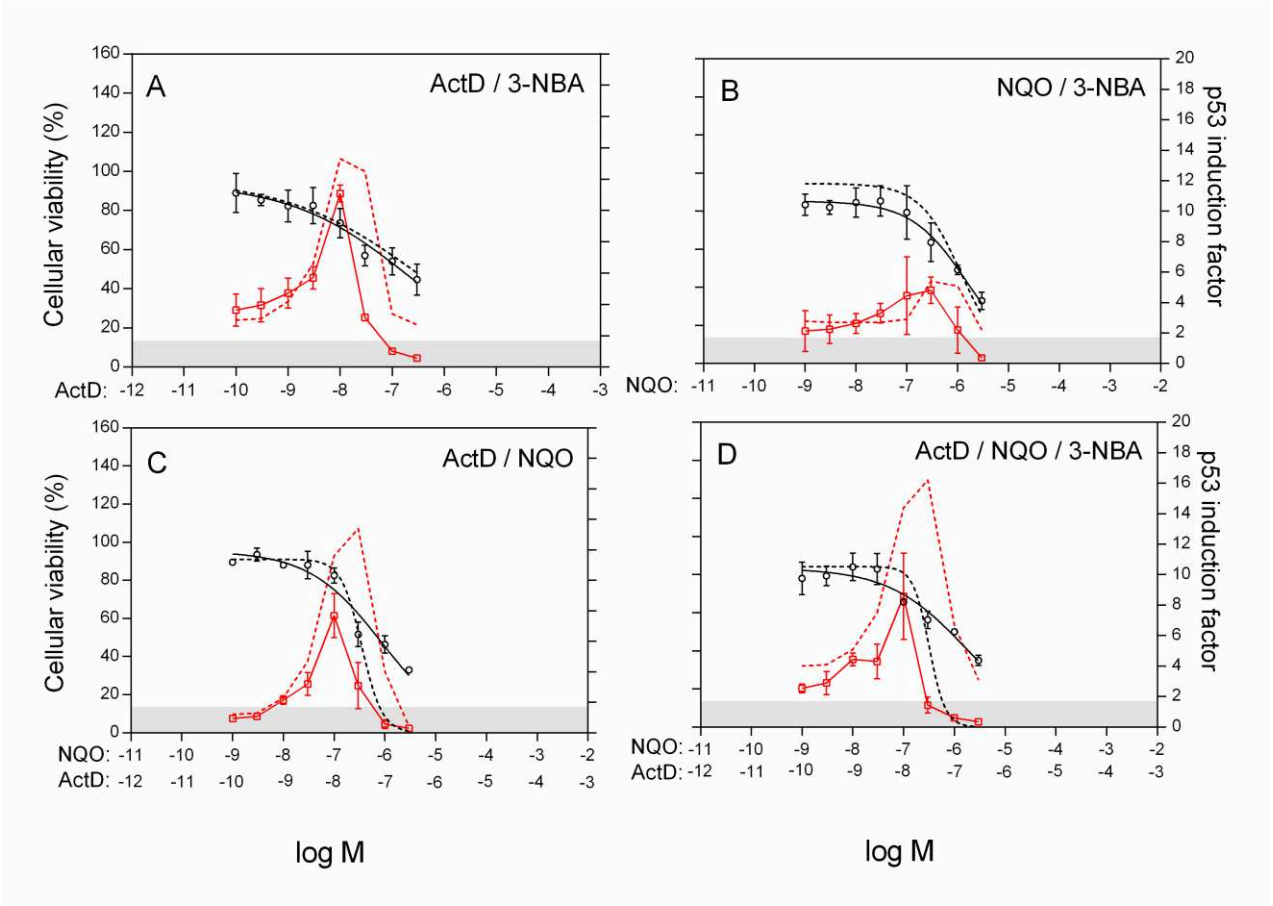


Figure 7.3: Measured (full lines) and additivity-predicted (dotted lines) values of cellular viability (black, as % in left Y axis) and p53 induction factor (red, in right Y axis) plotted versus concentrations (log M) of the binary mixtures (A) ActD/3-NBA, (B) NQO/3-NBA, (C) ActD/NQO; and of the tertiary mixture (D) ActD/NQO/3-NBA. The mixtures containing 3-NBA had 3×10^{-5} M of the chemical in all exposure conditions. Average values (viability: black circles, induction factor: red squares) and standard deviations are shown. The shadowed areas indicate threshold values for p53 induction activity of 1.7. For all mixtures, three independent experiments were performed for the MTT assay and for the p53 induction test, respectively.

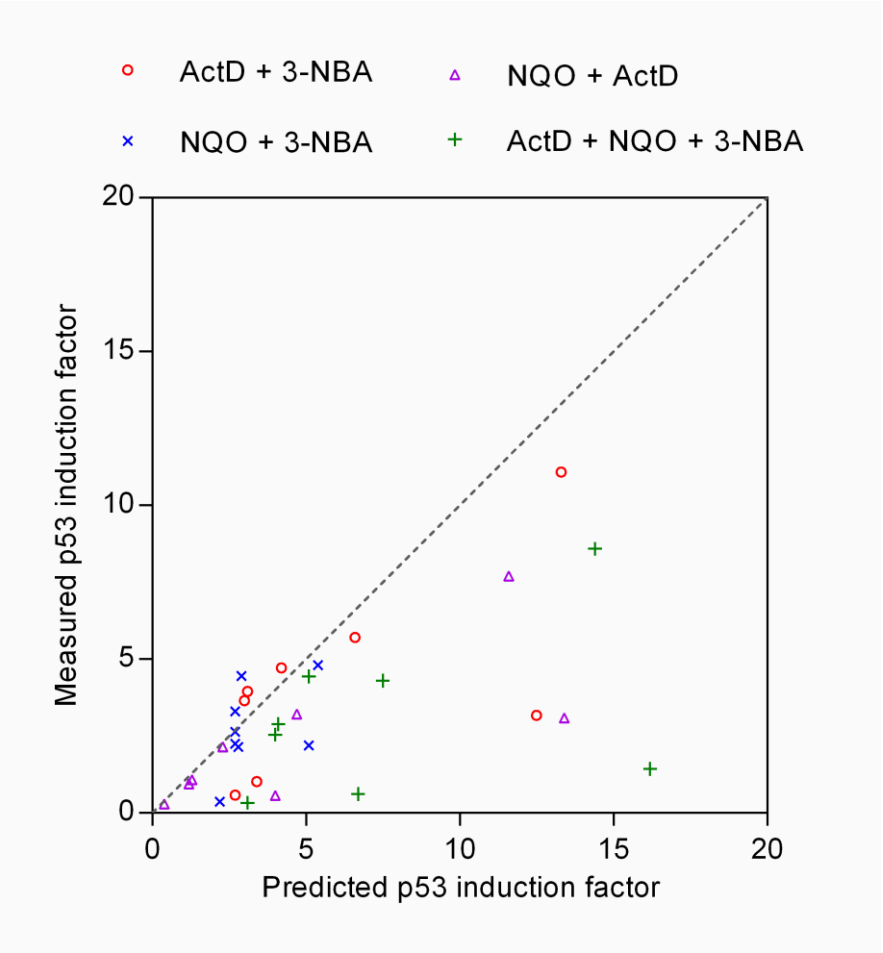


Figure 7.4: Measured (Y-axis) versus predicted (X-axis) p53 induction factor values for the binary and tertiary mixtures.

Our study indicates that, resembling the therapeutic use of pharmaceuticals, additivity of p53 response is obtained when cells are exposed to mixture of compounds presenting different mechanisms of toxicity. Such outcome supports the application of the p53 assay to investigate environmentally relevant mixtures or environmental samples. Similarly to chemical exposures, p53 peaks can occur at concentrations which cause cell viability reduction under the 80% value (Fig. 7.3). Therefore, when evaluating single chemicals, mixtures or samples for the occurrence of p53 induction, it is recommended to include also rather cytotoxic concentrations in evaluated test range, reaching up to 50% of reduction in cell viability. Concentrations that cause more than 50% of cell viability reduction should however be interpreted with caution since non-specific responses caused by general cell stress can also occur (van der Linden et al. 2014).

7.4 Conclusions

This study demonstrated the ability of the p53 assay to identify p53 induction through different mechanisms and both with and without metabolic activation system. Additionally the assay was efficient in identifying additivity of the p53 response when evaluating chemical mixtures, confirming its ability to identify mixture activity and potentially also of complex environmental samples. For such future applications, it is of relevance to consider in experimental planning and evaluation that p53 peaks can be lower and occur at lower test concentrations than for respective single chemicals. Further, cytotoxicity evaluation should be applied to support the selection of concentration ranges and also the interpretation of the p53 assay results. In this sense our study demonstrates that the often used minimum 80 % cell viability threshold to set the maximum exposure concentration for cell-based bioassays is not optimal for the p53 assay. Future evaluations of mixtures or environmental samples through the p53 assay are recommended to include concentrations that cause up to 50 % cell viability reduction in the evaluated test range. In this way, the identification of the LOEC and peak concentration will unlikely be missed for the compounds and mixture components that cause meaningful p53 induction concurrently with cytotoxic effects.

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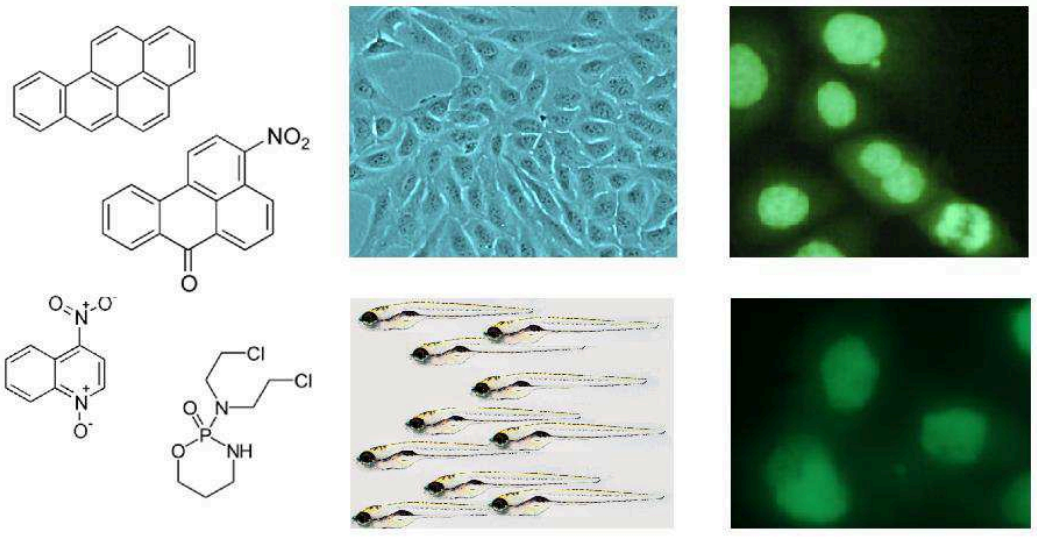
Chapter 8: Development of procedures for the assessment of micronucleus formation in zebrafish liver cell line and in early larvae



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Micronucleus formation in zebrafish liver cell line and larvae exposed to direct- and indirect-acting genotoxicants.

Abstract

Genotoxic compounds can cause DNA damage and lead to the formation of tumours, effects on growth and development, and reduced survival of different life stages. Therefore they are of high concern for human health and the environment, including aquatic environments and fish populations. The micronucleus (MN) test is one of the most traditional genotoxicity methods in eukaryotic organisms, having been applied also to a great variety of aquatic organisms, including the well-studied model zebrafish. However, very few studies have attempted to apply the MN test to zebrafish cell lines or non-protected life stages. In this study, we have developed the procedures to apply the MN test to the zebrafish liver ZFL cell line and to zebrafish early larvae. A testing strategy integrating acute toxicity assays was set with the MTT test and the fish embryo acute toxicity (FET) test. Tested compounds included the direct-acting genotoxicant 4-Nitroquinoline 1-oxide (NQO), the pro-drug cyclophosphamide (CPP), and the environmental contaminants 3-Nitrobenzanthrone (3-NBA) and benzo[a]pyrene (BaP). For the ZFL MN test, the cell doubling time was determined using a live cell time-lapse microscopy system. The larval MN test required the establishment of adequate procedures to obtain primary cell suspension from larvae followed by cell fixation. NQO and 3-NBA showed to be particularly cytotoxic to the ZFL cells, while CPP and BaP caused toxic effects only at the highest concentrations. BaP exposures were observed to present chemical precipitate in medium in both cell and larvae exposures, which compromised result interpretation. The ZFL MN was able to evaluate both the direct as indirect acting genotoxicants. NQO was the compound causing the highest induction of MN formation, reaching circa 9% of counted cells in the highest test concentrations of 6.2×10^{-8} M. 3-NBA and CPP also induced MN formation with significant differences from controls, even if at lower levels than NQO. NQO is therefore recommended to be used as the direct-acting positive control chemical, while CPP can be considered as the indirect-acting one. For the larval MN test, since only NQO and CPP produced robust results in the FET tests, these two compounds were evaluated. All the MN test steps were established with primary cells of zebrafish larvae, however the observation and scoring of MN was not as easily achieved as for the ZFL cells. Exposure to NQO and CPP led to tendency for increased MN formation, however there was high variation between experimental replicates and differences to controls were not significant. Recommendations for further improvement of the protocol are discussed.

Keywords: micronucleus, genotoxicity, zebrafish, ZFL cell line, larva.

8.1 Introduction

Genotoxic compounds can damage the DNA of organisms and cause impairments on the organism and population levels. Although cells are often able to repair DNA damage such as by the p53-pathway (as discussed in Chapter 7), these protecting mechanisms can fail, potentially resulting in formation of tumours, effects on growth and development, and reduced survival of different life stages (Theodorakis 2001, Hollert et al. 2003, Jha 2004). In consequence, genotoxic compounds are of high concern for human health and the environment, including aquatic systems and fish populations. Different methods are available to evaluate genotoxicity, including *in vitro* and *in vivo* approaches. The micronucleus (MN) test is one of the most traditional genotoxicity methods in eukaryotic organisms (Schmid 1975), having been applied to a great variety of aquatic organisms as reviewed by Bolognesi and Hayashi (2011).

The MN test method evaluates the formation of the so-called micronuclei, which consist of whole chromosomes or fragments that are formed as a result of aneugenic (whole chromosome loss) or clastogenic (chromosomal breakage) events, and can be modulated by epigenetic factors (Kirsch-Volders et al. 2011, Luzhna et al. 2013). Therefore micronucleus formation represents a robust chromosome damage and also a multi-target genotoxicity endpoint that is used as a predictor of cancer and of ecotoxicological effects (Bolognesi and Hayashi 2011, Kirsch-Volders et al. 2011). In addition, there are standard procedures for the application of MN tests in mammal *in vitro* and *in vivo* models (OECD 2014), which further supports the method application in regulatory assessment.

The MN test has been applied to diverse fish species, mostly adult organisms (Rodriguez-Cea et al. 2003, Cavas and Ergene-Gozukara 2005, Bolognesi and Hayashi 2011) and including also studies with zebrafish adults (Ramirez and Garcia 2005, Fassbender and Braunbeck 2013a). The application of genotoxicity methods such as the MN test to zebrafish is of particular interest, since this is a standard species for aquatic toxicity and a model organism in which many mechanisms of toxicity are investigated (Chakravarthy et al. 2014). Further, early life stage represent an alternative to adult tests (OECD 2013b). Although other genotoxicity methods such as the comet assay have been applied to zebrafish early life stages (Kosmehl et al. 2006, Kosmehl et al. 2008), only one study have described the MN test in zebrafish larvae (Böttcher 2012). Additionally, only one recent study have investigated the application of the zebrafish liver ZFL cell line to evaluate MN formation (Gajski et al. 2015).

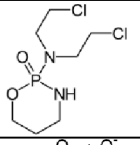
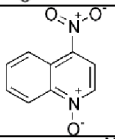
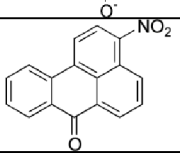
In this study, we have developed the procedures to apply the MN test to the zebrafish liver ZFL cell line and to zebrafish early larvae. For that, a testing strategy integrating acute toxicity assays was set, with the MTT test providing the basis for the selection of exposure concentrations in the ZFL MN test, while the fish embryo acute toxicity (FET) test provided input for the larval MN test. For the ZFL MN test, the cycle length of the ZFL line was determined. The larval MN test required the establishment of adequate procedures to obtain primary cell suspension from larvae followed by cell fixation. Tested compounds included the direct-acting genotoxicant 4-Nitroquinoline 1-oxide (NQO), the pro-drug cyclophosphamide (CPP), and the environmental contaminants 3-Nitrobenzanthrone (3-NBA) and benzo[a]pyrene (BaP). NQO (direct clastogen), BaP and CPP (clastogens requiring metabolic activation) are recommended as reference substances for the selection of positive control conditions (OECD 2010b). Results are discussed regarding the selection of chemicals for positive control conditions, and in terms of achievements or potential improvements of experimental procedures.

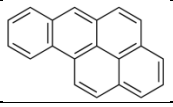
8.2 Materials and Methods

8.2.1 Chemicals

The test chemicals (Table 8.1) CPP and NQO were provided by Sigma Aldrich (Sigma Aldrich Chemie GmbH, Steinheim, Germany), and 3-NBA was provided by Chiron (Chiron AS, Trondheim, Norway). Stock solutions were prepared by chemical dilution in Dimethyl sulfoxide (DMSO, Sigma Aldrich Chemie GmbH, Steinheim, Germany) and stored at 4°C (NQO, 3-NBA) or -20°C (CPP, BaP). CPP was directly diluted in exposure medium in the FET tests.

Table 8.1: Test chemicals and respective CAS numbers, formulas and molecular weights.

Test chemicals	CAS number	Formula	Molecular weight	
Cyclophosphamide (CPP)	50-18-0	$C_7H_{15}Cl_2N_2O_2P.H_2O$	279.10	
4-Nitroquinoline 1-oxide (4-NQO)	56-57-5	$C_9H_6N_2O_3$	190.16	
3-Nitrobenzanthrone (3-NBA)	17117-34-9	$C_{17}H_9NO_3$	275.30	

Benzo[a]pyrene (BaP)	50-32-8	$C_{20}H_{12}$	252.31	

8.2.2 Cell culture

Zebrafish liver ZFL cell cultures were maintained in Leibovitz L-15 medium (Invitrogen Life Technologies, Darmstadt, Germany) supplemented with 10 % Fetal calf serum (FCS) (Th. Geyer GmbH, Renningen, Germany). Cultures were kept in a humidified atmosphere at 28 °C until reaching around 90 % confluence, when cells were used for tests or sub-cultures. If confluence was not reached after 3-4 days, the old medium was removed and 15 mL of fresh supplemented medium was added. For the use in tests or new cultures, cells were rinsed with 10 mL Dulbecco's Phosphate Buffered Saline (PBS), incubated during 20 min at 28 °C with 2 mL of trypsin, re-suspended in 10 mL of a 3:2 medium/PBS mixture, centrifuged for 5 min (1,010 rpm, 20 °C), and re-suspended in 15 mL of fresh supplemented medium.

8.2.3 Evaluation of doubling time of ZFL cells

The doubling time of the ZFL cells was determined by evaluating the cell growth during a defined time period using the BioStation IM-Q live cell time-lapse microscopy system (Nikon, Düsseldorf, Germany). The system was used to periodically capture pictures of the cells in real time, and the number of cells was counted every hour along a period of 48 h. Cell cultures used to determine the doubling time always presented 90 - 95 % confluence. After trypsin treatment, cells were re-suspended in fresh FCS supplemented medium, cell density was adjusted to 50,000 cells/ml, and 2 ml of the cell suspension were transferred to a culture dish with glass bottom. Cells were then incubated in the BioStation in a temperature controlled room at 26 °C, and of 5 different fields were taken every hour over a period of 72 h. Afterwards pictures were evaluated for the number of cells presented in each field. The number of cells at the beginning of incubation (t = 0 h) and after 24 h and 48 h were used for further calculations. Averages of the five fields for each of the three different time points were calculated, and the doubling time was determined through least square fit exponential regression using an online calculator (Roth 2006).

8.2.4 MTT test

Cell viability of single chemicals and mixtures was evaluated though a colorimetric microplate assay with 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT test), which is reduced to formazan by viable cells (Mosmann 1983, Berridge et al. 2005). After confluence was reached, cell cultures were treated with trypsin, re-suspended in FCS supplemented medium, and cell concentration was adjusted to 125,000 cells/mL (Bopp and

Lettieri 2008). The cell suspension was transferred to 96-well plates (200 µl or 25,000 cells per well), and external wells were filled instead with 200 µl of PBS. After 16 to 20 h, the medium was refreshed and cells were incubated for 48 h at 28°C with serial dilutions of the test chemicals (200 µl/well) NQO (1×10^{-9} to 1×10^{-5} M), BaP (1×10^{-7} to 1×10^{-3} M), 3-NBA (1×10^{-8} - 9×10^{-5} M) and CPP (1×10^{-7} to 1×10^{-3} M). Each plate contained also solvent controls (1% DMSO). All conditions were performed in triplicate wells in each test and contained final DMSO concentration of 1%. At the end of the incubation period, there was visual verification of no contamination evidence, medium was removed, cells were washed with 150 µL PBS, and each well received 100 µL of freshly prepared 0.5 mg/mL MTT in FCS-free medium. After 1 h of incubation at 28 °C, the MTT solution was discarded and DMSO was added (200 µL/well) to dissolve the blue formazan crystals. The plate was gently shaken for 25 min. The amount of formed formazan was measured with a microplate spectrophotometer (Tecan Infinite® M200, Tecan, Switzerland) at an absorbance wavelength of 492 nm.

8.2.5 Micronucleus test with zebrafish liver ZFL cells

The MN test followed the general principle described in the OECD TG 487 (OECD 2010b) with adaptations to the cell line characteristics. After confluence was reached, cell cultures were treated with trypsin, re-suspended in FCS supplemented medium, and cell concentration was adjusted to 50,000 cells/mL. Autoclaved cover glasses inserted under sterile conditions into 6-well microplates were used for cell seeding, done by addition of 2 mL of the cell suspension to each cover glass and well (i.e. 100,000 cells/well). After incubation for 16 h at 28°C, the medium was replaced with 2 mL of exposure medium containing the test chemicals NQO (1.9×10^{-9}, 6.2×10^{-9}, 1.9×10^{-8}, 6.2×10^{-8} M), 3-NBA (3.2×10^{-8}, 1.1×10^{-7}, 3.2×10^{-7}, 1.1×10^{-6} M), CPP (3.0×10^{-4}, 6.5×10^{-4}, 1.0×10^{-3} M) and BaP (1.0×10^{-4}, 3.0×10^{-4}, 1.0×10^{-3} M) or DMSO 1%. Cells were then incubated for 48 h at 28°C to ensure that 1.5 to 2 cell cycles occurred. For every exposure concentration and the solvent control condition, two internal replicates were performed. After the incubation period, cell fixation was done by transferring the cover glasses into a 1:1 mixture of PBS and methanol/acetic acid (4:1) for 10 min and afterwards in the pure 4:1 methanol/acetic acid mixture for another 10 min. Both slides of the internal replicates of each concentration were glued on one microscope slide using mounting agent Microscopy Aquatex (Merck, Darmstadt, Germany) and stored in darkness until evaluation. For cell staining, each cover glass received 16 µL of 0.0004 % acridine orange solution and was covered with another cover glass. In each exposure condition, 2,000 cells were observed (1,000 cells in each internal replicate) by use of a fluorescence microscope at 400x magnification (Nikon Eclipse 50i, Düsseldorf, Germany). Three experimental replicates were

done. The criteria for identification of micronuclei in ZFL cells were: only undamaged cells were considered, micronuclei should be next to but not touching the nucleus, micronuclei should be at the same plain as the nucleus, the size of micronuclei should not be more than 30 % of the nucleus, and cells with more than one micronucleus were counted as one (OECD 2010b). Additionally, the occurrence of mitosis was counted in the different conditions and replicates.

8.2.6 Maintenance of zebrafish and selection of eggs

Zebrafish adults were maintained following standard maintenance procedures, i.e. at room and water temperature of 26 ± 1 °C and light-dark cycle of 14:10 hours at the Fraunhofer IME Institute (Aachen, Germany). The fish are from a wildtype zebrafish stock maintained at the institute for more than 20 years, with original stocks obtained in 1992 from West Aquarium GmbH (Bad Lauterberg, Germany). Eggs were obtained from community mating, and fertilization rate was at least 70 % for all experiments. At the end of experiments, fish were euthanized by prolonged immersion in a solution of benzocaine 40 g/L.

8.2.7 Fish Embryo Acute Toxicity test

The Fish Embryo Acute Toxicity (FET) tests, which were based on the OECD TG 236 (OECD 2013b), were performed to identify effect-concentrations for acute toxicity of the test compounds. These provided the basis for the selection of the exposure concentrations applied in the MN test with zebrafish larvae. Fertilized eggs at around 2 hours post fertilization (hpf) were exposed in ISO medium water (ISO 2007a) to NQO (0.47, 0.66, 0.93, 1.31, 1.58, 1.87, 2.63, 3.73 and 5.26 µM), BaP (0.3125, 0.625, 1.25, 2.5, 5 and 10 µM), 3-NBA (0.04, 0.07, 0.14, 0.28, 0.40, 0.57, 0.80, 1.14, 2.27, 4.54 and 9.08 µM) or CPP (0.3125, 0.625, 1.25, 2.5, 5.0, 5.95, 7.07, 8.41 and 10 mM). Exposure solutions were freshly prepared by dilution of respective stock solutions in medium water, except for CPP with was diluted directly in exposure solution. Embryos were exposed in glass crystallization dishes (BaP and 3-NBA with final 0.1% DMSO) or in 24-well plates (NQO with final 0.01% DMSO and CPP), with one embryo in 2 mL of solution per well or dish. For BaP and 3-NBA, the glass crystallization dishes were pre-soaked with respective exposure solutions for circa 18 h before exposure start. Solvent controls contained either 0.01 % (NQO) or 0.1 % (BaP and 3-NBA) DMSO, water controls the water medium only, and positive controls 3,4-Dichloroaniline 3.7 mg/L. In one experiment, 10 embryos were exposed for each chemical concentration; 20 embryos for the positive controls; and at least 36 embryos for the water and solvent controls. Dead fish were removed daily. At 48 and 120 h after the exposure start, lethal and sublethal effects were

scored. Lethal effects were lack of heartbeat, coagulation, non-detachment of tail, and lack of somite formation; and sublethal effects were reduced heartbeat, reduced or no blood circulation, occurrence of edema in yolk or heart, reduced or no pigmentation, underdevelopment, and occurrence of spine malformation (Nagel 2002, Hollert et al. 2003, Lammer et al. 2009, Selderslaghs et al. 2009). At the end of the test, surviving larvae were euthanatized using benzocaine 40 g/L solution. Effect-concentration values were obtained with basis on the cumulative occurrence of lethal and sublethal effects (i.e. occurrence of any lethal or sublethal endpoint, EC values) or lethality only (LC values).

8.2.8 Total larval cell suspension and fixation of cells for the micronucleus test

(i) Total larval body cell suspension

After the test of different procedures, a protocol to obtain total cell suspensions from zebrafish larvae was developed with basis in similar methods with medaka (Morin et al. 2011, Le Bihanic et al. 2014). After exposure and euthanasia, larvae were transferred in groups of 10 fish of the same exposure condition into crystallization dishes on ice. Mechanical dissociation was done through mincing with razor blades. Minced tissues were submitted to enzymatic treatment by incubation with 1 mL of either 1.25 mg/mL dispase II; or of a mixture of 1.25 mg/mL dispase II and 0.5 mg/mL type IV collagenase from *Clostridium histolyticum*. Further, the enzyme solutions were prepared either in Leibovitz L-15 or in PBS. Incubation times of 30 to 120 min were evaluated, all performed at 37°C in plate shaker (400 rpm, Titramax 1000 / Inkubator 1000, Heidolph Instruments, Schwabach, Germany), with gently mixing of contents by tube inversion every 15 min. After incubation, tubes were centrifuged at 1,000 rpm for 10 min, the supernatant containing the enzymes was removed, and dissociated cells were suspended with 1 mL of either PBS or L-15 medium. Next, the cell viability was determined by trypan blue exclusion by mixing 10 µL of the cell suspension with 10 µL of trypan blue on a microscope slide, and observation under 400 x magnifications. Afterwards, tubes were centrifuged at 1,000 rpm for 10 min, and submitted to hypotonic treatment or directly to fixation.

(ii) Hypotonic treatment

An additional procedure applying hypotonic treatment of cells was tested. After centrifugation the medium was reduced to circa 100 µL in each tube, 500 µL of KCl 0.075 M or 0.11 M KCl was added, and incubation at 4°C during different times (10 to 45 min) was tested. Afterwards, centrifugation at 2,000 rpm for 5 min was done, and cells were submitted to fixation step.

(iii) Fixation

After centrifugation, part of the supernatant was removed until circa 500 µL was left in the tube. Cells were re-suspended and 500 µL of 4:1 methanol/acetic acid-mixture was added. After 10 min, during which gently mixing by tube inversion was done, tubes were centrifuged at 2,000 rpm for 5 min. The supernatant was removed, and each tube received 1 mL of 4:1 methanol/acetic acid-mixture. A last centrifugation step was applied, after which the supernatant was removed until circa 100 µL was left, which was used to re-suspend cells and transfer then to a microscope slide. Cells were then submitted to additional fixation on the slides using the 4:1 methanol/acetic acid-mixture.

(iv) Final protocol

Considering the alternative procedures mentioned above, the final protocol utilized PBS instead of L-15 medium; enzymatic treatment was done by 30 min incubation with 1.25 mg/mL dispase II and 0.5 mg/mL collagenase; no hypotonic treatment was applied; and fixation was done as described above.

8.2.9 Micronucleus test with primary cells of zebrafish larvae

Zebrafish embryos were exposed to NQO (0.55, 0.72 and 0.9 µM) or CPP (5.04, 7.48 and 10.0 mM) and respective water and solvent control conditions for 120 h following the FET test procedures. These exposure concentrations were equivalent to the FET EC_{10} for cumulative effects (lowest), LC_{25} (highest), plus an intermediate concentration. At the end of exposure period, larvae were euthanized with tricaine 1 g/L solution. The procedures to obtain cell suspension and fixate them on slides described above were performed. One slide was produced for each 10 larvae. Per slide, 1,000 cells were evaluated using a fluorescence microscope at a 1,000 x magnification (Nikon Eclipse 50i, Düsseldorf, Germany). Immediately before scoring, the slides were stained with 20 µl of 0.0004 % Acridine orange solution, covered with a cover glass, and received immersion oil. Criteria for micronuclei in ZF cells were the same as mentioned for the ZFL cells.

8.2.10 Data and statistical analysis

For ZFL cell viability, results are expressed as fold changes of measurements obtained from treated cells in comparison to cells exposed to DMSO only, after subtraction of average background signal from all conditions. Fold changes were then converted to cell viability percentage values, considering the solvent control to present 100%. Effect concentration values for the MTT and FET tests were obtained by four-parameter logistic non-linear

regression curve fit using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). MN occurrence for both ZFL cells and zebrafish larvae are expressed as the number of MN per 1,000 cells, as the percentage of cells containing micronuclei relative to the total number of counted cells, and as induction factors (IF) obtained by normalizing MN numbers in each condition to those in water (CPP in FET) or solvent (all other conditions) controls. Statistical analysis for the MN test was done with R package version 3.2.3 (R Development Core Team, 2015) using chi-square test to compare, in each experiment, the exposed conditions to respective control. Bonferroni correction was applied to the determined p-values. Differences between each exposure condition and respective control were considered as significant if at least 2 of 3 experimental replicates showed significant results. Afterwards, the data was analysed using GraphPad Prism 5 with 1-way ANOVA followed by Dunnett's test.

8.3 Results and Discussion

8.3.1 Viability of ZFL cells and selection of MN test exposure concentrations

Viability of ZFL cells after exposure to the test compounds indicated relatively high cytotoxicity of NQO and 3-NBA when compared to BaP and CPP (Fig. 8.1). Exposure to increasing NQO (Fig. 8.1A) and 3-NBA (Fig. 8.1C) concentrations led to cell viability reduction on a dose-dependent manner, reaching down to circa 40% at the respective highest concentrations. Instead, the highest test concentrations of BaP and CPP reduced cell viability only down to circa 80 % (Fig. 8.1B) and 90 % (Fig. 8.1D), respectively. Such relatively low cytotoxicity of BaP to the ZFL cells is in agreement with a previous study (Bopp and Lettieri 2008).

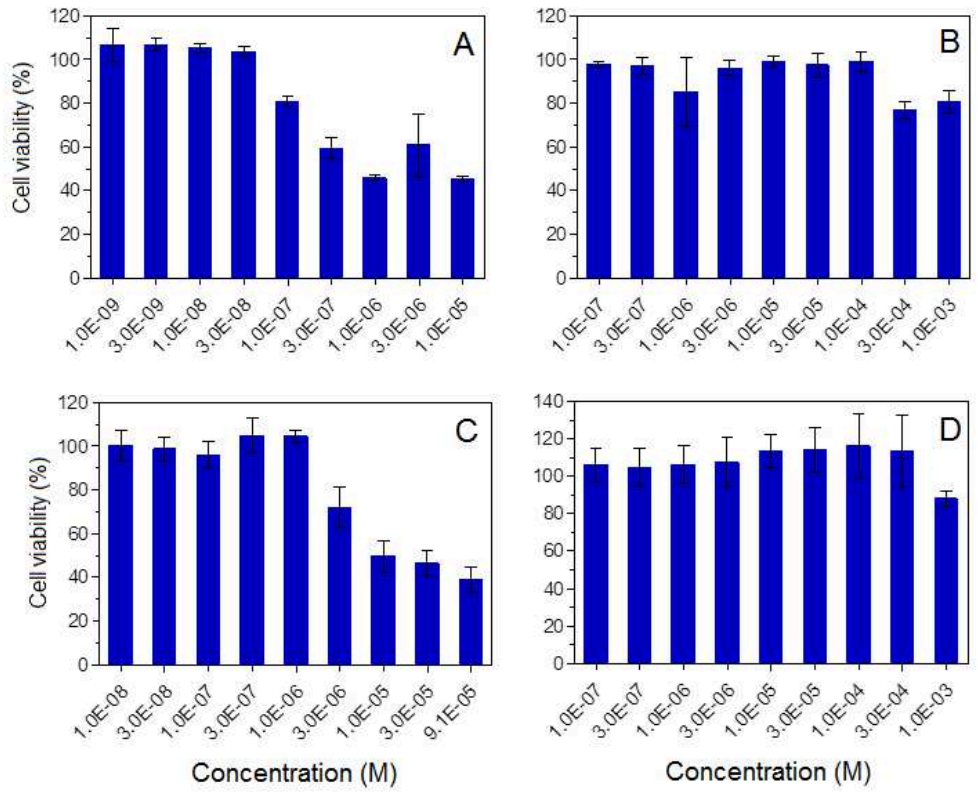


Figure 8.1: Cell viability in the MTT assay plotted versus the concentrations (M) of NQO (A), BaP (B), 3-NBA (C) and CPP (D). Average and standard deviation values for 3 experiments.

The MTT test results provided the basis for selection of exposure concentrations in the MN test. The initial approach aimed to select a concentration range producing from none up to circa 55 % of cytotoxicity (OECD 2010b). For CPP and BaP, at least two concentrations causing some cytotoxic effects, plus one that caused no viability reduction, were aimed. For BaP that corresponded to the highest three exposure concentrations in the MTT, while for CPP the two highest plus an intermediate one was selected. For NQO and 3-NBA, concentrations causing circa 55 % of cytotoxicity were selected as the highest exposure concentrations; the lowest concentrations were instead the highest concentration that caused no viability reduction in the MTT test; plus an intermediate concentration was included. However, these concentrations showed to be too cytotoxic in the MN test (as discussed below). Therefore cell viability IC_{20} values for both compounds (NQO: 0.14 µM, 3-NBA: 2.45 µM) was instead applied as the highest MN test concentrations, and three lower concentrations were included maintaining the same dilution factors. Finally, since cytotoxicity in MN could be related due to the different cell densities in the two tests (MTT: 25,000 cells/well, MN: 100,000 cells/well), the NQO and 3-NBA MTT-based exposure

concentrations were corrected for the different cell numbers between MTT and MN. The final exposure concentrations applied in the MN are presented in Table 8.2.

Table 8.2: MTT-based exposure concentrations (M) for the different test compounds selected for the ZFL MN test.

CPP (M)	**NQO** (M)	**BaP** (M)	**3-NBA** (M)
3.0×10^{-4}	1.9×10^{-9}	1.0×10^{-4}	3.2×10^{-8}
6.5×10^{-4}	6.2×10^{-9}	3.0×10^{-4}	1.1×10^{-7}
1.0×10^{-3}	1.9×10^{-8}	1.0×10^{-3}	3.2×10^{-7}
	6.2×10^{-8}		1.1 10^{-6}

A possible reason for the higher NQO and 3-NBA cytotoxicity in the MN test compared to the MTT test could be due to higher chemical adsorption to the surface of 96-well (MTT) plates material when compared to the 6-well (MN) plates. However since no chemical analysis was performed this hypothesis was not verified. The two compounds are recognized to cause cytotoxic effects to other cell types. As discussed in Chapter 7, the exposure of human osteoblastic osteosarcoma cells to the two compounds led to MTT cell viability IC_{20} values (NQO -/+S9: 0.25 / 5.1 µM, 3-NBA -/+S9: 76 / 73 µM) that were higher than the obtained for ZFL cells (NQO: 0.14 µM, 3-NBA: 2.45 µM). The present NQO IC_{20} for ZFL was also lower than the IC_{20} obtained for rainbow trout liver RTL-W1 cells with the neutral red retention assay (1 µM) (Brinkmann et al. 2014a), indicating higher sensitivity of the ZFL cell line.

8.3.2 Micronucleus test with zebrafish liver ZFL cell line

(i) ZFL cells doubling time

The number of cells increased along the incubation time, with each counted field (Fig. 8.2) presenting 19.2 ± 4.0, 33.8 ± 10.4 and 64.6 ± 18.2 cells at the time points 0, 24 h and 48 h. The calculated doubling time for the ZFL cells was of 27.1 h at 26°C. For the MN test, the incubation period after the exposure start should be of 1.5-2 times the doubling time (2010b), being set at 48 h for the present study. Although the MN was conducted at a slightly higher temperature (28 °C instead of 26 °C), this difference is expected only to cause minor increase in cell growth, if any.

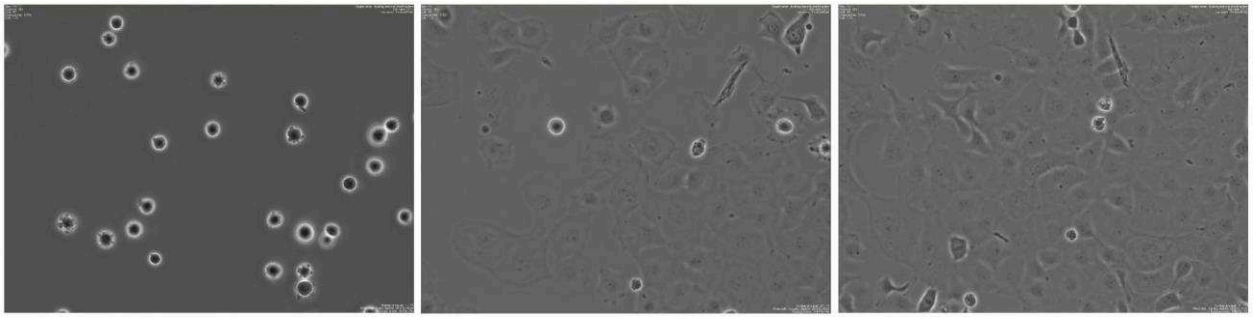


Figure 8.2: A same image field of a ZFL cell culture registered with the BioStation over a period of 72 h. Pictures taken at the time points zero (lefts), at 24 h (center) and at 48 h (right).

(ii) Micrunucleus observation and scoring

As previously described, the first range of MTT-based concentrations for NQO (3.0×10^{-8}, 1.0×10^{-7}, 1.0×10^{-6} M) and 3-NBA (1.0×10^{-6}, 3.0×10^{-6}, 3.0×10^{-6} M) produced very high cytotoxic effects in the MN test, with the cells not even remaining attached to the cover glasses. Instead, the final selected concentrations for the different chemicals (Table 8.2) produced satisfactory results, allowing scoring of MN formation (Fig. 8.3, Fig. 8.4). Additionally, mitosis occurrence was confirmed in all conditions (Table 8.3).

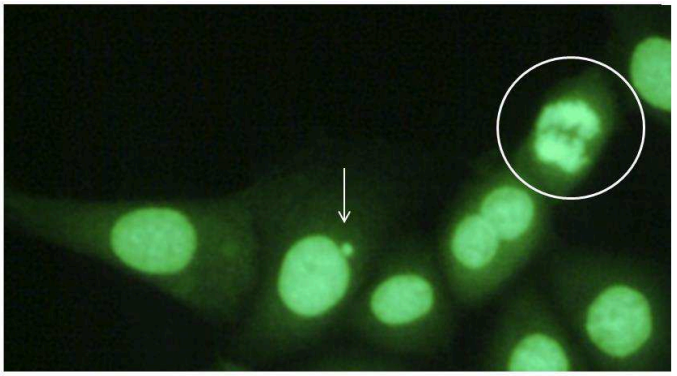


Figure 8.3: Micronucleus (arrow) and mitosis (circle) in the ZFL cells. Fluorescence microscope, 400 x magnification.

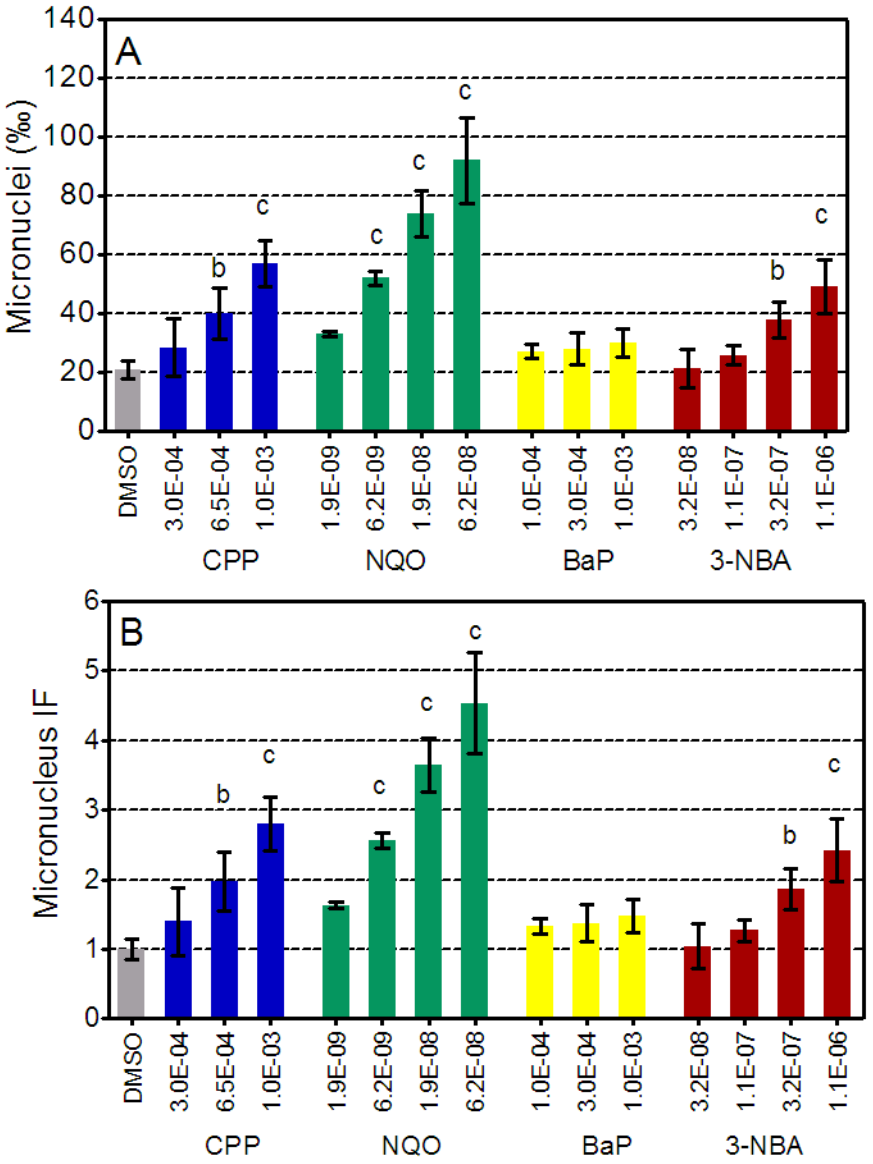


Figure 8.4: Micronuclei occurrence per 1,000 cells (‰, A) or as induction factors (IF, B) in ZFL cells exposed to the different test conditions. Averages and standard deviations. All bars marked with a/b were significantly different ($p<0.05$) from respective control in the chi-square test in all three experiments. One-way ANOVA followed by Dunnetts's test, b: $p<0.01$, c: $p<0.001$.

Table 8.3: Mitosis occurrence for the different test conditions. Average and standard deviation values in 2,000 cells.

	Concentration (M)	Average	Standard deviation
DMSO		22.8	4.6
CPP	3.0×10^{-4}	25.3	5.3
	6.5×10^{-4}	27.0	5.0
	1.0×10^{-3}	26.3	6.6
NQO	1.9×10^{-9}	29.3	1.2
	6.2×10^{-9}	26.0	1.6
	1.9×10^{-8}	36.7	8.2

	6.2×10^{-8}	17.7	11.7
BaP	1.0×10^{-4}	22.3	1.2
	3.0×10^{-4}	20.3	2.9
	1.0×10^{-3}	16.7	0.9
3-NBA	3.2×10^{-8}	22.3	2.5
	1.1×10^{-7}	20.0	3.6
	3.2×10^{-7}	21.3	3.7
	1.2 10^{-6}	13.7	8.2

DMSO solvent controls caused MN formation of 2.0 ± 0.3 %, in agreement with the OECD TG 487 (2010b) requirement of a frequency between 0.5 % and 2.5 % for control conditions in human peripheral blood lymphocytes. MN formation presented dose-response trend for CPP, NQO and 3-NBA, with MN occurrence being significantly higher ($p<0.01$ or 0.001) than in solvent controls in the highest three NQO concentrations and in the two highest concentrations of CPP and 3-NBA (Fig. 8.4). Among the tested compounds, NQO presented the highest induction of micronuclei, reaching 9.2 % at the highest concentration. Contrary to the other substances, BaP caused no significant increase of the MN formation, with MN formation for each concentration ranging between 2.7 and 3.0%. However, during experiments precipitate formation was observed in the exposure medium, which is considered to be precipitation of the test compound itself. Therefore for BaP it is considered that the chemical was not available to the cells as planned.

NQO, BaP and CPP are all recommended as reference substances for the selection of positive control conditions, with NQO being a clastogen that is active without metabolic activation; and CPP and BaP being clastogens requiring metabolic activation (OECD 2010b). In fact, CPP and NQO are already used as positive controls in the MN test with V79 and RTL-W1 cells, respectively (ISO 2004, OECD 2010b, Brinkmann et al. 2014a). NQO acts both as a direct- and an indirect-acting genotoxic compound, being shown to cause MN formation in different cell types, such as human lymphoblastoid cell line, human primary hepatocytes, and rainbow trout liver cells (Brinkmann et al. 2014a, Hégarat et al. 2014, Brüsehafer et al. 2016). In this study study, NQO was the compound inducing higher MN formation, which supports its use as a positive control chemical in the ZFL MN test.

Comparatively lower MN induction values were caused by CPP and 3-NBA. That can be related to the fact that these compounds require metabolic activation in order to cause any (CPP) or increased (3-NBA) genotoxic effects. Although 3-NBA can also directly cause MN formation, its metabolites were shown to in general induce higher numbers that the parent

compound (Arlt et al. 2004). For CPP metabolic activation is required for genotoxic activity, which is usually performed by incubation of cells with rodent S9 liver fraction. The metabolic capacity of the ZFL cell line is considered to be limited, since although cyp1a-like proteins were induced by exposure to TCDD 2,3,7,8,-tetrachlorodibenzodioxin (Ghosh et al. 1994), general low potential for transcriptional induction was reported when compared to primary zebrafish hepatocytes (Eide et al. 2014). Therefore it is considered that the relatively low metabolic capacity of the ZFL cells interfered with MN formation after exposure to CPP and 3-NBA, which would also explain the lower sensitivity to 3-NBA of ZFL when compared to the human hepatocellular carcinoma HepG2 cells (Lamy et al. 2004). Another aspect to consider is that both compounds but particularly CPP can modulate the cell cycle and induce p53 (see Chapter 7). It is in fact recommended by the MN test guidelines that if a test chemical can affect the cell cycling time the sampling times can be extended up to total 3-4 cell cycles (OECD 2010b). Therefore in future investigations of such and similar acting chemicals the test can be ended after 96 h of exposure.

8.3.3 Fish Embryo Acute Toxicity test

Similar to the experiments with ZFL cells, BaP exposure solutions showed chemical precipitate formation. As a result, no evident toxic effects were observed. 3-NBA caused effects already after 48 h exposure in embryos of *Danio rerio*, with most of the affected individuals presenting reduced or no pigmentation compared to control condition. At 120 h exposure, additional sublethal effects were observed, such as: underdevelopment, reduced heartbeat, reduced or no blood circulation, oedema (mainly of yolk and heart but also of tail), and in column malformations. However, high variability occurred between experiments, resulting in unsatisfactory non-linear regressions and unreliable LC/EC values. Based on that, BaP and 3-NBA were excluded from the MN test with primary zebrafish larval cells.

CPP and NQO presented consistent results between experimental replicates, with results allowing the calculation of effect-concentration values that served as basis for the selection of respective exposure concentrations for the zebrafish larval MN test. After 120 h of exposure to CPP, sublethal effects were evident, particularly in individuals exposed to the highest concentration (10 mM) that presented oedemas (70 %), reduced heartbeat (57 %), reduced (10 %) or no blood circulation (60 %), no pigmentation (23 %), column malformation (17 %), and lack of hatching (23 %). Lethality reached up to circa 20 % also at 10 mM CPP. The obtained EC_{10} and LC_{25} were 5.04 and 11.1 mM, respectively (Table 8.4). NQO exposure also caused evident effects at 120 h, with occurrence of reduced (20 %) or no heartbeat (15 %), no

blood circulation (20 %), and edemas (55 %) already at 0.93 µM. At the highest concentration (5.26 µM) there was mortality of 93%. NQO EC_{10} and LC_{25} were 0.55 and 0.9 µM, respectively (Table 8.4). In the present study, CPP was directly dissolved in water medium. For the highest test concentration of 10 mM that was achieved with the use of heat (warm water bath) and agitation for 1 h. Hence, 10 mM was used as the highest exposure concentration for the MN test with zebrafish larvae instead of the calculated LC_{25}.

Table 8.4: Selected exposure concentrations for the zebrafish larval MN with basis on the effect-concentration values EC_{10} and LC_{25} (plus respective 95% confidential intervals, CI) obtained for CPP (mM) and NQO (µM) in 3 experiments. An additional concentration intermediate between the EC_{10} and LC_{25} was selected.

	EC_{10}	**95 % CI**	**LC_{25}**	**95 % CI**	**Intermediate concentration**
CPP (mM)	5.04	4.4 - 5.6	11.1 (10) [a]	9.6 - 12.9	7.48
NQO (µM)	0.55	0.4 - 0.7	0.9	0.8 - 1.0	0.72

a: 10 mM CPP was applied as highest test concentration.

The present CPP effect-concentration values are slightly lower than previous literature values. Zebrafish embryo 72 h EC_{50} of 4.7 mM and LC_{50} of 8.4 mM were determined for continuous exposure (Weigt et al. 2011). Another study demonstrated that, with the addition of metabolic activation, a short 60 min exposure to CPP 3.5-28 mM caused teratogenic effects after 24-48 h (Busquet et al. 2008). However, these studies applied Tris-buffer to increase the embryo permeability to chemicals, which was either contained in medium (Weigt et al. 2011) or as a pre-treatment (Busquet et al. 2008).

8.3.4 Micronucleus test with primary zebrafish larval cells

(i) Optimization of the cell isolation

Different procedures were tested for the establishment of a protocol to obtain a high yield of undamaged primary cells from zebrafish larvae on a microscope slide. Primary cell isolation was adapted from procedures described in previous studies for investigations in 48 or 72 h post hatching medaka larvae (Morin et al. 2011, Le Bihanic et al. 2014). PBS and L-15 were tested as medium for the cell isolation. It was observed that with the use of PBS the cells maintained their tonicity along the procedures and presented well defined shape and cellular membranes under microscopic observation. Instead, with the use of L-15 cell shrinkage was observed at the end of the process, which would interfere with later MN scoring. The ideal osmolar condition for the culture zebrafish embryonic cells was reported to

be around 315 mOsm (Perez-Camps and Garcia-Ximenez 2008). L-15 medium has an osmolarity of 300-340 mOsm and PBS of 270-300 mOsm (Thermo Fisher Scientific , HiMedia Laboratories 2011). It is considered that L-15 acted as a low hypertonic solution, while PBS as low hypotonic solution; the latter can improve MN scoring, since with slightly increased intercellular fluids the cell shape is maintained at the end of the fixation procedure, which is required for MN scoring (Perez-Camps and Garcia-Ximenez 2008). In consequence, the additional hypotonic treatment was considered unnecessary, since KCl incubation step provided no improvement independently of used concentrations.

The enzymatic treatment performed in this study utilized dispase II and collagenase type IV according to the Tissue Dissociation Enzymes Product Selection Guide (Roche 2013), which describes dispase II as suitable to obtain cell suspensions embryos body and tissues. Collagenase type IV was added since insufficient digestion was achieved by dispase II only, plus it minimizes damages to membrane proteins and receptors (HiMedia Laboratories 2012). No reduction in cell viability was observed after enzymatic treatment. Collagenase type H has been described to provide adequate digestion of zebrafish embryos but also undesired DNA fragmentation, however the used concentrations (20 mg/mL) (Kosmehl et al. 2006) was much higher than in the present study (0.5 mg/mL). However in the present study there were remains of undigested tissues on some slides, which interfered with MN observation. That could be improved by incorporation of a filtration step as described by Kosmehl et al. (2006).

(ii) Micronucleus assay with primary zebrafish larval cells

For the primary zebrafish larval cells, MN visualization was not easily achieved. Larval cells showed to be rather small, requiring 1,000 x magnification and use of oil immersion microscope objectives for their observation. The fluorescent staining was not as intense as for the ZFL cells. Consequently, cytoplasm visualization was not always possible, limiting the cells that could be evaluated for MN formation. Still, it was possible to perform MN observation (Fig. 8.5) and scoring for the CPP and NQO exposures (Fig. 8.6). MN occurrence in water and solvent controls were between 2-4 and 3-5 per 1,000 cells, respectively. Larvae exposed to NQO or CPP presented higher MN occurrence than respective controls, however no significant differences occurred.

A protocol for assessment of MN in primary zebrafish larvae cells has been developed by Böttcher (2012) using 7 days old exposed zebrafish and mechanical dissociation to obtain cell suspension (Kosmehl et al. 2006). However this study investigated relatively high concentrations (0.25 – 4 µM), and the LOEC was nevertheless reported to be 4 µM. In the

present study, concentrations at comparable range caused high lethality in the FET tests, which should not be included in concentration range according to *in vivo* MN test guidelines (OECD 2014).

Although the MN assay with primary cells of larvae presented low MN induction in the present study, the relevance of the experimental model for aquatic toxicology justifies that further studies are conducted to if possible increase the method sensitivity. The present results demonstrated that it is possible to obtain enough cells for MN scoring utilizing 5 days post fertilization (dpf) zebrafish larvae. The method can provide an organism-level assay that does not require adult fish and neither animal test authorization.

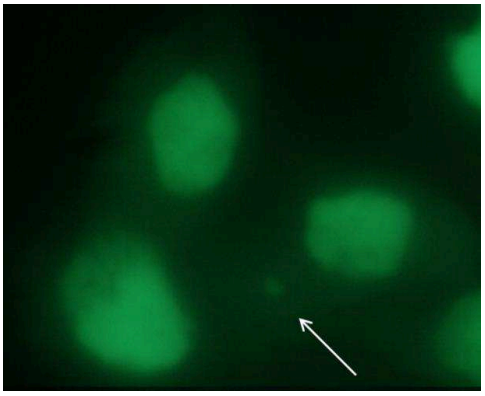


Figure 8.5: Micronucleus (arrow) in primary zebrafish larval cells. Fluorescence microscope, 1,000 x magnification.

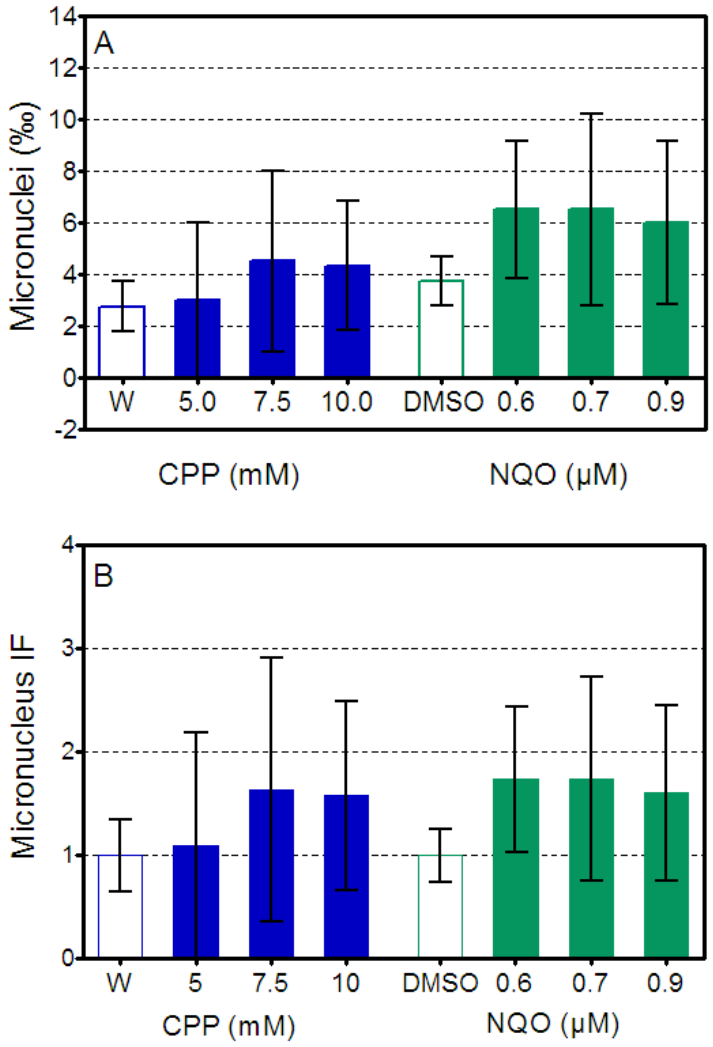


Figure 8.6: Micronuclei occurrence per 1,000 cells (‰, A) or as induction factors (IF, B) in primary cell suspensions from zebrafish larvae exposed to the different test conditions (CPP: 2-4 experiments, NQO: 4 experiments). Averages and standard deviations. No significant differences occurred.

8.4 Conclusions

In the present study, new *in vitro* (ZLF cell line) and organism-level (larvae) protocols for MN tests using zebrafish models were developed. Further, a testing strategy integrating acute toxicity assays was applied: the MTT test provided the basis for the selection of test concentrations for the ZFL MN, while the FET test provided input for the larval MN. The MN test with the ZFL cell was successfully performed and identified increase of MN formation in cells exposed to the candidate positive control chemicals NQO and CPP and the environmental contaminant 3-NBA. NQO was the compound eliciting higher MN formation, being therefore recommended as a direct-acting positive control chemical. MN formation after CPP and 3-NBA was relatively lower, indicating limited but present metabolic capacity

of the ZFL cell line. Still, both compounds induced significantly MN formation and could be considered as indirect acting positive control chemicals, particularly CPP. Further, in this way the method was shown to be able to evaluate also chemicals and samples that require metabolic transformation for genotoxic activity. The MN assay with primary cells of 5 dpf zebrafish larvae was also established, however the observation and scoring of MN was not as easily achieved as for the ZFL cells. Also, the cytoplasm of cells was often not fully visible, which restricted MN scoring. Exposure to NQO and CPP led to tendency for increased MN formation, however there was high variation between experimental replicates and differences were not significant. It is recommended that additional investigations are performed to further improve the present protocol, since this assay can provide an organism-level approach to investigate the genotoxic potential of chemicals and samples to fish.

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Chapter 9: Zebrafish larvae behavioural responses following exposure to neuroactive and neurotoxic compounds

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Zebrafish larvae behavioural responses following exposure to neuroactive and neurotoxic compounds - part A: Evaluation of candidate positive control conditions and chemicals.

Abstract

The neurotoxic and neuroactive activities of aquatic contaminants present a serious threat to vertebrates and invertebrates inhabiting aquatic environments, ultimately leading to behavioural changes that can have ecologically-relevant effects. Therefore bioassays that investigate effects of neurotoxic and neuroactive compounds in aquatic organisms are needed. The investigation of behavioural effects in fish can benefit of the fact that zebrafish, a well-studied experimental model, is rapidly becoming a main model organism in neurosciences, neurotoxicology and behavioural sciences. Further, zebrafish early life stages are increasingly being applied in behavioural methods, such as in the light-dark transition test. Although many studies have applied this test to investigate effects of diverse chemicals, no standardized procedure or positive control conditions are available yet. In this study, we applied 5 and 6 days post fertilization (dpf) zebrafish larvae to investigate extrinsic factors that can interfere with behavioral responses; followed by the investigation of positive control conditions applying ethanol, caffeine and nicotine; and by the evaluation of the pesticides alpha-cypermethrin, chlorpyrifos and its metabolite chlorpyrifos-oxon regarding behavioral effects. In addition, most of the experiments were replicated in two laboratories, each one having a distinct zebrafish strain. The behavioural test protocol for light-dark transition test assessment applied in this study was able to identify effects of neuroactive and neurotoxic chemicals on the behaviour of zebrafish 5-6 dpf larvae. Results produced in the two laboratories were in general consistent, and although some differences occurred these were more likely related to different fish strain use. The new parameter peak:basal ratio, together with distance moved and burst activity, supported the interpretation of responses to the different chemicals. The 5 dpf larvae showed to be a suitable model presenting sensitivity and consistent behavioral responses. Summed to the fact that these are not protected stages, behavioural assessment should, always that possible, be performed with zebrafish up to 5 dpf of age. Among tested candidate positive control chemicals, ethanol 1-3% produced the more robust responses, eliciting either stimulation or inhibition of responses depending on concentration; and presenting consistent responses even after 40 min of exposure. Caffeine and nicotine instead presented very brief stimulatory effects, followed by inhibitory effects only. The tested insecticides presented less evident patterns of behavioral responses, which can be related to inadequate exposure scenarios. It is recommended that future studies investigate the temporal profiled of alpha-cypermethrin and chlorpyrifos effects on early life stage behaviour.

Keywords: behavior, zebrafish, larva, locomotion, swimming, ethanol, insecticides.

9.1 Introduction

The neurotoxic and neuroactive activities of aquatic contaminants present a serious threat to vertebrates and invertebrates inhabiting aquatic environments. However, the assessment of the neurotoxicity of chemicals is limited to few compounds and target organisms. OECD guidelines for neurotoxicity assessment in vertebrates focus on mammals (OECD 1997, US-EPA 1998, OECD 2007) and birds (OECD 1995a, b); and so far there is no European regulatory framework for environmental neurotoxicity assessment. Even the human risk assessment of neurotoxicity requires improvement: by 2009 only 90 chemicals had been tested for developmental neurotoxicity (Makris et al. 2009, Crofton et al. 2012). In consequence, known neuroactive and neurotoxic compounds, such as pesticides, pharmaceuticals, and heavy metals, occur in the environment together with thousands of chemicals of unknown neurotoxic potential.

There is therefore the need to develop methods to investigate the effects of neurotoxic and neuroactive compounds on aquatic organisms. Behavioral changes are particularly interesting endpoints to evaluate effects of such compounds since they represent early organism-level responses to exogenous conditions that can be investigated by non-invasive methods, and that potentially can indicate impairment of ecologically relevant processes like foraging, escaping and competition (Fent 2007, MacPhail et al. 2009, Vignet et al. 2013, Raley-Susman 2014). Also on the regulatory level, fish behavioral endpoints are proposed as being highly sensitive and ecologically-relevant , with their assessment already being recommend in test guidelines (ASTM 2012, OECD 2012a). This demand can benefit of the fact that the zebrafish *Danio rerio* is rapidly becoming a main model organism in neurosciences, neurotoxicology and behavioral sciences (Ton et al. 2006, Levin and Tanguay 2011, Kalueff et al. 2013, Kalueff et al. 2014).

Initially, behavioral studies that utilized zebrafish as model organisms applied mostly adults (Tierney 2011). More recently, zebrafish early life stages such as embryos and larvae are being recognized to produce many of the responses observed in adults, leading to increasing number of studies utilizing these life stages (Brustein et al. 2003, Kokel et al. 2010, Ahmad et al. 2012, Ali et al. 2012). At 5 days post fertilization (dpf), there is inflation of the swim-bladder and development of the sensorial and motor systems (Goolish and Okutake 1999, Levin et al. 2006, Ahmad et al. 2012), with these early larvae being capable of performing slow and high swimming activities (Budick 2000). A behavioral test that has been

adapted from rodents to zebrafish adults and larvae is the light-dark transition test, which is related to the dark/light preference by the animal and can be used to investigate anxiety modulation but also escape response (Maximino et al. 2010, Colwill and Creton 2011). This test is based on the tendency for zebrafish larvae to present low locomotion in light, which gradually increases to a stable level; and to present a sudden increase in locomotion following transition from light to dark, since the larvae present escape response and will try to avoid dark areas (scotophobia) (MacPhail et al. 2009, Irons et al. 2010, Colwill and Creton 2011, Padilla et al. 2011, Ali et al. 2012, Ellis et al. 2012, Kalueff et al. 2013). Although many studies have applied such method to investigate effects of diverse chemicals, no standardized procedure or positive control conditions are available yet.

In this study, we have first investigated extrinsic factors that can interfere with behavioral responses, such as raising density, solvent presence, age of larvae and acclimation in light or darkness. Afterwards, we investigated ethanol, caffeine and nicotine in different concentrations and exposure scenarios, regarding their use in positive control conditions. After short or prolonged exposure to each compound, zebrafish larvae aged 5 or 6 dpf were submitted to the light-dark transition test. Further, the pesticides α-cypermethrin (aCM), chlorpyrifos (CPO) and its metabolite chlorpyrifos-oxon were also evaluated regarding behavioral effects. The selection of exposure concentrations was based on the literature and preliminary tests for the candidate positive control chemicals; and on experimental evaluation of teratogenic effects through the fish embryo toxicity tests for the pesticides. In addition, most of the experiments were replicated at two laboratories, each one having a distinct zebrafish strain. Results are discussed in comparison to the literature, regarding the usefulness to set a positive control condition, and in terms of applicability of the assay to evaluate behavioral effects of environmental contaminants.

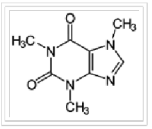
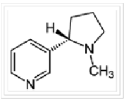
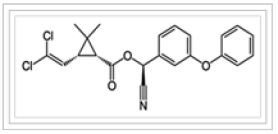
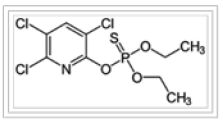
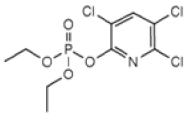
9.2 Materials and Methods

9.2.1 Chemicals

The test chemicals (Table 9.1) α-cypermethrin (aCM, CAS number 67375-30-8), caffeine (CAS number 58-08-2), chlorpyrifos (CHP, CAS number 2921-88-2), chlorpyrifos-oxon (CHPO, CAS number 5598-15-2), ethanol (CAS number 64-17-5) and nicotine (CAS number 22083-74-5), as well as dimethlysulfoxide (DMSO), were purchased from Sigma Aldrich (Sigma Aldrich Chemie GmbH, Steinheim, Germany). Chlorpyrifos-oxon (CAS number 5598-15-2) was purchased from AccuStandard (New Haven, CT; USA). Stock solutions were

prepared by dissolving the compounds in DMSO and were stored at -20°C, except for caffeine and ethanol which were freshly dissolved in test medium before every test.

Table 9.1: Physical properties of the test compounds.

	CAS number	Chemical structure	Molecular weight
Ethanol	64-17-5		46.1
Caffeine	58-08-2		194.2
Nicotine	22083-74-5		162.2
α-cypermethrin (aCM)	67375-30-8		416.3
Chlorpyrifos (CHP)	2921-88-2		350.6
Chlorpyrifos-oxon (CHPO)	5598-15-2		334.5

9.2.2 Zebrafish maintenance and embryo collection

Zebrafish adults were maintained following standard maintenance procedures, i.e. at room and water temperature of 26°C ±1 and light-dark cycle of 14:10 hours. Different zebrafish strains were used in each institute. RWTH used a wildtype zebrafish stock maintained for more than 20 years at the Fraunhofer IME Institute, with original stocks obtained in 1992 from the West Aquarium GmbH (Bad Lauterberg, Germany). The IVM fish were a mix between AB (ZFIN ID: ZDB-GENO-960809-7) and petshop wildtype zebrafish. Eggs were obtained from community mating, and fertilization rate was at least 70 % for all experiments.

9.2.3 Exposure of embryos and larvae

(i) General raising protocol

Fertilized embryos were transferred to pre-aerated ISO water medium (ISO 2007a) in crystallization dishes (circa 50 embryos per dish in 10 mL of medium, i.e. 200µL/fish) and maintained at 26±1°C in a 14:10 light:dark regime. Dead fish were removed daily. All

experiments presented survival of fish in water and solvent control conditions of at least 90 %. At the end of experiments, fish were euthanized by prolonged immersion in a solution of benzocaine 40 g/L and freezing at -20 °C.

(ii) Preliminary tests for extrinsic factors

Preliminary tests were performed to characterize the effects of extrinsic factors on behavioral responses evaluated in the newly established behavioral system at RWTH, to ensure consistency and reproducibility of results (Padilla et al. 2011). The general raising protocol was followed, except with the adaptions regarding investigated factors, which were (Table 9.2): (1) age of larvae at the testing day, i.e. 5 or 6 dpf; (2) influence of solvent, i.e. addition of DMSO to medium water; (3) density of larvae during raising, i.e. group or single raising; and (4) acclimation in dark before behavior analysis. Afterwards, the larvae were submitted to behavior analysis. Additionally, the comparability of the EthoVision and ViewPoint software regarding video analysis outcomes was evaluated by analyzing a video recorded with the ViewPoint system utilizing both softwares.

Table 9.2: Investigated extrinsic factors and respective exposure setups in preliminary tests.

	DMSO	**Density**	**Acclimation before measurement**	**Age during measurement**
General protocol	*No solvent*	*Group raising: 50 larvae in 10 mL medium (200µL/fish) in a crystallization dish*	*In light*	*5 dpf*
Investigated factors				
1) Age				**6 dpf**
2) Solvent	**0.1 %**			
3) Density		**Isolated raising: in 96-well plates, with one embryo per well in 200 µL medium**		
4) Acclimation in dark			**30 min in darkness**	

(iii) Time course measurement of candidate positive control chemicals

Short exposures (10 - 90 min) to caffeine (0.4, 0.8 and 1.1 mM), ethanol (1, 2 and 3 %) and nicotine (50, 75 and 100 µM, with final 0.1 % DMSO) were evaluated as positive control conditions using 5 dpf larvae. At the morning of 5 dpf, circa 5 hours before the exposure start,

normally developed larvae were randomly transferred to 96-well plates, with one larva per well in 200 µL medium, and maintained under normal light conditions. For the exposure, half of the medium volume (100 µL/well) was exchanged for 100 µL of medium containing the test compounds. For the negative controls, medium only (caffeine and ethanol) or with DMSO 0.1 % (nicotine) were used. After exposure periods of 10, 40 or 90 minutes, the larvae were submitted to behavior analysis. In addition, prolonged exposure to ethanol until 5 or 6 dpf was also evaluated, using however lower exposure concentrations in order to minimize acute toxicity occurrence. For that, embryos up to 2 hours post fertilization (hpf) were exposed under static conditions to ethanol 0.25, 0.5 and 1 %. Larvae were raised following the general protocol.

(iv) Teratogenic effects of chlorpyrifos and α-cypermethrin

Teratogenicity assessment followed adapted fish embryo toxicity test procedures (ISO 2007a, OECD 2013b) with exposure until 5 or 6 dpf and observation of lethal and sublethal morphological effects (Nagel 2002, Lammer et al. 2009, Selderslaghs et al. 2009). Embryos up to 2 hpf were exposed under static conditions to chlorpyrifos (CHP, 71.3, 45.6, 28.5, 17.8, 11.4, 7.1, 4.6 and 2.9 µM) or α-cypermethrin (aCM, 600, 240, 96, 38.4, 15.1, 6, 2.4 and 0.96 nM) in glass crystallization dishes, with all conditions containing final DMSO 0.1 %. Testing vessels were pre-soaked with respective exposure solutions for a minimum of 16 h before the exposure start. Positive (3,4-Dichloranilin 3.7 mg/L), solvent (0.1 % DMSO) and water controls were performed. Dead fish were scored and removed daily. At the end of the exposure period, the larvae were examined using an inverted microscope, and lethal and sublethal effect occurrences (%) were registered. Effect-concentrations for sublethal effects were obtained, considering the data only for the exposure conditions that did not present mortality. In each experiment, there was exposure of 30 (pesticide concentrations and positive control) or 60 (water and solvent controls) embryos per condition divided in three vessels, which contained respectively 10 embryos in 2mL or 20 embryos in 4 mL of exposure solutions (200 µL/fish). For each insecticide, experiments were performed three times at RWTH, and one time at IVM.

(iv) Exposure tests to assess behavioral effects of insecticides

Zebrafish embryos were exposed until 5 or 6 dpf following the same procedure as for the teratogenicity assessment. Exposure concentrations were based on effect-concentration values for sublethal effect occurrence in the teratogenicity assays. For CHP and aCM, these corresponded to the EC_{10}, EC_{25} and EC_{50} values obtained at RWTH (which did not present

significant difference from respective IVM values). For CHPO, exposure concentrations corresponded to NOEC and LOEC previously obtained at IVM (personal communication by Jessica Legradi). Briefly, embryos were submitted to static exposure to CHP (11.1, 7.4 and 5.1 µM), CHPO (0.1 and 0.5 µM) and aCM (15.4, 6.7 and 3.1 nM) in pre-soaked glass crystallization dishes, with all conditions containing final DMSO 0.1 %. Water and solvent controls were performed. Exposure to ethanol (1.0, 0.5 and 0.25 %) was run in parallel as a candidate positive control for behavioral effects. In each experiment, three replicate vessels per condition were performed, containing 40 (pesticides and positive control) or 25 (water and solvent controls) embryos per vessel in 8 or 5 mL of exposure solution, respectively (200 µL/fish). Dead fish were scored and removed daily. In addition, three mixtures of CHP and aCM were evaluated at RWTH, consisting of: (i) a-CM EC_{10} plus CHP EC_{10}; (ii) a-CM EC_{10} plus CHP EC_{25}; and (iii) a-CM EC_{25} plus CHP EC_{25}.

At 5dpf, the larvae were examined using an inverted microscope, and morphologically non-affected larvae were transferred to 96-well plates, one fish per well in 200 µL test solution per fish. The larvae which were measured for behavior at 5 dpf larvae were transferred to 96-well plates circa 5 hours before the behavioral assessment. The larvae which were measured for behavior at 6 dpf were transferred to 96-well plates at 5 dpf afternoon, i.e. the day previous to the behavioral evaluation. Each 96-well plate contained 20 larvae per insecticide treatment, 10 for the water control, and 10 for the solvent control condition. For each insecticide, experiments were performed three times at RWTH and three times at IVM.

9.2.4 Behavior analysis systems

(i) RWTH

The video recording system of RWTH was built as part of this study. The system consists of a wooden cabinet covered internally with a thin layer of isolating foam, in which an infrared (IR) sensitive USB 2.0 monochrome industrial camera (DMK 31AU03, Imaging Source Europe GmbH, Bremen, Germany) with a varifocal IR sensitive lens (T5Z8513CS-IR, Computar, CBC America Corp., New York, USA) was installed at the top. An IR spotlight lamp with 96 LEDs, covered by a milky-white acrylic plate for light diffusion, was placed at the basis of the system. Visible light was provided by a dimmable 12 W light bulb (Philips, Hamburg, Germany), with the intensity being set at 230 lux using the smart phone application LuxMeterPro (AM PowerSoft). For the recording of videos, a multiwell plate was placed above the infrared light source. The camera was attached to a computer and videos were recorded using the software IC Capture (Imaging Source Europe GmbH, Bremen, Germany)

using frame width/height of 1024/768 and frame rate of 30 frames per second. The videos were saved as Audio Video Interleave (.avi) files using mpeg4 compression. The system was maintained in a temperature controlled room at 26 ±1°C. Quantitative analysis of locomotor behavior in zebrafish larvae was performed with the EthoVision software XT 8 or 10 (Noldus Information Technology, Leesburg, VA, USA). Previously recorded videos were analyzed using 30 frames per second utilizing multiwell arena setup. The scale of the video was calibrated by the diameter of wells, set to 8 mm. The detection settings were adjusted according to subject size (10-110 pixels) and to contrast between subjects (darker) and the background, with contrast in the range of 15-120 pixels. Background subtraction was done at the beginning of analysis versus a reference image. Further, dynamic background subtraction method that accounts for changing light settings during each video recording was used. No minimum distance moved filter was set. The data for single individuals were exported as excel files for the time bins of 1 and 4 minutes.

(ii) IVM

At the IVM the commercially available system ZebraBox (Viewpoint Life Sciences Inc., Montreal, Quebec, Canada) was utilized. The recording cabinet consists of a plastic box holding a CCD camera, which is connected to a video track system that performs live analyses. The plate is placed on top of an infrared light source, while visible light comes from a light source from above. The intensity of visible light was 222 lux, measured using the smart phone application LuxMeterPro (AM PowerSoft). Constant temperature was maintained with a flow-through water bath that kept the water in wells at 26°C. Settings for analysis were adjusted using the ViewPoint Application Manager. The location count was set to multiwell arena setup. The frame rate for image capturing was set to 25 images per second. Calibration of the scale unit was done by drawing a line of 7 cm over the plate using a ruler, with the scale being set to its corresponding length. Larvae were also detected by contrast (darker) with the background, with threshold in the grey level scale set to 104. Background subtraction method was used versus a reference image. A detection sensitivity threshold of 20 was used to avoid artificial effects. At the end of experiments, data were stored as avi files, result files (.vtr and .xls), and raw data files (.raw).

9.2.5 Assessment of larval response to the light-dark transition test

The larval response to sudden light-dark transition was applied to evaluate behavioral responses of normally developed larvae only, with fish presenting any malformations being excluded. This well characterized behavioral response is related to the fact that zebrafish

larvae maintained in light show relatively low locomotion; however, when a stimulus of sudden darkness is given, locomotion immediately increases until reaching a peak, followed by gradual decrease along time (Bilotta et al. 2002, Irons et al. 2010, Padilla et al. 2011, Ali et al. 2012, Ellis et al. 2012). Considering previous studies and own preliminary tests, the selected behavioral assessment followed a 2 min acclimation period in the test system, and periods of 4 min in light or darkness for the sudden transitions (Bilotta et al. 2002, Ali et al. 2012). All experiments were performed in the afternoon between 13:00 and 16:00, since that is reported as the time of the day when zebrafish larvae present the most stable activity (MacPhail et al. 2009, Vignet et al. 2013). Evaluated parameters for each individual larva included total distance moved per light or dark period; burst activity; and peak:basal ratio.

(i) Distance moved during light or dark periods

The total distance moved by individual larvae per minute and in each of the 4 min periods of light or dark was quantified.

(ii) Burst activity

To calculate the burst activity, fish locomotion above 2 cm/sec was classified as high activity (Winter et al. 2008, Ellis et al. 2012) through setting a threshold in software analysis. The amount of time that each larva spends in high activity was then converted to a percentage of the respective light or dark cycle.

(iii) Peak:basal ratio

The ratio between the peak activity in dark and the basal activity in light was proposed as a new parameter to evaluate alterations of the light-dark transition response. For that, a ratio was calculated between the distance moved during the first minute in dark (considered as peak activity) and the average distance moved per minute during the previous period in light (considered as basal activity).

9.2.6 Data and statistical analysis

Effect-concentration values EC_{10}, EC_{25} and EC_{50} for sublethal effect occurrence (%) were obtained by probit analysis using a maximum likelihood regression with ToxRat (ToxRat Solutions GmbH, Alsdorf, Germany) for experiments conducted at RWTH and at IVM separately. For the behavioral parameters, statistical analysis was done with GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Average and standard deviation values were calculated from individual fish data from pooled experiments. For distance moved and burst activity, responses obtained in first or second period of light or darkness were analyzed

separately, and compared to respective water or solvent control response in the same period. For peak:basal ratio, values obtained for the first or second light-dark transition were compared to responses in respective controls. In addition, for peak:basal ratio the data was initially corrected for outliers using the cutoff value of twice the respective standard deviation, i.e. excluding values higher or lower than respective average +/- 2 times the standard deviation. Data for distance moved was first analyzed for normal distribution with a Shapiro-Wilks test, and then for homoscedasticity with a Bartlett's test. When both conditions were met, one-way ANOVA followed by Dunnetts's test was applied; when not, Kruskal-Wallis test followed by Dunn's post hoc test was applied. For burst activity and peak:basal ratio, no normal distribution was assumed, with Kruskal-Wallis test followed by Dunn's multiple comparison being performed. For the evaluation of extrinsic factors the two conditions being compared were evaluated using a t-test.

9.3 Results and Discussion

9.3.1 Influence of extrinsic factors

Results for the preliminary tests performed at RWTH to evaluate interference of extrinsic factors on the parameters distance moved, burst activity and ratio peak:basal are presented in Table 9.3. No evident effects were observed when comparing responses from larvae raised in groups or isolated. Regarding effects of solvent, larvae exposed to DMSO 0.1 % presented only higher ratio peak:basal ($p \leq 0.01$) for the first cycle than the larvae in medium only. Larval age showed to be a clear factor of interference, with 6 dpf larvae presenting higher distance moved values in light periods ($p \leq 0.001$ or 0.01) when compared to respective 5 dpf fish response. As a result, the peak:basal ratio was lower in 6 dpf than in 5 dpf larvae ($p \leq 0.01$). The acclimation in light or darkness prior to testing also affected the evaluated parameters. Dark-adapted larvae presented lower values of distance moved ($p \leq 0.01$ or 0.001) and peak:basal ratio ($p<0.001$), and lower burst occurrence ($p<0.001$) when compared to larvae kept in light.

Although the present results did not identify consistent changes regarding raising density and exposure to DMSO, recent studies have shown them to be important factors. Zellner et al. (2011)) reported a higher level of activity in grouped-raised larvae when compared to individually-raised ones. Also, one study identified hyperactivity in 6 dpf larvae after exposure to DMSO in concentrations as low as 0.01 % (Chen et al. 2011). Therefore these aspects are recommended to be maintained stable when performing a set of experiments to

avoid potential interferences on behavioral responses. Regarding the age of larvae, it has already been demonstrated that zebrafish larvae in different stages present distinct locomotion patterns (Selderslaghs et al. 2009, Padilla et al. 2011). Although 6 dpf has been indicated as a rather stable stage for behavioral assessment (Padilla et al. 2011, Vignet et al. 2013), our results indicate that the response to the dark stimulus is more clear at 5 dpf. Further, at this age the larvae are not a protected life stages yet, which can facilitate experimental planning (Strahle et al. 2012). Also, it is of relevance to consider that at 5 dpf there is transition to external feeding, and any differences in yolk energy or nutrition content might present an additional interference (Di Paolo et al. 2015a). Finally, acclimation either in light or darkness prior to behavioral testing was also found to be an important factor of interference. Previously, prolonged dark periods reduced subsequent locomotion in light when compared to light-acclimated larvae, while resulting in less variation between individuals (Burgess and Granato 2007). Nevertheless, the response to the dark stimulus was also shown to decrease, making the response less pronounced (Burgess and Granato 2007). Since the experimental setup was focused on the response to the dark stimulus, light acclimation was selected.

Table 9.3: Effects of extrinsic factors at RWTH. Distance moved (cm) or burst activity occurrence (%) in first of second light (L1, L2) or dark (D1, D2) periods, and ratios peak:basal activities for the first and second cycles (C1, C2) for the different tested factors. Significant differences are highlighted.

RWTH		**Raising density**						**Solvent use**					
		Single			**Grouped**			**None**			**DMSO**		
		Mean / SD / n			**Mean / SD / n**			**Mean / SD / n**			**Mean / SD / n**		
Distance moved (cm)	**L1**	18.2	23.3	22	14.8	22.6	31	19.7	29.1	21	18.2	23.3	22
	D1	58.1	36.2	22	72.5	30.1	32	59.1	25.7	21	58.1	36.2	22
	L2	17.2	15.9	22	24.3	29	32	27.3	38	21	17.2	15.9	22
	D2	60.2	34.9	22	68	33.6	32	59.2	29.5	21	60.2	34.9	22
Burst activity (%)	**L1**	1.1	2.6	22	0.4	1.3	32	0.7	1.4	21	1.1	2.6	22
	D1	3.5	2.8	22	4.2	1.4	32	3.2	1.8	21	3.5	2.8	22
	L2	0.7	1.4	22	1.4	2.5	32	1.2	2.1	21	0.7	1.4	22
	D2	3.6	2.5	22	3.5	2.1	32	3.1	1.8	21	3.6	2.5	22
Peak:basal ratio	**C1**	5.6	3.8	23	7.1	5.5	22	4.0 [b]	1.9	18	5.2 [b]	4.4	17
	C2	4.4	3.5	23	5.6	5.2	22	2.7	1.5	17	4.4	2.4	20
RWTH		**Age of larvae**						**Acclimation lighting**					
		5 dpf			**6 dpf**			**Light**			**Dark**		
		Mean / SD / n			**Mean / SD / n**			**Mean / SD / n**			**Mean / SD / n**		
Distance moved (cm)	**L1**	18.2	23.3	22	49.9 [c]	30.5	30	12.6	23	21	9.9	3.4	33
	D1	58.1	36.2	22	74.4	23	30	56.3 [c]	21.1	22	37.8	13.7	33
	L2	17.2 [b]	15.9	22	38.8 [b]	28	30	17.8 [c]	12.4	22	9.8 [c]	4.1	33
	D2	60.2	34.9	22	74.1	24.9	30	58.7 [b]	19.1	22	44.5 [b]	15.3	33
Burst activity	**L1**	1.1	2.6	22	2.9	2.1	30	0.5	1.6	22	0.4	0.4	33
	D1	3.5	2.8	22	4.2	1.5	30	4.7 [b]	2	22	3.4 [b]	1.6	33

(%)													
	L2	0.7	1.5	22	2	2.1	30	0.8	1	22	0.9	3	33
	D2	3.6	2.5	22	5.3	1.7	30	4.8	1.9	22	4.3	1.8	33
Peak:basal	**C1**	5.2 [b]	4.4	17	2.2 [b]	1.6	30	6.0 [c]	4.1	15	1.6 [c]	0.7	15
ratio	**C2**	4.4 [c]	2.4	20	1.8 [c]	0.7	24	2.1	1.5	10	1.6	0.5	5

a: p<0.05, b: p<0.01, c: p<0.001.

The comparability of EthoVision and ViewPoint regarding video analysis, done through evaluation of a same video using both softwares, indicated some differences between respective outcomes. Although both systems identified similar locomotion profile (Fig. 9.1A), EthoVision results indicated tendency for lower activity than the identified by the ViewPoint software. This was reflected in the respective total distances moved (Fig. 9.1B) and peak:basal ratios (Fig. 9.1C). Such differences can be related to different calibration scales and methods performed with each software. Therefore for future comparisons it is recommended to always apply the same calibration approach. For burst activity there was an inverse tendency than the previous parameters, with EthoVision indicating values circa one fold higher than ViewPoint (Fig. 9.1D). Although the same thresholds for high activity were set, the final identification of high activity still differed, indicating that future comparison studies should not only follow same setups but also verify the final results comparability.

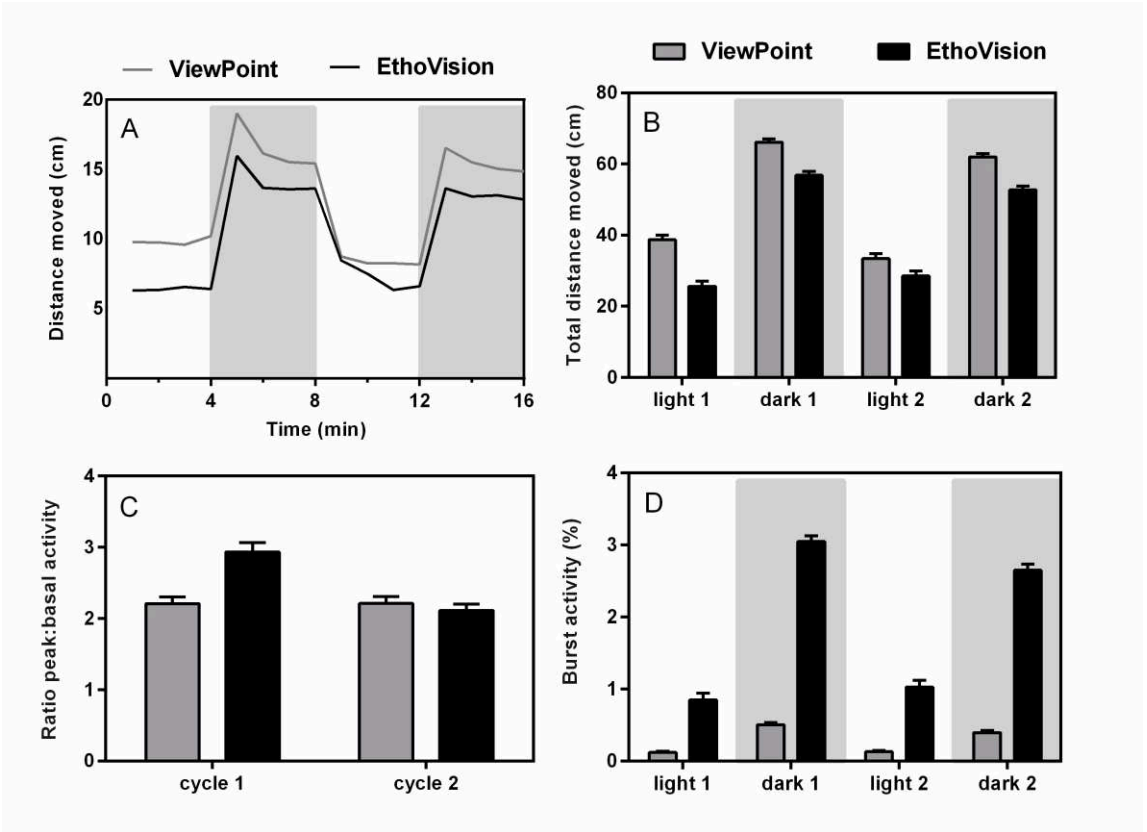


Figure 9.1: Distance moved profiles along time (A), total distance moved in light or dark cycles (B), ratios peak:basal activities in cycles 1 and 2 (C) and burst activities in light or dark cycles (D) obtained using ViewPoint (gray) or EthoVision (black) for the analysis of a same video. Average and standard error of the mean values (n=96 larvae). No statistical analysis was applied since the comparison was done for one video only.

9.3.2 Prolonged exposure to ethanol until 5 or 6 dpf

While no malformations were observed in larvae exposed to ethanol at IVM, at RWTH there was sublethal effect occurrence (mostly edema) of 10 and 40 % for larvae exposed to ethanol 0.5 and 1 %, respectively. Individuals with malformations were not included in behavioral analysis.

Behavioral responses of fish after prolonged exposure to ethanol up to 5 dpf (IVM) or 6 dpf (IVM and RWTH) are summarized in Table 9.4. At IVM, behavioral changes were more evident for tests conducted until 5 dpf. During both dark periods, fish from all three ethanol concentrations presented higher distance moved values when compared to water control ($p<0.01$ or 0.001); and also increased burst activity in ethanol 0.25 and 0.5 % ($p<0.05$ or 0.01). At 6 dpf, in contrast, no clear effects were observed. At RWTH, while no behavioral effects were observed for the 0.25 % ethanol condition, larvae exposed to 0.5% ethanol showed decreased distance moved when compared to controls for all light and dark periods ($p<0.05$ or 0.001). In larvae treated with the highest concentration, the parameters distance moved and burst activity were decreased in the first light period ($p<0.01$), resulting in respective lower ratio peak:basal when compared to water control ($p<0.01$).

Our results indicate that the two strains presented different sensitivity to ethanol acute toxicity, since morphological effects were observed only at RWTH tests. Further, larvae aged 5 dpf seem to be more suited for behavioral assessment of ethanol effects than those from 6 dpf, as indicated by IVM experiments. However, the fact that prolonged exposure to ethanol caused morphological effects is not a characteristic desired in a positive control condition for behavioral effects. The occurrence of sublethal effects includes an additional step in experimental procedures, which is to select the normally developed larvae to be evaluated in experiments. That increases the experimental workload and additionally increases the interference of analyzer subjectivity and interference of inter-individual fish sensitivity.

Table 9.4: Behavioral responses following prolonged exposures to ethanol (0.25, 0.5 or 1 %) at IVM (up to 5 or 6 dpf) and RWTH (up to 6 dpf). Distance moved (cm) or burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios for the first and second cycles (C1, C2) for the different exposure concentrations. Significant differences versus respective water control are highlighted.

IVM		**Water**			**0.25 % Ethanol**			**0.5 % Ethanol**			**1 % Ethanol**		
5 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
Distance moved (cm)	**L1**	35.9	16.4	72	36.0	24	72	37.0	19	72	40.0	25	72
	D1	64.4	10.3	72	73.6 [c]	21	72	74.5 [c]	17	72	72.5 [c]	17	72
	L2	33.4	14.2	72	37.0	24	72	35.0	18	72	35.0	20	72
	D2	59.8	11.0	72	67.7 [b]	16	72	71.6 [c]	18	72	68.7 [c]	18	72

	L1	0.1	0.2	72	0.2	0.3	72	0.2	0.3	72	0.2	0.3	72
Burst activity	**D1**	0.7	0.4	72	1.0 [a]	0.7	72	1.0 [a]	0.7	72	0.9	0.5	72
(%)	**L2**	0.1	0.2	72	0.2	0.3	72	0.2	0.3	72	0.2	0.2	72
	D2	0.5	0.4	72	0.7 [b]	0.5	72	0.7 [a]	0.5	72	0.6	0.4	72
Peak:basal	**C1**	2.4	1.2	71	3.8	5.1	69	3.2	3.4	70	2.4	1.3	69
ratio	**C2**	2.0	0.7	70	2.0	1.1	61	2.0	0.9	61	2.1	1.1	65
IVM		**Water**			**0.25 % Ethanol**			**0.5 % Ethanol**			**1 % Ethanol**		
6 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
	L1	39.8	13.9	72	43.8	15.4	72	34.9 [a]	14.5	72	36.0	15.6	72
Distance	**D1**	69.4	10.6	72	72.2	13.2	72	67.7	10.9	72	66.4	15.2	72
moved (cm)	**L2**	36.6	13.5	72	39.2	12.8	72	34.5	12.7	72	35.5	14.0	72
	D2	65.5	9.6	72	67.9	13.6	72	65.1	10.9	72	66.0	14.0	72
	L1	0.2	0.2	72	0.2	0.3	72	0.2	0.4	72	0.2	0.2	72
Burst activity	**D1**	0.5	0.3	72	0.6	0.4	72	0.5	0.3	72	0.5	0.3	72
(%)	**L2**	0.2	0.2	72	0.2	0.3	72	0.3	0.3	72	0.3	0.3	72
	D2	0.4	0.2	72	0.5	0.3	72	0.4	0.3	72	0.5	0.4	72
Peak:basal	**C1**	2.1	0.8	71	1.1 [c]	0.2	8	1.7	0.3	35	2.1	0.7	67
ratio	**C2**	1.5	0.3	34	1.4	0.3	33	1.5	0.2	26	1.2	0.3	24
RWTH		**Water**			**0.25 % Ethanol**			**0.5 % Ethanol**			**1 % Ethanol**		
6 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
	L1	42.8	24.6	26	35.5	22.3	34	17.3 [c]	24.5	50	21.8 [b]	26.7	32
Distance	**D1**	59.4	17.8	26	63.5	18.3	34	34.2 [a]	34.0	50	52.6	42.9	32
moved (cm)	**L2**	22.3	15.3	26	25.5	19.3	34	14.1 [a]	19.5	50	20.9	26.1	32
	D2	57.2	17.4	26	59.3	18.8	34	30.4 [a]	31.4	50	47.0	39.9	32
	L1	2.4	2.2	26	1.9	1.6	34	2.2	3.6	50	0.8 [b]	1.2	32
Burst activity	**D1**	3.5	1.6	26	3.6	1.8	34	3.9	2.2	50	3.9	3.1	32
(%)	**L2**	1.0	0.9	26	1.7	2.2	34	1.6	2.1	50	1.1	1.5	32
	D2	3.5	1.7	26	3.4	1.8	34	3.8	2.3	50	3.4	2.9	32
Peak:basal	**C1**	1.7	1.0	23	2.4	1.8	28	2.9	2.5	40	4.6 [b]	3.9	28
ratio	**C2**	3.5	2.1	24	4.0	2.8	47	4.0	2.8	47	3.8	5.7	34

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

9.3.3 Behavioral alterations of candidate positive control chemicals after short exposures at IVM and RWTH

Exposure to the candidate positive control chemicals tended to present biphasic response pattern, with low concentrations eliciting stimulatory effects and higher concentrations causing instead inhibition of locomotion. Among the tested compounds, this response profile was more evident for ethanol. Responses obtained at 90 min were always very similar to those at 40 min, therefore are not discussed in details. Peak:basal ratios were always reduced following caffeine and nicotine exposure, therefore results are presented in details only for ethanol. The parameter burst activity is presented for IVM experiments only. The responses obtained in the two laboratories were in general consistent, and although some differences

occurred these are more likely related to the fact that different fish strains were used (Vignet et al. 2013). The new parameter peak:basal ratio, together with distance moved and burst activity, supported the interpretation of responses to the different chemicals.

(i) Ethanol

Acute exposure to ethanol caused concentration and time dependent effects in 5 dpf zebrafish larvae of the IVM and RWTH strains (Table 9.5, Fig. 9.2, Fig. 9.3). Ethanol exposure affected the locomotion of larvae already after 10 min (Fig. 9.2A/B) and up to 40 min (Fig. 9.2C/D). At 10 and 40 min, ethanol 1 % caused general locomotion increase while exposure to 2 and 3% disrupted the response pattern, with the larvae not presenting the clear increase in locomotion following the dark stimulus. Larvae exposed to ethanol 1% presented, in both time points, increased distance moved in dark (IVM and RWTH, $p<0.05$ or 0.001) and in light (RWTH only, $p<0.05$ or 0.001) when compared to water controls. Exposure to ethanol 2% instead caused in most cases increased distance moved in light ($p<0.01$ or 0.001) but normal or reduced response in dark ($p<0.05$ or 0.001); while ethanol 3 % caused evident decrease of activity in dark ($p<0.01$ or 0.001), which was even lower than respective activity in light (Fig. 9.2). Considering the peak:basal ratios (Fig. 9.3), values were decreased when compared to respective water controls in both time points, with significant differences for 2 and 3 % at IVM (Fig. 9.3A/C) and for all concentrations at RWTH experiments (Fig. 9.3B/D). That indicates that, despite increased activity in some of the periods, the response to the dark stimulus was reduced in fish exposed to all conditions when compared to those maintained in water. Burst activity results measured for the IVM strain were more relevant at 40 min, being increased at 1 and 2 % and reduced at 3 % (Table 9.5).

Table 9.5: Behavioral responses following short exposures (10, 40, 90 min) of 5 dpf larvae to ethanol (1, 2 or 3 %) at IVM. Burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios activities for the first and second cycles (C1, C2) for the different exposure concentrations. Significant differences versus respective water control conditions are highlighted.

IVM		**Water**			**1 % Ethanol**			**2 % Ethanol**			**3 % Ethanol**		
10 min		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
Burst activity (%)	**L1**	0.19	0.27	72	0.94	1.23	72	0.13	0.3	72	0.16	0.3	72
	D1	1.1	0.57	72	1.37	1.03	72	0.26 [c]	0.44	72	0.09 [c]	0.22	72
	L2	0.24	0.35	72	0.41	0.69	72	0.43	0.55	72	0.03 [b]	0.07	72
	D2	0.74	0.39	72	1.22	1.01	72	0.8	0.7	72	0.03	0.1	72
IVM		**Water**			**1 % Ethanol**			**2 % Ethanol**			**3 % Ethanol**		
40 min		**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Burst activity	**L1**	0.11	0.19	72	0.24	0.47	72	1.00 [c]	0.66	72	0.23	0.38	72
	D1	0.48	0.4	72	1.42 [c]	0.88	72	1.06 [c]	0.71	72	0.23 [c]	0.37	72

(%)	**L2**	0.13	0.19	72	0.30 [c]	0.28	72	1.12 [c]	0.82	72	0.21	0.34	72
	D2	0.46	0.32	72	1.29 [c]	0.76	72	1.05 [c]	0.77	72	0.26 [b]	0.44	72
IVM		**Water**			**1 % Ethanol**			**2 % Ethanol**			**3 % Ethanol**		
90 min		**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Burst activity (%)	**L1**	0.11	0.18	72	0.04	0.13	72	1.02 [c]	0.76	72	0.61 [b]	0.72	72
	D1	0.87	0.44	72	1.61 [a]	0.94	72	0.94	0.7	72	0.47	0.52	72
	L2	0.19	0.24	72	0.15	0.22	72	1.35 [c]	0.71	72	0.51	0.55	72
	D2	0.74	0.32	72	1.37	0.77	72	0.75	0.72	72	0.56	0.58	72
Peak:basal ratios	**C1**	2.3	1.1	64	4.1	3.2	66	1.2 [c]	0.4	70	0.9 [c]	0.2	71
	C2	1.8	0.5	53	2.7	1.3	55	0.8 [c]	0.3	54	1.0 [c]	0.3	65

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

In our experiments, larval behavior was affected by ethanol exposure already at 10 min, with a stable response being reached after 40 minutes. That is in agreement with the previous finding that ethanol concentrations in adult zebrafish brain reach a plateau after 40 min of exposure (Chatterjee and Gerlai 2009). The tendency for dose-response biphasic response, i.e. hyperactivity due to lowest concentrations and disruption of behavioral response in higher concentrations, is in agreement with previous studies (Gerlai et al. 2006, MacPhail et al. 2009, Irons et al. 2010). In fact, the cortisol response to stress was shown to be reduced in ethanol-exposed zebrafish (Baiamonte et al. 2015), with ethanol causing different zebrafish stages to present less anxiety-like behaviour and reduced response to light/dark stimulus (Baiamonte et al. 2016). The differences observed for behavioral responses after the prolonged (i.e. up to 5-6 dpf) or short exposures to ethanol are similar to profiles observed in other zebrafish studies (Mathur and Guo 2011). Considering the overall results, the acute, short exposure to ethanol is considered to be a more suitable condition to be applied as positive control.

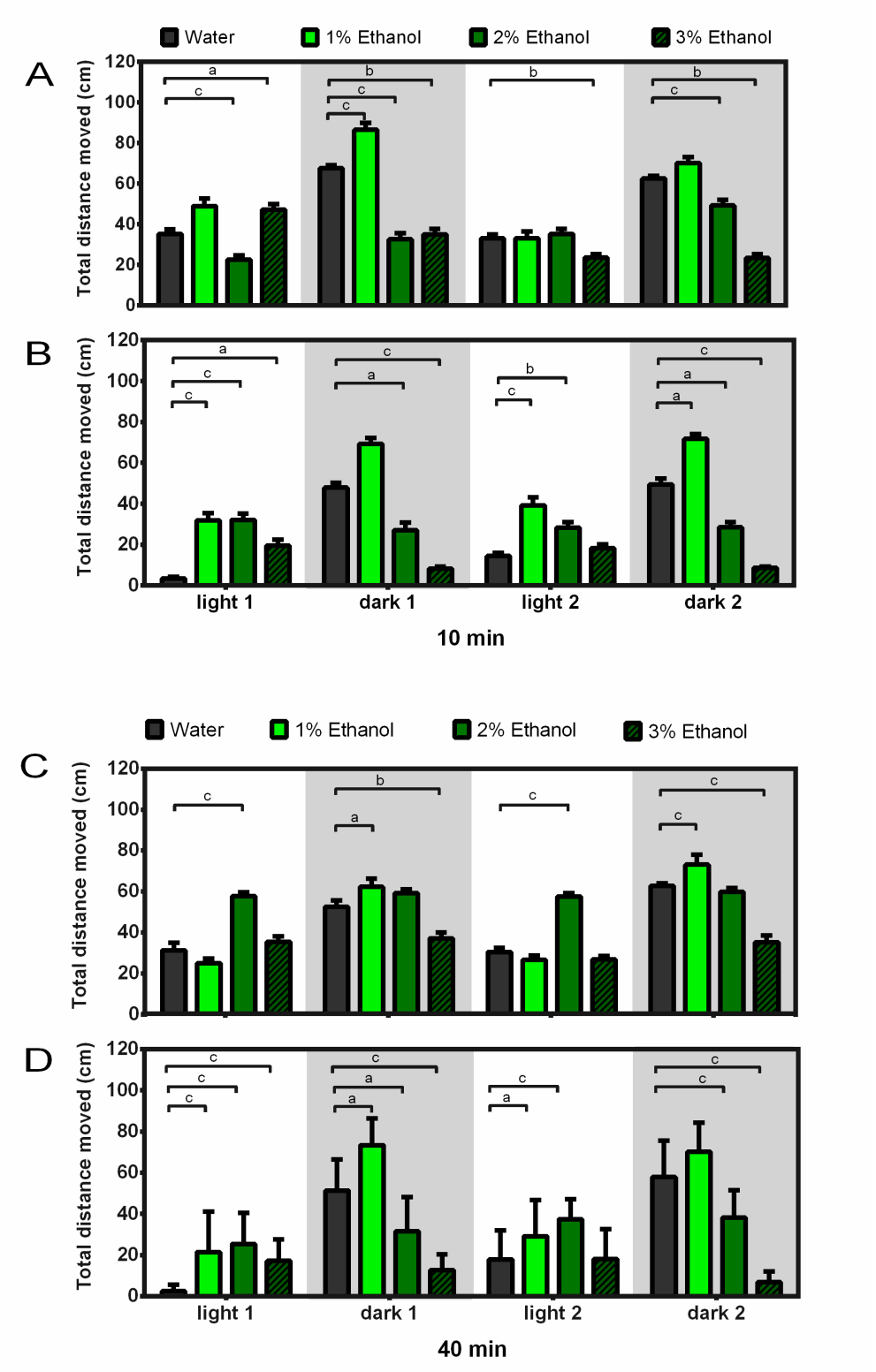


Figure 9.2: Distance moved (cm) in first (1) or second (2) light or dark periods after larval exposure to water control or ethanol 1, 2 or 3% for 10 min (A: IVM, B: RWTH) or 40 min (C: IVM, D: RWTH). Average and standard error of the mean values for 72 (IVM) or 30 (RWTH) larvae. Significant differences evaluated by one-way ANOVA followed by Dunnetts's test (a: $p<0.05$, b: $p<0.01$, c: $p<0.001$).

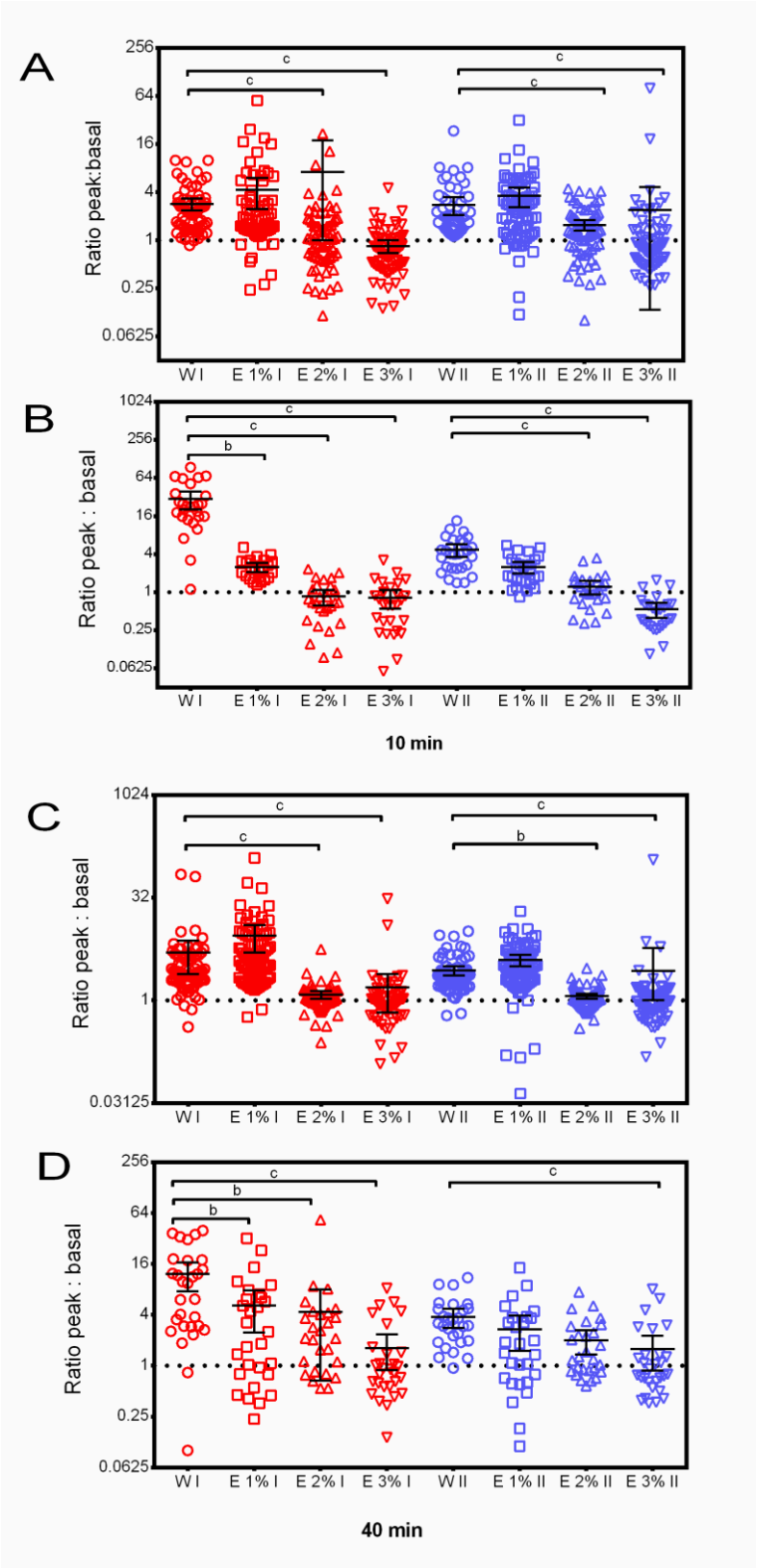


Figure 9.3: Distance moved (cm) in first (I, red) or second (II, blue) light or dark periods after larval exposure to water control (W) or ethanol (E) 1, 2 or 3 % for 10 min (A: IVM, B: RWTH) or 40 min (C: IVM, D: RWTH). Symbols stand for individuals, with mean and 95 % confidence intervals being represented by error bars. Values are for 72 (IVM) or 30 (RWTH) larvae. Significant differences evaluated by Kruskal-Wallis test followed by Dunn's test (a: $p<0.05$, b: $p<0.01$, c: $p<0.001$).

(ii) Caffeine

Acute exposure to caffeine at IVM also caused concentration and time dependent effects in 5 dpf zebrafish larvae (Table 9.6, Fig. 9.4). Caffeine exposure affected the locomotion of larvae very quickly, with effects already after 10 min (Fig. 9.4A/B) and up to 40 min (Fig. 9.4C/D). Responses obtained at 90 min were very similar to those at 40 min, therefore are not discussed in details. Increased distance moved was only observed after 10 min exposure to 75 mg/L in first light and dark periods at IVM (Fig. 9.4A), followed by reduction in second cycle. Reduced distance moved values were observed at RWTH already at 10 min exposure for all concentrations (Fig. 9.4B); and for both strains and all concentrations at 40 min exposure (Fig. 9.4C/D). In consequence, the peak:basal ratio was increased only for IVM 75 mg/L exposure at first cycle (Table 9.6), while it was reduced in all other conditions. That indicates that the larval response to the dark stimulus was impaired by caffeine exposure. Burst activity measured for the IVM strain was increased at 10 min for 75 and 150 mg/L, while it was decreased at longer exposures (Table 9.6).

Acute exposure of zebrafish adults to caffeine produces anxiogenic behavioral responses (Egan et al. 2009). At the same time, caffeine pre-treatment was shown to protect zebrafish from stress-induced anxiety caused by exposure to the synthetic glucocorticoid dexamethasone (Khor et al. 2013). Therefore caffeine can act both as anxiogenic and as anxiolytic depending on exposure concentration and duration. The present observed inhibitory effects at longer exposure times were consistent with a previous study that described reduced swim speeds in 7 dpf larvae after 2 h exposure (Richendrfer et al. 2012).

Table 9.6: Behavioral responses following short exposures (10, 40, 90 min) of 5 dpf larvae to caffeine (75, 150 or 200 mg/L) at IVM. Distance moved (cm) or burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios for the first and second cycles (C1, C2) for the different exposure concentrations. Significant differences versus respective water control conditions are highlighted.

IVM		**Water**			**Caffeine 75 mg/L**			**Caffeine 150 mg/L**			**Caffeine 200 mg/L**		
10 min		Avg.	SD	N	Avg.	SD	N	Avg.	SD	N	Avg.	SD	N
Burst activity (%)	**L1**	0.05	0.1	66	0.23 [c]	0.25	72	0.43 [c]	0.45	72	0.42 [c]	0.38	72
	D1	0.58	0.4	66	0.96 [c]	0.56	72	0.98 [c]	0.54	72	0.61	0.38	72
	L2	0.17	0.23	66	0.33 [c]	0.28	72	0.27 [c]	0.21	72	0.21	0.19	72
	D2	0.44	0.26	66	0.83 [c]	0.43	72	0.41	0.37	72	0.14 [c]	0.15	72
Peak:basal ratios	**C1**	3.7	2.4	66	2.1 [b]	1.1	65	1.8 [c]	0.7	60	1.4 [c]	0.5	67
	C2	2.0	0.6	51	2.7	1.6	67	1.3 [c]	0.6	53	0.8 [c]	0.3	42
IVM		**Water**			**Caffeine 75 mg/L**			**Caffeine 150 mg/L**			**Caffeine 200 mg/L**		
40 min		Avg.	SD	N	Avg.	SD	N	Avg.	SD	N	Avg.	SD	N
Burst activity	**L1**	0.07	0.16	66	0.09 [b]	0.11	72	0.03	0.05	72	0.04	0.07	72
	D1	0.42	0.27	66	0.27 [b]	0.24	72	0.06 [c]	0.13	72	0.04 [c]	0.09	72

(%)	**L2**	0.13	0.18	66	0.13	0.17	72	0.05 [a]	0.08	72	0.04 [c]	0.07	72
	D2	0.40	0.24	66	0.33	0.29	72	0.08 [c]	0.23	72	0.03 [c]	0.06	72
Peak:basal ratios	**C1**	2.8	1.6	65	2.0	1.3	70	0.9 [c]	0.3	50	0.7 [c]	0.2	49
	C2	2.4	1.1	66	1.7 [a]	0.8	65	0.9 [c]	0.3	61	0.6 [c]	0.3	40
IVM		**Water**			**Caffeine 75 mg/L**			**Caffeine 150 mg/L**			**Caffeine 200 mg/L**		
90 min		Avg.	SD	N	Avg.	Avg.	SD	N	Avg.	Avg.	SD	N	Avg.
Burst activity (%)	**L1**	0.05	0.07	66	0.15 [b]	0.31	72	0.04	0.07	72	0.03	0.07	72
	D1	0.47	0.25	66	0.17 [c]	0.2	72	0.05 [c]	0.17	72	0.02 [c]	0.07	72
	L2	0.11	0.14	66	0.07	0.09	72	0.03 [c]	0.07	72	0.05 [b]	0.09	72
	D2	0.38	0.21	66	0.22 [c]	0.21	72	0.06 [c]	0.19	72	0.03 [c]	0.13	72
Peak:basal ratios	**C1**	1.4	0.5	19	1.1	0.3	45	0.7 [c]	0.3	56	0.8 [c]	0.5	70
	C2	1.7	0.6	44	0.9 [c]	0.5	43	0.7 [c]	0.3	53	0.6 [c]	0.3	61

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

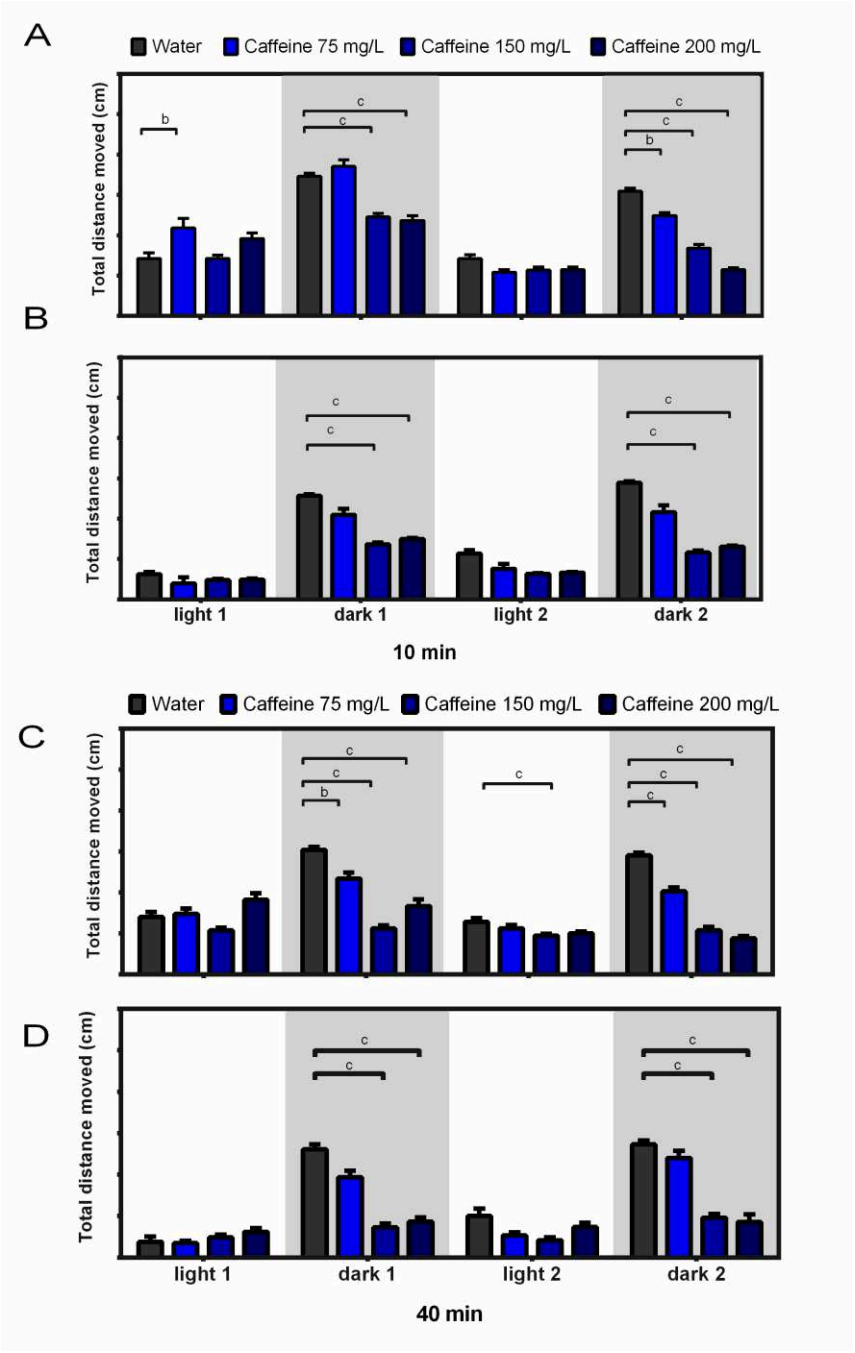


Figure 9.4: Distance moved (cm) in first (1) or second (2) light or dark periods after larval exposure to water control or caffeine 75, 150 or 200 mg/L for 10 min (A: IVM, B: RWTH) or 40 min (C: IVM, D: RWTH). Average and standard error of the mean values for 72 (IVM) or 30 (RWTH) larvae. Significant differences evaluated by one-way ANOVA followed by Dunnetts's test (a: $p<0.05$, b: $p<0.01$, c: $p<0.001$).

(iii) Nicotine

Short exposure to nicotine caused responses similar to those found for caffeine (Table 9.7, Fig. 9.5). Tendency for increased locomotion was observed in nicotine 8 mg/L after 10 min exposure in both strains (Fig. 9.5); while it was observed in 12 and 16 mg/L only at RWTH. In higher concentrations (Fig. 9.5) and longer exposure periods, nicotine always caused locomotion inhibition, which reflected in reduction of all measured parameters. Burst activity

measured for the IVM strain was increased only at 10 min for 8 and 12 mg/L exposure, while it was decreased in all other conditions (Table 9.7). The peak:basal ratio was always reduced, indicating that larvae exposed to nicotine responded less effectively to the dark stimulus.

Nicotine is a neurotoxic compound that can affect neurodevelopment in zebrafish early life stages (Welsh et al. 2009, Muth-Köhne et al. 2012). The early onset of behavioral responses following nicotine exposure has been demonstrated for zebrafish embryos, rapidly accumulating and reaching steady state in only 10 minutes, and with peak of behavioural responses occurring at only 5 min of exposure (Thomas et al. 2009b). This very short latency time before behavioural effects can help to explain the occurrence of stimulatory effects only at first light cycle at 10 min. Although the neurotoxicity relevance of nicotine is clear, its use in behavioral modulation studies has the drawback of a very short time spam for effect occurrence.

Table 9.7: Behavioral responses following short exposures (10, 40, 90 min) of 5 dpf larvae to nicotine (8, 12 or 16 mg/L) at IVM. Distance moved (cm) or burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios for the first and second cycles (C1, C2) for the different exposure concentrations. Significant differences versus respective water control conditions are highlighted.

IVM		DMSO			Nicotine 8 mg/L			Nicotine 12 mg/L			Nicotine 16 mg/L		
10 min		**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Burst activity (%)	**L1**	0.12	0.19	36	0.26 [c]	0.26	72	0.22 [a]	0.27	72	0.13	0.22	72
	D1	0.68	0.43	36	1.01	0.69	72	0.65	0.64	72	0.08 [c]	0.17	72
	L2	0.2	0.2	36	0.17	0.2	72	0.07 [b]	0.11	72	0.05 [c]	0.11	72
	D2	0.59	0.41	36	0.57	0.71	72	0.07 [c]	0.19	72	0.06 [c]	0.14	72
Peak:basal ratios	**C1**	2.0	1.0	38	1.9	0.7	54	1.9	0.9	62	1.0 [c]	0.5	72
	C2	1.7	0.8	21	1.6	0.6	60	1.1 [b]	0.8	74	1.1 [b]	0.7	77
IVM		DMSO			Nicotine 8 mg/L			Nicotine 12 mg/L			Nicotine 16 mg/L		
40 min		**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Burst activity (%)	**L1**	0.11	0.2	36	0.14	0.29	72	0.07 [a]	0.24	72	0.05 [b]	0.14	72
	D1	0.51	0.36	36	0.08 [c]	0.11	72	0.06 [c]	0.13	72	0.04 [c]	0.09	72
	L2	0.09	0.09	36	0.06 [a]	0.11	72	0.06 [c]	0.15	72	0.09 [c]	0.29	72
	D2	0.45	0.33	36	0.12 [c]	0.15	72	0.07 [c]	0.16	72	0.07 [c]	0.22	72
Peak:basal ratios	**C1**	2.1	0.9	35	1.8	1.2	70	1.5 [a]	0.7	61	0.8 [c]	0.4	57
	C2	1.3	0.4	9	1.6	0.5	46	1.4	0.8	54	0.6	0.4	22
IVM		DMSO			Nicotine 8 mg/L			Nicotine 12 mg/L			Nicotine 16 mg/L		
90 min		**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Burst activity (%)	**L1**	0.11	0.13	36	0.11	0.19	72	0.05 [a]	0.1	72	0.08	0.14	72
	D1	0.53	0.35	36	0.24 [c]	0.29	72	0.09 [c]	0.16	72	0.06 [c]	0.12	72
	L2	0.1	0.09	36	0.09	0.16	72	0.06 [c]	0.18	72	0.05 [c]	0.23	72
	D2	0.49	0.29	36	0.18 [c]	0.25	72	0.08 [c]	0.13	72	0.04 [c]	0.11	72
Peak:basal ratios	**C1**	2.2	0.9	34	2.3	1	67	1.7	0.8	55	1.1 [c]	0.6	64
	C2	1.1	0.2	9	1.8 [a]	0.6	52	1.2	0.6	40	0.8	0.4	59

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

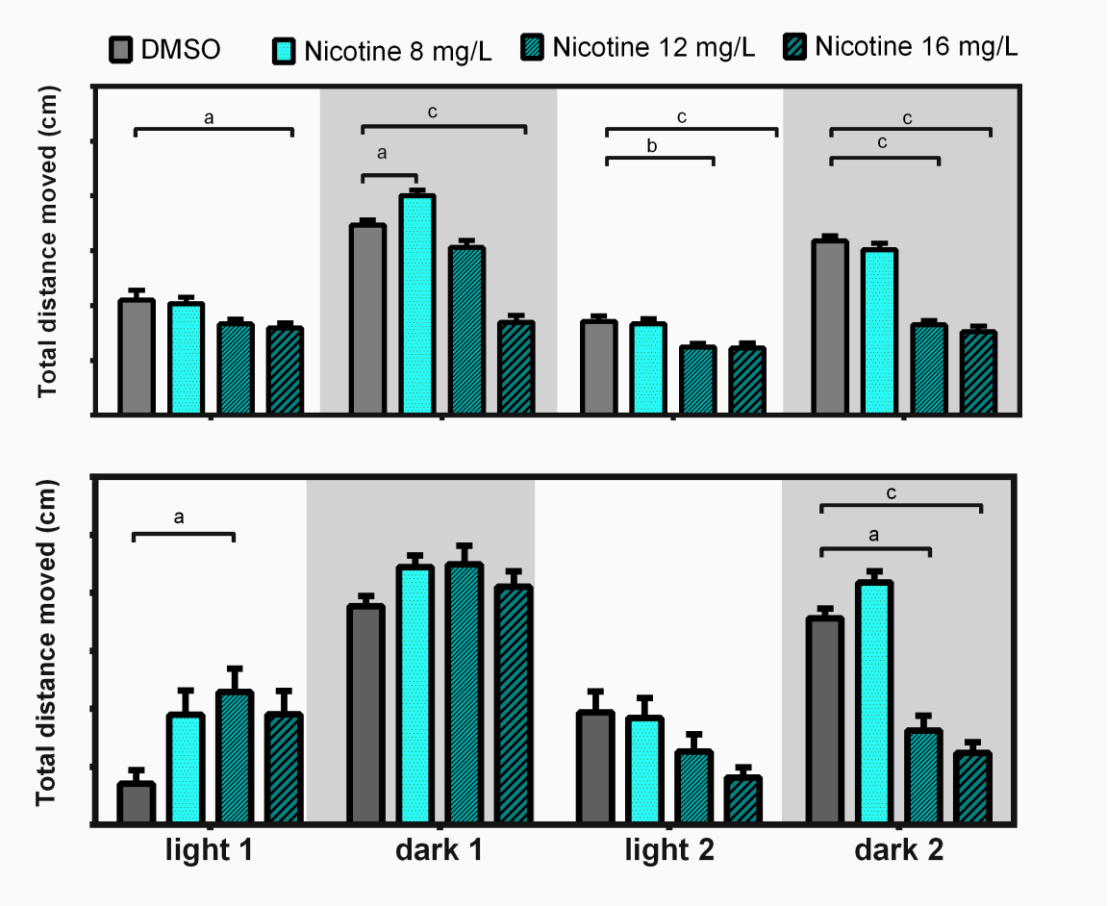


Figure 9.5: Distance moved (cm) in first (1) or second (2) light or dark periods after larval exposure to water control or caffeine 75, 150 or 200 mg/L for 10 min (upper: IVM, lower: RWTH). Average and standard error of the mean values for 72 (IVM) or 30 (RWTH) larvae. Significant differences evaluated by one-way ANOVA followed by Dunnetts's test (a: $p<0.05$, b: $p<0.01$, c: $p<0.001$).

9.3.4 Chlorpyrifos and α-cypermethrin teratogenicity

Effect-concentration values EC_{10}, EC_{25} and EC_{50} as well as the NOEC and LOEC values for aCM and CHP are presented in Table 9.8, and respective dose response curves are presented in Fig. 9.6. Observed malformations included spinal cord deformations for both insecticides, reduced pigmentation in larvae exposed to aCM, and heart edema for larvae exposed to CHP. Additionally, aCM caused twitching movement of larvae at 96 hpf. Although effect-concentration values presented some variation between RWTH and IVM results, most often there was overlap between 95 % confidence intervals. Further, it is of relevance to consider that only one experiment was performed at IVM. Therefore the EC values obtained at RWTH were selected as the exposure concentrations for next experiments for behavioral effects conducted at IVM and at RWTH.

For aCM, a 96 h LC_{50} of 1.84 µg/L was reported (FAO 2013), being in similar range to the obtained 120 h EC_{50} value of 6.4 µg/L. The observed sublethal endpoints of body axis curvature, spasms and reduced pigmentation were reported for cypermethrin (DeMicco et al.

2009, Shi et al. 2011), indicating that type II pyrethroids cause similar malformations in zebrafish larvae. The present CHP EC_{50} (3.9 mg/L) is in same range of nominal exposure concentrations reported to cause malformations in zebrafish exposed to CHP up to 6 dpf (Selderslaghs et al. 2009). Our value is however lower than a previously determined 96 h EC_{50} under semi-static conditions (Pérez et al. 2013), indicating that chemical bioavailability was reduced under static exposure. The observed sublethal of effects of spine malformation and heart edema are in agreement with previous studies (Kienle et al. 2009, Selderslaghs et al. 2009).

Table 9.8: Effect-concentration values EC_{10}, EC_{25} and EC_{50} and NOEC / LOEC values for α-cypermethrin and chlorpyrifos obtained for experiments conducted at RWTH and IVM.

		α-cypermethrin (µg/L)				Chlorpyrifos (mg/L)			
		RWTH		IVM		RWTH		IVM	
EC_{10}	95% C.I.	1.3	0.7-1.9	3.0	1.2-4.4	1.8	1.4-2.2	2.4	1.5-2.9
EC_{25}	95% C.I.	2.8	1.9-3.8	4.2	2.3-5-9	2.6	2.2-3.1	2.9	2.1-3.4
EC_{50}	95% C.I.	6.4	4.7-8.6	6.1	4.1-9.1	3.9	3.4-4.5	3.6	2.9-4.3
NOEC		1.0		1.0		1.6		1.6	
LOEC		2.5		3.5		2.5		2.5	

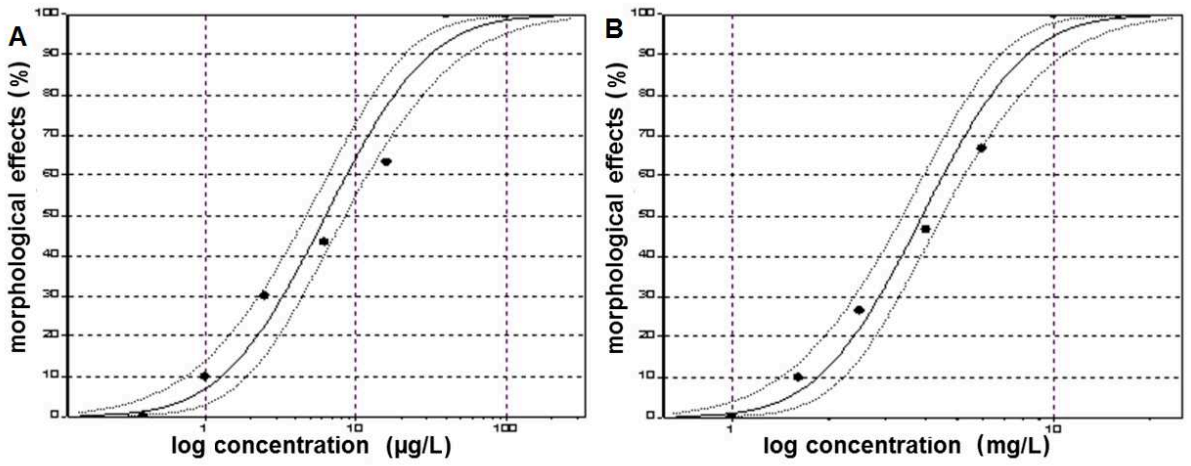


Figure 9.6: Dose- response- curves obtained for α-cypermethrin (left, A) and chlorpyrifos (right, B) for experiments conducted at RWTH. Maximum likelihood fit and 95 % confident bands for occurrence of morphological effects (%) plotted versus log concentration values of α-cypermethrin (µg/L) and chlorpyrifos (mg/L) obtained in three experiments.

9.3.5 Behavior effects of insecticides after prolonged exposure

(i) α-cypermethrin

Prolonged exposure to α-cypermethrin affected the behavioral of 5 dpf larvae at IVM experiments for the lowest concentration of 1.3 µg/L (Table 9.9), leading to increased distance moved values during light and dark periods and increased burst activities during light

cycles. Still, the peak:basal ratio was decreased in the first cycle, indicating decreased response of the larvae to the dark stimulus. In the older larvae at 6 dpf, for experiments conducted at IVM and at RWTH, no significant differences were observed between the different parameters of exposed larvae versus those in respective DMSO control conditions (Table 9.9). Therefore, it was not possible to compare present results to effects observed in other organisms such as rodents (Wolansky et al. 2006). Still, since hyperactivity was observed for IVM 5 dpf larvae, and visual observations identified increased activity in 4 dpf aCM-exposed larvae when compared to controls, it is considered that temporal profiles of exposure scenarios and behavioral effect occurrence play an important role in aCM toxicity assessment.

Table 9.9: Behavioral responses at 5 or 6 dpf larvae after prolonged exposures to α-cypermethrin (aCM) 1.3, 2.8 or 6.4 µg/L. Distance moved (cm) or burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios for the first and second cycle (C1, C2) for the different exposure concentrations. Significant differences versus respective DMSO control conditions are highlighted.

IVM		**DMSO**			**aCM 1.3 µg/L**			**aCM 2.8 µg/L**			**aCM 6.4 µg/L**		
5 dpf		Avg.	SD	N	Avg.	SD	N	Avg.	SD	N	Avg.	SD	N
Distance moved (cm)	**L1**	33.3	13.2	36	43.7 [a]	22.9	72	37.9	14.6	72	40.0	15.8	72
	D1	62.6	8.7	36	69.8 [b]	11.9	72	67.9	11.4	72	64.6	9.4	72
	L2	30.7	13.7	36	43.9 [b]	29	72	33.6	13	72	35.5	14.3	72
	D2	58.3	9.6	36	65.6 [b]	9.5	72	62.5	11.9	72	61.0	10.6	72
Burst activity (%)	**L1**	0.08	0.07	36	0.22 [a]	0.26	72	0.13	0.15	72	0.13	0.16	72
	D1	0.66	0.4	36	0.83	0.41	72	0.92 [b]	0.62	72	0.76	0.46	72
	L2	0.08	0.13	36	0.21 [a]	0.39	72	0.14	0.17	72	0.14	0.17	72
	D2	0.52	0.38	36	0.64	0.36	72	0.65	0.43	72	0.6	0.52	72
Peak:basal ratio	**C1**	2.3	0.9	34	1.3 [c]	0.3	32	1.9	0.5	59	1.2 [c]	0.2	19
	C2	1.5	0.3	15	1.8	0.7	70	1.6	0.4	37	1.4	0.2	30
IVM		**DMSO**			**aCM 1.3 µg/L**			**aCM 2.8 µg/L**			**aCM 6.4 µg/L**		
6 dpf		Avg.	SD	N	Avg.	SD	N	Avg.	SD	N	Avg.	SD	N
Distance moved (cm)	**L1**	40.7	12.8	36	44.7	16.8	72	44.2	16.6	72	39.3	14.4	72
	D1	67.6	11.4	36	70.3	12.9	72	71.3	12.8	72	67.0	12	72
	L2	33.6	11.5	36	36.8	13.0	72	36.9	18.5	72	33.1	15.9	72
	D2	62.1	9.3	36	65.2	12.4	72	65.5	13.2	72	61.5	11.9	72
Burst activity (%)	**L1**	0.22	0.24	36	0.21	0.29	72	0.18	0.24	72	0.12	0.15	72
	D1	0.54	0.35	36	0.57	0.45	72	0.46	0.36	72	0.38	0.27	72
	L2	0.19	0.26	36	0.16	0.18	72	0.17	0.22	72	0.14	0.23	72
	D2	0.43	0.27	36	0.44	0.33	72	0.38	0.29	72	0.3	0.2	72
Peak:basal ratio	**C1**	1.3	0.2	7	1.3	0.3	28	1.2	0.2	12	1.5	0.3	32
	C2	1.5	0.2	11	1.3	0.3	25	1.4	0.3	31	1.9	0.7	64
RWTH		**DMSO**			**aCM 1.3 µg/L**			**aCM 2.8 µg/L**			**aCM 6.4 µg/L**		
6 dpf		Avg.	SD	N	Avg.	SD	N	Avg.	SD	N	Avg.	SD	N

Distance moved (cm)	**L1**	33.8	19.4	26	38.9	20.2	53	34.8	19.8	52	38.2	24	52
	D1	63.6	17.6	26	58.8	18.5	53	57.5	19.1	52	68.7	18.1	52
	L2	32.1	21.8	26	24.6	17.3	53	25.8	22.3	52	27.1	26.1	52
	D2	62.1	20.3	26	53.1	20.3	53	55.3	20.8	52	64.4	25.2	52
Burst activity (%)	**L1**	2.0	1.8	26	2.4	2.0	53	3.2	3.8	52	4.8	16.0	52
	D1	4.0	1.7	26	3.5	1.9	53	4.0	1.9	52	13.6	67.7	52
	L2	2.8	4.5	26	1.5	1.6	53	2.0	2.7	52	4.0	14.3	52
	D2	3.6	1.3	26	3.3	2.1	53	3.9	2.6	52	14.7	78.2	52
Peak:basal ratio	**C1**	2.6	1.7	24	1.9	0.9	49	2.6	2.9	45	2.3	1.4	46
	C2	2.9	1.9	24	3.6	3.4	48	3.5	3.2	45	3.4	2.6	43

a: p<0.05, b: p<0.01, c: p<0.001.

(ii) Chlorpyrifos

CHP caused general decrease in larval activity at 5 and 6 dpf for the IVM tests. That is reflected in reduced distance moved and burst activity (p<0.05, 0.01 or 0.001) versus respective control values (Table 9.10). The ratio peak:basal instead did not present significant changes except for the highest concentration at 5 dpf. That indicates that, despite the larvae presented reduced activity, they were still capable of reacting to the dark stimulus. For RWTH 5 dpf larvae, CHP also caused reduction in distance moved and burst activity values when compared to solvent control values. In contrary, the ratio peak:basal was increased in the lowest concentration of CHP 1.8 mg/L (p<0.05 or 0.01). That indicates that, despite the general tendency for reduced larval locomotion, not only the response to the dark stimulus was maintained but it was in fact increased after the pesticide exposure. The effects of CHP on zebrafish behavior have already been investigated by previous studies, with reduced locomotion being described in zebrafish larvae (Kienle et al. 2009). Also reduced responses of CHP exposed larvae to the light-dark transition were reported (Jin et al. 2015b). In fact, behavioral effects of CHP exposure during early life stages were shown to persist until the adult stage (Levin et al. 2003), confirming their ecological relevance.

Table 9.10: Behavioral responses at 5 or 6 dpf larvae after prolonged exposures to chlorpyrifos (CHP) 1.8, 2.6 or 3.9 mg/L. Distance moved (cm) or burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios for the first and second cycle (C1, C2) for the different exposure concentrations. Significant differences versus respective DMSO control conditions are highlighted.

IVM		**DMSO**			**CHP 1.8 mg/L**			**CHP 2.6 mg/L**			**CHP 3.9 mg/L**		
5 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
Distance moved (cm)	**L1**	42.6	15.7	36	44.4	26.1	72	47.6	16.6	72	38.7	13.1	72
	D1	56.1	16.2	36	50	21.8	72	48.4	15.5	72	41.3 [c]	15.1	72
	L2	44.1	18	36	47.4	25.4	72	48.9	24.4	72	38.9	11.7	72
	D2	58.4	10.9	36	48.7 [b]	10.9	72	48.4 [c]	20.4	72	42.4 [c]	15.3	72

Burst activity (%)	**L1**	0.18	0.16	36	0.21	0.31	72	0.31	0.31	72	0.08 [b]	0.11	72
	D1	0.81	0.43	36	0.81	0.47	72	0.45 [c]	0.47	72	0.22 [c]	0.4	72
	L2	0.18	0.2	36	0.2	0.28	72	0.28	0.32	72	0.09 [b]	0.15	72
	D2	0.6	0.3	36	0.61	0.28	72	0.36 [c]	0.35	72	0.15 [c]	0.22	72
Peak:basal ratio	**C1**	1.1	0.3	12	1.2	0.6	61	1.1	0.3	55	1.1	0.3	61
	C2	1.3	0.3	21	1.3	0.7	63	1.2	0.5	65	0.9 [c]	0.2	46
IVM		**DMSO**			**CHP 1.8 mg/L**			**CHP 2.6 mg/L**			**CHP 3.9 mg/L**		
6 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
Distance moved (cm)	**L1**	44.5	13.5	36	35.5 [c]	24.9	72	35.7 [a]	15.5	58	47.1	24.3	30
	D1	69.5	10.8	36	49.5 [c]	9	72	47.1 [c]	15	58	48.6 [c]	21.8	30
	L2	38.9	11.4	36	30.8 [b]	22.7	72	34.3	15.6	58	46.5	20.1	30
	D2	63.1	11.3	36	45.0 [c]	10.7	72	46.0 [c]	14	58	53.0 [b]	24.4	30
Burst activity (%)	**L1**	0.23	0.28	36	0.16	0.28	72	0.16	0.16	58	0.32	0.37	31
	D1	0.67	0.39	36	0.52	0.3	72	0.38 [c]	0.34	58	0.42 [c]	0.44	31
	L2	0.19	0.19	36	0.14	0.34	72	0.12	0.17	58	0.21	0.22	31
	D2	0.48	0.28	36	0.42	0.21	72	0.35	0.24	58	0.42	0.42	31
Peak:basal ratio	**C1**	1.1	0.1	6.0	1.3	0.4	38	1	0.3	29	1	0.5	29
	C2	1.1	0.1	5.0	2.3	1.5	68	1.2	0.5	45	0.8	0.4	20
RWTH		**DMSO**			**CHP 1.8 mg/L**			**CHP 2.6 mg/L**			**CHP 3.9 mg/L**		
6 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
Distance moved (cm)	**L1**	46.1	22.1	28	12.9 [c]	7.6	41	24.1 [c]	18.4	48	40.9	29	28
	D1	68.7	28.3	28	25.0 [c]	13.3	41	38.5 [c]	23.7	48	48.0 [a]	36.3	28
	L2	34.4	17.4	28	10.1 [c]	8.1	41	26.2	22	48	45.8	35.9	28
	D2	71.5	27.9	28	27.8 [c]	15.9	41	43.4 [b]	27.2	48	54.0 [a]	39.3	28
Burst activity (%)	**L1**	3.2	1.9	28	1.0 [c]	2.7	41	1.1 [c]	1.2	48	3	4.1	28
	D1	5.0	2.0	28	2.3 [c]	3.4	41	2.4 [c]	1.8	48	3.1 [b]	2.5	28
	L2	2.3	1.5	28	0.4 [c]	0.6	41	1.1 [b]	1.5	48	2.6	2.2	28
	D2	5.1	1.8	28	2.4 [c]	3.5	41	2.5 [c]	2.2	48	3.3 [a]	2.4	28
Peak:basal ratio	**C1**	1.6	0.8	25	3.1 [b]	1.8	36	2.8	2.3	45	1.3	0.7	25
	C2	2.3	1.5	24	4.4 [a]	3.2	35	2.8	1.5	45	1.3 [a]	0.6	25

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

(iii) Chlorpyrifos-oxon

Exposure to CHPO until 5 or 6 dpf caused decreased distance moved and burst activity values versus respective control in some of the light or dark periods, but no clear tendency was observed (Table 9.11). The peak:basal ratio in general tended to be similar or higher than respective controls, indicating that despite the general reduction in activity the larvae were still capable of reacting to the dark stimulus.

The present results do not support the hypothesis that the metabolite CHPO would present higher neuromodulation than the parent compound CHP due to higher modulation of acetylcholinesterase activity (Qiao et al. 2001). However a factor that might have interfered is the short-life of the compound, reported to be of only one day (Jacobson et al. 2010). Indeed,

semi-static exposure to CHPO led to impairment of axonal growth and touch-evoked response in 72 hpf zebrafish (Yang et al. 2011). Therefore it is recommended that future investigations of behavioral effects of the compound are performed following daily exposure solution changes.

Table 9.11: Behavioral responses at 5 or 6 dpf larvae after prolonged exposures to chlorpyrifos-oxon (CHPO) 33 or 167 µg/L. Distance moved (cm) or burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios for the first and second cycle (C1, C2) for the different exposure concentrations. Significant differences versus respective DMSO control conditions are highlighted.

IVM		DMSO			CHPO 33 µg/L			CHPO 167 µg/L		
5 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
Distance moved (cm)	**L1**	36.5	14.2	36	34.2	16.1	72	33.1	23.2	72
	D1	66.2	9.4	36	66.2	12.5	72	57.5 [a]	14.8	72
	L2	37.5	12.6	36	27.8	15.7	72	26.3 [a]	14.3	72
	D2	60.7	9.3	36	59.6	12.3	72	54.3	15	72
Burst activity (%)	**L1**	0.18	0.31	36	0.11	0.18	72	0.16	0.34	72
	D1	0.87	0.38	36	0.72	0.51	72	0.55 [c]	0.41	72
	L2	0.14	0.14	36	0.1	0.18	72	0.09	0.1	72
	D2	0.57	0.32	36	0.42	0.32	72	0.36 [c]	0.24	72
Peak:basal ratio	**C1**	1.9	0.5	25	2.3	0.9	65	2.4	1.6	66
	C2	1.3	0.3	13	1.9	0.7	54	1.9	0.7	55
IVM		**SK**			**0.1 µM CHPO**			**0.5 µM CHPO**		
6 dpf		**Mean**	**SD**	**N**	**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Distance moved (cm)	**L1**	46	15.4	36	31.9 [c]	12.6	69	35.5 [c]	17.3	70
	D1	65.1	10.1	36	67.3	11.6	69	62.6	9.7	70
	L2	32.4	10.5	36	30.3	15.2	69	29.2	14.4	70
	D2	60.3	9.8	36	61.4	12.6	69	57	9.6	70
Burst activity (%)	**L1**	0.21	0.27	36	0.12 [a]	0.21	69	0.13	0.22	70
	D1	0.49	0.34	36	0.44	0.4	69	0.37	0.27	70
	L2	0.11	0.1	36	0.11	0.2	69	0.08	0.21	70
	D2	0.4	0.26	36	0.38	0.34	69	0.29	0.25	70
Peak:basal ratio	**C1**	1.2	0.1	9	2.0 [c]	0.6	54	1.4	0.3	27
	C2	1.8	0.4	23	1.5	0.5	28	1.0 [c]	0.3	12

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

(iv) Mixtures of α-cypermethrin and chlorpyrifos

Exposure to the aCM and CHP mixtures caused more severe acute effects than respective single compounds, leading to high occurrence of sublethal effects in mixtures containing aCM EC_{10} combined with CHP EC_{10} (60%) or EC_{25} (90%); and reaching 100% of mortality in both mixtures containing aCM EC_{25} combined with CHP EC_{10} or EC_{25} (Table 9.12).

Behavior was therefore assessed only in larvae exposed to the $EC_{10\text{-}aCM}$ and $EC_{10\text{-}CHP}$ mixture, as presented in Table 9.12. Distance moved of exposed larvae was increased at 5 dpf in light periods ($p<0.05$) similarly to the aCM single exposure; but was in general it decreased at 6 dpf ($p<0.01$ or 0.001), as it happened for CHP single exposure. Burst activity was less evidently affected, but it tended to present the same temporal profile as distance moved (Table 9.12). Ratios peak:basal were reduced at 5 dpf ($p<0.001$) and either reduced or increased at 6 dpf ($p<0.05$), indicating that the exposure to the mixture affected the response of the larvae to the dark stimulus. The present results indicate that exposure to mixture of compounds, even if at low-levels such as respective EC_{10} for sublethal effects, can highly impair not only acute toxicity but also behavioral responses in zebrafish larvae.

Table 9.12: Behavioral responses at 5 or 6 dpf larvae after prolonged exposures to the mixture aCM EC_{10} and CHP EC_{10}. Distance moved (cm) or burst activity occurrence (%) in first or second light (L1, L2) or dark (D1, D2) periods, and peak:basal ratios for the first and second cycle (C1, C2) for the different exposure concentrations. Significant differences versus respective DMSO control conditions are highlighted.

IVM		DMSO			Mixture aCM EC_{10} and CHP EC_{10}		
5 dpf		**Avg.**	**SD**	**N**	**Avg.**	**SD**	**N**
Distance moved (cm)	**L1**	39.7	21.00	36	57.06 [a]	31.0931	72
	D1	64.41	11.20	36	58.07	12.2378	72
	L2	34.14	11.77	36	49.66 [a]	26.4935	72
	D2	60.19	11.86	36	54.14	12.8855	72
Burst activity (%)	**L1**	0.18	0.31	36	0.30 [a]	0.36	70
	D1	0.87	0.38	36	0.98	0.54	70
	L2	0.14	0.14	36	0.22	0.27	70
	D2	0.57	0.32	36	0.65	0.36	70
Peak:basal ratio	**C1**	3.02	5.07	36	1.67 [c]	1.19	70
	C2	2.06	0.91	36	1.41 [c]	0.79	70
IVM		DMSO			Mixture aCM EC_{10} and CHP EC_{10}		
6 dpf		**Mean**	**SD**	**N**	**Mean**	**SD**	**N**
Distance moved (cm)	**L1**	46.01	15.16	36	32.22 [c]	14.38	69
	D1	65.05	9.94	36	55.37 [b]	13.99	69
	L2	32.81	18.36	36	30.26	15.06	69
	D2	60.30	9.69	36	49.35 [c]	11.87	69
Burst activity (%)	**L1**	0.21	0.27	36	0.14 [a]	0.19521	64
	D1	0.49	0.34	36	0.60	0.41	64
	L2	0.11	0.10	36	0.13	0.19	64
	D2	0.40	0.26	36	0.48	0.33	64
Peak:basal ratio	**C1**	1.79	0.67	36	2.94 [a]	4.93	64
	C2	2.36	0.85	36	1.96 [a]	1.30	64

a: $p<0.05$, b: $p<0.01$, c: $p<0.001$.

9.4 Conclusions

The behavioural test protocol for light-dark transition test assessment applied in this study was able to identify effects of neuroactive and neurotoxic chemicals on the behaviour of zebrafish 5-6 dpf larvae. The new parameter peak:basal ratio, together with distance moved and burst activity, supported the interpretation of responses to the different chemicals. The self-built system produced at RWTH presented in general consistent results with those obtained at IVM. Even though some differences occurred, it is important to consider that other factors such as different strains can play a role. In order to properly investigate interlaboratory differences, a specific study should be designed and performed. Regarding life stage for behavioural assessment, 5 dpf larvae showed to be a suitable model presenting sensitivity and consistent behavioural responses. Summed to the fact that these are not protected stages, behavioural assessment should, always that possible, be performed with zebrafish up to 5 dpf of age. Among tested candidate positive control chemicals, ethanol produced the more robust responses, eliciting either stimulation or inhibition of responses depending on concentration; and presenting consistent responses even after 40 min of exposure. Caffeine and nicotine instead presented very brief stimulatory effects, followed by inhibitory effects only. Therefore short exposures to ethanol 1 to 3 % are recommended to be applied in positive control conditions for light-dark transition tests with zebrafish 5 dpf larvae. The tested insecticides presented less evident pattern of behavioural responses, which can be related to inadequate exposure scenarios. It is recommended that future studies investigate the temporal profiled of α-cypermethrin and chlorpyrifos effects on early life stage behaviour. Exposure to mixtures of the compounds led to much higher acute toxicity than observed for single chemicals, and also affected substantially the behaviour of exposed larvae.

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Chapter 10: Bioassay battery interlaboratory investigation of emerging contaminants in spiked water extracts – towards the implementation of bioanalytical monitoring tools in water quality assessment and monitoring

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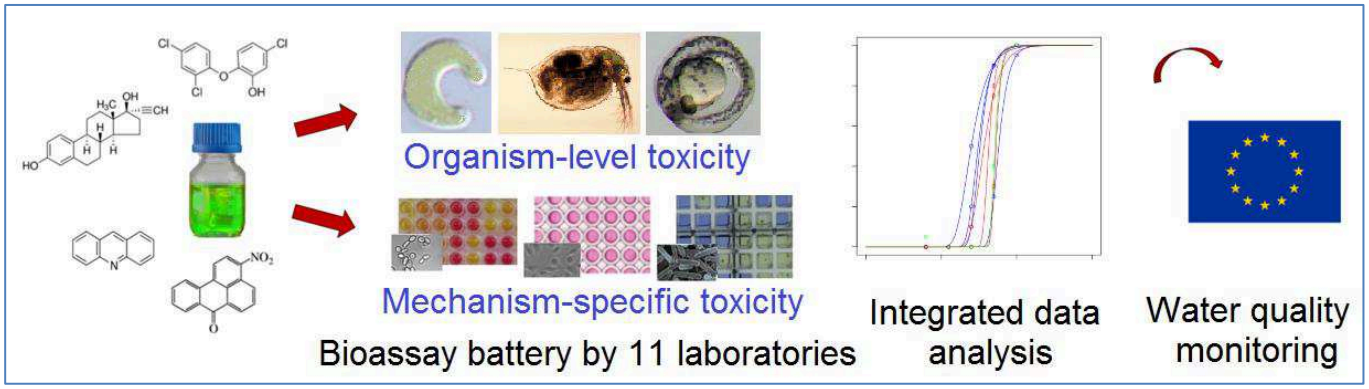

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Abstract

Bioassays are particularly useful tools to link the chemical and ecological assessments in water quality monitoring. Different methods cover a broad range of toxicity mechanisms in diverse organisms, and account for risks posed by non-target compounds and mixtures. Many tests are already applied in chemical and waste assessments, and stakeholders from the science-police interface have recommended their integration in regulatory water quality monitoring. Still, there is a need to address bioassay suitability to evaluate water samples containing emerging pollutants, which are a current priority in water quality monitoring. The presented interlaboratory study (ILS) verified whether a battery of miniaturized bioassays, conducted in 11 different laboratories following their own protocols, would produce comparable results when applied to evaluate blinded samples consisting of a pristine water extract spiked with four emerging pollutants as single chemicals or mixtures, i.e. triclosan, acridine, 17α-ethinylestradiol (EE2) and 3-nitrobenzanthrone (3-NBA). Assays evaluated effects on aquatic organisms from three different trophic levels (algae, daphnids, zebrafish embryos) and mechanism-specific effects using in vitro estrogenicity (ER-Luc, YES) and mutagenicity (Ames fluctuation) assays. The test battery presented complementary sensitivity and specificity to evaluate the different blinded water extract spikes. Aquatic organisms differed in terms of sensitivity to triclosan (algae>daphnids>FET) and acridine (FET>daphnids>algae) spikes, confirming the complementary role of the three taxa for water quality assessment. Estrogenicity and mutagenicity assays identified with high precision the respective mechanism-specific effects of spikes even when non-specific toxicity occurred in mixture. For estrogenicity, although differences were observed between assays and models, EE2-spike relative induction EC50 values were comparable to the literature, and E2/EE2 equivalency factors reliably reflected the sample content. In the Ames, strong revertant induction occurred following 3-NBA-spike incubation with the TA98 strain, which was of lower magnitude after metabolic transformation and when compared to TA100. Differences in experimental protocols, model organisms, and data analysis can be sources of variation, indicating that respective harmonised standard procedures should be followed when implementing bioassays in water monitoring. Together with other ongoing activities for the validation of a basic bioassay battery, the present study is an important step towards the implementation of bioanalytical monitoring tools in water quality assessment and monitoring.

Keywords: bioassay test battery; interlaboratory study; emerging pollutants; water quality assessment; water quality monitoring.

10.1 Introduction

Water quality investigation and monitoring in Europe and worldwide is facing a challenge. There is societal, regulatory and scientific consensus on the urgent need to achieve good water quality in national and transboundary river basins. Meanwhile, an immense variety of contaminants is constantly reaching aquatic systems, which complicates the identification of drivers of chemical toxicity to be routinely monitored (von der Ohe et al. 2011). Further, there is a lack of direct indicators on the regulatory level to verify the biological relevance of chemical monitoring in different water bodies. While the ecological status assessment is certainly of high environmental relevance, it is based primarily on biodiversity indices that often do not present consistency with respective chemical monitoring (Wernersson et al. 2015). Therefore, complementary monitoring strategies are required to achieve the Water Framework Directive (WFD) aim to maintain and improve water quality in Europe (European_Union 2000).

Effect-based tools such as bioassays and biomarkers are particularly useful to bridge the gap between chemical contamination and ecological status, since they can cover a broad range of toxicity mechanisms in diverse organisms, and account for additional risks posed by non-target compounds and mixtures. Bioassays already provide the regulatory basis to derive environmental quality standards (EQS) (Weigt et al. 2011) and to evaluate pelagic toxicity under the REACH authorization process (ECHA 2014). They are also applied to assess effluents from domestic wastewater treatment plants and industrial sectors (OSPAR 2007, Gartiser et al. 2009). Moreover, the recommendation to integrate bioassays in regulatory water quality monitoring (Hecker and Hollert 2011, Hamers et al. 2013) is supported by many tests being available as standardized methods (OECD guidelines, ISO standards). However, there are still open questions that prevent their application in effect-based monitoring of water bodies. A major issue is whether reliable results can be achieved when evaluating effects of samples containing diverse aquatic pollutants and chemical mixtures. Particularly, the evaluation of emerging contaminants, such as pharmaceuticals, personal care and disinfection products, is a current priority in regulatory water quality monitoring (Loos et al. 2009, Brack et al. 2012).

In response to that, the present interlaboratory study (ILS) investigated weather a battery of miniaturized bioassays would produce consistent results for the evaluation of blinded samples containing pristine water extract spiked with representative emerging pollutants as single-chemicals or mixtures. These included:

(i) Triclosan, a chlorinated phenoxy phenol used as biocide in personal care and household products, already suggested as candidate priority substance (von der Ohe et al. 2012);

(ii) 17α-ethinylestradiol (EE2), a synthetic estrogenic human and veterinary pharmaceutical recently included in the European chemical watch list for water quality monitoring (EC 2013, Kunz et al. 2015);

(iii) Acridine, an heterocyclic aromatic hydrocarbon of industrial origin and a carbamazepine transformation product found in aquatic sediments and groundwater (Hartnik et al. 2007, de Voogt and Laane 2009);

(iv) 3-Nitrobenzanthrone (3-NBA), a potent mutagenic diesel exhaust component that occurs in aquatic sediments and rainwater (Murahashi et al. 2003, Lübcke-von Varel et al. 2012).

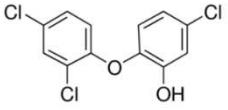
The water extract included a realistic environmental matrix as a sample component, increasing the relevance of the study for water quality assessment. Methods evaluated effects on organisms from three trophic levels (algae, daphnids, fish); and mechanism-specific effects using *in vitro* estrogenicity and mutagenicity assays. The resulting interlaboratory trial brings a novel approach since, with very few exceptions (Carvalho et al. 2014, Escher et al. 2014), previous bioassay ILS focused on only one or few methods, a single mode of action, or single chemical or sample (Hoss et al. 2012, Reifferscheid et al. 2012, Feiler et al. 2014). Finally, a unique aspect of this study that is reflected in the discussion is the clear aim to promote the regulatory use of bioassays for water quality monitoring at the European policy-makers level.

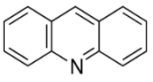
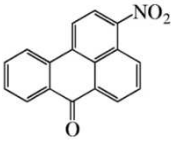
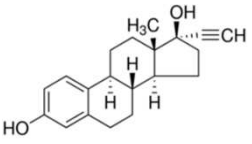
10.2 Material and Methods

10.2.1 Chemicals

Information on the test chemicals is provided in Table 10.1.

Table 10.1: Chemical properties of the compounds used for water extract spiking.

Chemical	CAS number	Formula	Supplier	Purity	Structure	Molecular weight (g mol^{-1})	logK_{ow}	Solubility in water (mgL^{-1})
Triclosan	3380-34-5	$C_{12}H_7Cl_3O_2$	Sigma-Aldrich (Germany)	≥97%		289.6	4.76 [a]	10 (20°C) [a]

Acridine	260-94-6	$C_{13}H_9N$	Merck (Germany)	>98%		179.2	3.40 [a]	38.4 (24°C) [a]
3-Nitrobenzanthrone (3-NBA)	17117-34-9	$C_{17}H_9NO_3$	Chiron AS (Norway)	>98%		275.3	4.5 [b]	0.025 [b]
17α-Ethinylestradiol (EE2)	57-63-6	$C_{20}H_{24}O_2$	Sigma-Aldrich (Germany)	≥98%		296.4	3.67 [a]	11.3 (27°C) [a]

a: National Center for Biotechnology Information. PubChem Compound Database (September 2015)

b: Predicted data, US Environmental Protection Agency's EPISuite™, KOWWIN v1.67 estimate.

10.2.2 Participant institutes

The study was coordinated by the Department of Ecosystem Analysis, Institute for Environmental Research, RWTH Aachen University, Germany. The 11 participant laboratories (Table 10.2) are associates of the NORMAN working group on bioassays and biomarkers.

Table 10.2: Laboratories that performed bioassays within the interlaboratory study.

Code number	Name of institution
1	Department Biochemistry and Ecotoxicology, Federal Institute of Hydrology - BfG, Koblenz, Germany
2	Swiss Centre for Applied Ecotoxicology Eawag-EPFL, Dübendorf, Switzerland
3	National Research Centre for Environmental Toxicology - Entox, the University of Queensland, Brisbane, Australia
4	Laboratoire d'Ecotoxicologie, Ifremer, L'Houmeau, France
5	INERIS, Verneuil-en-Halatte, France
6	Scientific Institute of Public Service - ISSeP, Liège, Wallonia, Belgium
7	Department of Applied Environmental Science - ITM, Stockholm University, Stockholm, Sweden
8	Institute for Environmental Studies - IVM, VU University Amsterdam - VUA, the Netherlands
9	Research Centre for Toxic Compounds in the Environment - RECETOX, Faculty of Science, Masaryk University, Brno, Czech Republic;
10	Institute for Environmental Research, RWTH Aachen University, Aachen, Germany
11	Waternet Institute for the Urban Water Cycle Division of Technology Research & Engineering, Amsterdam, Netherlands; Waterproef Laboratory, Edam, the Netherlands.

10.2.3 Battery of bioassays

The bioassay battery (Tables 10.3 and 10.4) evaluated effects on organisms from different trophic levels: unicellular green algae growth inhibition (Algae), daphnid immobilization (*Daphnia*), and zebrafish embryo lethality and morphological effects (FET). Mechanism-specific assays evaluated estrogenicity (ER-Luc and YES) and mutagenicity (Ames). Experiments were performed in miniaturized format following static exposure without vessel pre-incubation with test solutions.

Table 10.3: Performed bioassay and respective method title, endpoints, model organisms, exposure duration and different protocols.

Bioassay	Method title	Endpoints / expressed results	Model organism	Exposure duration (h)	Exposure vessels	Medium per vessel or well (mL)	Followed protocols
Algae test	Freshwater algal growth inhibition test	Growth inhibition / Growth inhibition normalized to solvent control	*Pseudokirchneriella subcapitata*	72	96-well plates	0.2	OECD Test No. 201 or ISO 8692:2012 modified to 96-well plate
	Combined algae assay	Inhibition of microalgae growth and photosynthesis / Growth and photosynthesis inhibition normalized to solvent control	*P. subcapitata*	24	96-well plates	0.3	(Escher et al. 2008)
Daphnia test	*Daphnia sp.* acute immobilisation test	Immobilization of daphnids / Immobilization occurrence	*D. magna*	48	96-well plates Glass tubes Glass beakers	0.2 10 20	OECD Test No. 202 or ISO 6341:2012
FET test	Fish embryo acute toxicity test	Fish embryo lethality and occurrence of morphological sublethal endpoints / Occurrence of survival and cumulative occurrence of lethal and sublethal morphological endpoints	*Danio rerio*	96	96-well plates	0.2	OECD Test No. 236 with observation of sublethal morphological endpoints modified to 96-well plate
YES assay	Yeast estrogen screening assay	Estrogen receptor binding activity / Induction values converted to % of standard maximum response (after subtracting the solvent response from both sample and standard)	Recombinant yeast cells	18-72	96-well plates	0.2	ISO/TC 147/SC 5 N 804 or (Routledge and Sumpter 1996)
				2.5	96-well plates	0.2	(Leskinen et al. 2003, Leskinen et al. 2005)
ER-Luc assay	Estrogen receptor luciferase reporter-gene assays with permanent cell lines	Estrogen receptor binding activity / Induction values converted to % of standard maximum response (after subtracting the solvent response from both sample and standard)	T47D-kbLuc human breast cancer cells	19-24	96-well plates	0.2	(Wilson et al. 2004)

Table 10.4: Protocols followed by each laboratory for the different bioassays.

Bioassay short-name and method tile		Protocols by laboratories
Algae test	Freshwater algal growth inhibition test with unicellular green algae	10, 9 and 11: OECD Test No. 201 (OECD 2011) or ISO 8692:2012 (ISO 2012b) modified to 96-well plate 2 and 3: Combined Algae assay (Escher et al. 2008)
Daphnia test	*Daphnia magna* acute immobilisation test	5, 6, 7, 10 and 11: OECD Test No. 202 (OECD 2011) or ISO 6341:2012 (ISO 2012a) performed in 96-well plates, glass tubes or glass beakers
FET test	Fish Embryo Acute Toxicity Test with *Danio rerio*	4, 9 and 10: OECD Test No. 236 (OECD 2013b) modified to 96-well plate and with observation of sublethal morphological endpoints (Nagel 2002, Lammer et al. 2009)
YES	Yeast Estrogen Screen	1: β-galactosidase recombinant yeast (McDonnell et al. 1991), ISO/TC 147/SC 5 N 804 (ISO 2013) 6: β-galactosidase recombinant yeast (Routledge and Sumpter 1996) 9: Luciferase recombinant yeast (Leskinen et al. 2003, Leskinen et al. 2005)
ER-Luc assay	Cell-based estrogen receptor reporter gene assay	5: T47D-kbLuc human breast cancer cells (Wilson et al. 2004) 8: BG1Luc4E2 human ovarian cancer cells (Rogers and Denison 2000, OECD 2012d) 10: ER-CALUX assay (Maletz et al. 2013, Besselink 2015)
Ames assay	Ames fluctuation assay	1, 8 and 10: ISO standard 11350 (ISO 2012a) 3: (Reifferscheid et al. 2012, Escher et al. 2014).

10.2.4 Water sample extract spiking

A 180 L water sample was collected at the pristine creek Wormsgraben (Harz Mountains, Germany), transported to the laboratory in stainless steel drums, extracted using large-volume solid phase extraction (Schulze et al. in preparation), and concentrated in 18 mL methanol. The water extract was evaluated in some bioassays (Table 10.4) by the coordinator.

Chemicals for spiking (Table 10.1) were selected due to relevance as emerging pollutants and bioactivity. Effect-data from previous studies and own preliminary tests (Table 10.5) provided the basis for spiking composition decision.

Table 10.5: Bioeffects of test chemicals described in the literature. Except where indicated, reported effect-data are the EC_{50} values.

Bioassay	**Triclosan** (µg/L, except where indicated)	**Acridine** (mg/L)	**3-Nitrobenzanthrone** (revertants / nmol, or µg/L) [c]	**17-α-Ethinylestradiol** (ng/L, except where indicated) [c]
Algae test (72 h growth, except where indicated) *Desmodesmus subspicatus*	2.8 [b] (Orvos et al. 2002)	4.5 [c] / 2.1 [b] (Eisentraeger et al. 2008)	No data available	0.84 mg/L (24h biomass) (van Vlaardingen PLA 2008), 12.35 mg/L (24 h photosynthesis) (Escher et al. 2005)
Pseudokirchneriella subcapitata (Raphidocelis subcapitata / Selenastrum capricornutum)	4.5 [b] (Orvos et al. 2002), 12 [c] (96h) (Harada et al. 2008), 37 [c] (Rosal et al. 2010), 5.1 [c] (Tamura et al. 2013), 4.7 [c] (Tatarazako et al. 2004) , 0.53 [c] (Yang et al. 2008)	0.27 [b] (96h) (Dijkman et al. 1997) 0.9 [b] (96h) (Blaylock et al. 1985)	No data available	
Daphnia test with *D. magna* (48 h, except where indicated)	390 [b] (Orvos et al. 2002), 260 [c] (Harada et al. 2008), 330 [c] (Peng et al. 2013), 856.8 [b] (Silva et al. 2015), 180 [c] (Tamura et al. 2013)	6.8 [c] / 4.6 [b] (Eisentraeger et al. 2008), 2.3 [b] (Parkhurst et al. 1981), 3.1 (96 h) (Blaylock et al. 1985), 4.2 [c] (24 h), 2.4 (Feldmannová et al. 2006)	No data available	NOEC 5 mg/L (Goto and Hiromi 2003) , 5.7 mg/L (24h) (van Vlaardingen PLA 2008))
FET test *D. rerio*, (lethality)	420 [c] (96h) (Oliveira et al. 2009)	0.66 (48 h) (Peddinghaus et al. 2012)	No effect [a]	1.7 mg/L (96h adult)
ER-Luc assay				
T47DKB-Luc	<LOD for 1 µg/mL [a,c]	No data available	No data available	2.5 (Wilson et al. 2004), 0.07 (Bermudez et al. 2012)
BG1Luc ERTA	No data available	No data available	No data available	2.0 (OECD 2012d)
ER-CALUX, T47D.Luc	<LOD for 1 µg/mL [a,c]	EEF50: 3.18 x 10^{-5} [c] (Brinkmann et al. 2014b)	No effect [a]	1.5 (Legler et al. 2002), 1.5 (Murk et al. 2002)
YES assay β-galactosidase - Routledge and Sumpter 1996	4.2 [c] (Svobodová et al. 2009)	No data available	No data available	51.6 (Van den Belt et al. 2004), 57.2 (Balsiger et al. 2010), 166 (Schultis and Metzger 2004), 24.7 (Murk et al. 2002)
β-galactosidase - McDonnell	No data available	No data available	No data available	80.4 [a]
LYES-assay (modified Routledge and Sumpter)	No data available	Not active (Brinkmann et al. 2014b)	No data available	20 (Schultis and Metzger 2004)
Ames assay Plate (revertants / nmol)	Not mutagenic, cytotoxic (SCCP 2009)	Not mutagenic (Eisentraeger et al. 2008)	TA98-S9: 208,000, TA100-S9: 29,700 (Enya et al. 1997)	No data available
Fluctuation (LOEC, µg/L)	Not mutagenic, cytotoxic [a]	Not mutagenic [a]	TA98-S9: 0.075 [a], TA98+S9: 6 [a], TA100 –S9: 1.5 [a]	No data available

a: own results; b: measured chemical concentrations were the basis to determine the effect-concentration values; c: nominal chemical concentrations were the basis to determine the effect-concentration values.

Two or three spikes were designed per assay (Table 10.6) having either the most active toxicant(s) for each method; or a final chemical mixture containing a fixed ratio of respective single chemical(s). Concentrations aimed to produce full dose-response curves considering as maximum test concentration 1 $\mu L_{extract}/mL_{medium}$, corresponding to an enrichment factor of 10 (10 $mL_{water\text{-}equivalent}/mL_{medium}$).

Spikes for *Daphnia*, FET, ER-Luc and Ames were prepared by water extract evaporation to dryness, addition of DMSO as carrier, and spiking of chemicals using stock solutions in DMSO; followed by separation in aliquots for each participant. For algae and YES, the water extract was spiked with the chemicals in methanol, divided in aliquots, and evaporated to dryness. Aliquots were coded and shipped at room temperature to the laboratories, who were not informed on sample composition during the testing period. DMSO was also provided for solvent control conditions.

Table 10.6: Composition of the spiked water samples for each bioassay, consisting of one or two single-chemical spiking and a chemical mixture for each bioassay

Bioassay	Sample	Composition of spiking of 10,000 times concentrated water extract				Exposure setup		
		Triclosan (mg/mL extract)	**Acridine** (mg/mL extract)	**EE2** (µg/mL extract)	**3-NBA** (µg/mL extract)	**Maximal test concentration** (mL extract / L medium)	**Serial dilution steps**	**Number of tested dilutions**
Algae test	Triclosan	0.1	-	-	-	1-3 [a] 50-33 [b]	1 : 2 (2-fold)	5-7 [a] 16 [b]
	Acridine	-	10	-	-			
	Mixture	0.1	10	100	-			
***Daphnia* test**	Triclosan	1	-	-	-	1	1 : 2 (2-fold)	4-5
	Acridine	-	15	-	-			
	Mixture	1	15	100	2			
FET test	Triclosan	3	-	-	-	0.77	1: 1.3 (1.3-fold)	5
	Acridine	-	2	-	-	1		
	Mixture	3	2	100	2	0.58		
YES assay	EE2	-	-	100	-	0.1-2	3 : 10 and 1 : 3 (3.3 and 3-fold)	9-16
	Mixture	1	2	100	-			
ER-luc assay	EE2	-	-	1	-	0.5-1	1 : 10 (10-fold)	7
	Mixture	1	2	1	-			
Ames assay	3-NBA	-	-	-	2	1	1 : 2 (2-fold)	6
	Mixture	0.1	2	100	2			

a: Freshwater algal growth inhibition test with unicellular green algae

b: Combined algae assay

10.2.5 Exposure setup and tested concentration ranges

Experiments were repeated mostly three times per bioassay, in each test with 3-4 replicate wells/vessels for each test condition following exposure setups described in Table 10.6.

10.2.6 Integrated data and statistical analysis

Bioassay results (expressed as described in Table 10.3) were evaluated following the same data preparation and statistical analysis methods. Results from experimental replicates were pooled and EC_{50} values were calculated for grouped experiments either by 2-parameter Weibull function using R language package (*Daphnia*), two parameter log-logistic curve from 0 to 100% with the two adjustable parameters being slope and EC_{50} by GraphPad Prism 6 (algae, FET, Ames), or four-parameter log-logistic function with GraphPad (ER-Luc, YES) (GraphPad Software, San Diego, CA, USA). Differences between logEC_{50} values from different laboratories were compared by t-test or one-way ANOVA followed by Tukey's multiple comparisons test. EC_{50} values obtained in $\mu L_{extract}/mL_{bioassay}$ (S.I.) were converted to nominal concentrations of individual chemicals contained in each sample. For algae, *Daphnia* and FET, ratios between EC_{50} ($\mu L_{extract}/mL_{bioassay}$) values of single-chemical and mixture spikes ($EC_{50\text{-single}}$:$EC_{50\text{-mixture}}$) were calculated. That allowed comparing single- and mixture-spike effects, since the mixture contained a fixed ratio of triclosan and acridine. For ER-Luc and YES, toxic-equivalent factors to respective standard chemical, 17β-estradiol (E2) or EE2, were obtained. Relative estrogenic potencies are expressed as E2 or EE2 equivalents (EEQ), calculated as a ratio between the EC_{50} of the reference compound and the EC_{50} of the spiked sample: EEQ= $EC_{50\text{-E2 or EE2}}/EC_{50\text{-sample}}$. The only exception was the water extract, for which the EEQ was obtained with the PC10 approach (Besselink 2015).

10.3 Results and Discussion

Result differences are indicated either as not significant (n.s.) or according to p values.

10.3.1 Toxic effects on aquatic organisms

Aquatic organisms differed in terms of sensitivity to triclosan (algae>daphnids>FET) and acridine (FET>daphnids>algae) spikes. Present EC_{50} nominal ($EC_{50\text{-nom}}$) for single-chemical spikes (Fig.10.1, Table 10.7) were in same range as literature data for tests performed in microtiter plates (Table 10.5) but tended to be higher than literature values based on measured

concentrations or for experiments in higher medium volume. $EC_{50\text{-single}}$:$EC_{50\text{-mixture}}$ ratios (Fig. 10.2) are discussed for each bioassay.

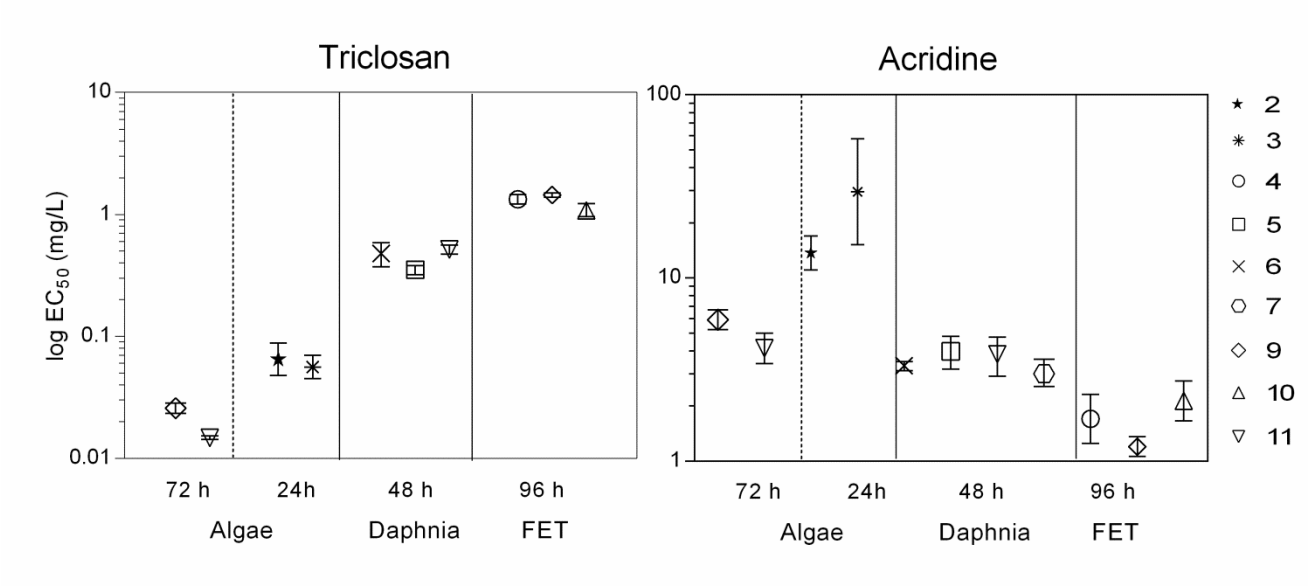


Figure 10.1: Effect-concentration values (log EC_{50} and 95% C.I., mg/L) obtained for pooled data from one to three experiments for each assay for the triclosan (left) and acridine (right) spikes in the algae (72 h or 24 h growth inhibition), Daphnia (48 h immobilization) and FET (96 h cumulative effects) tests. Y-axes correspond to laboratory codes (Table 10.2).

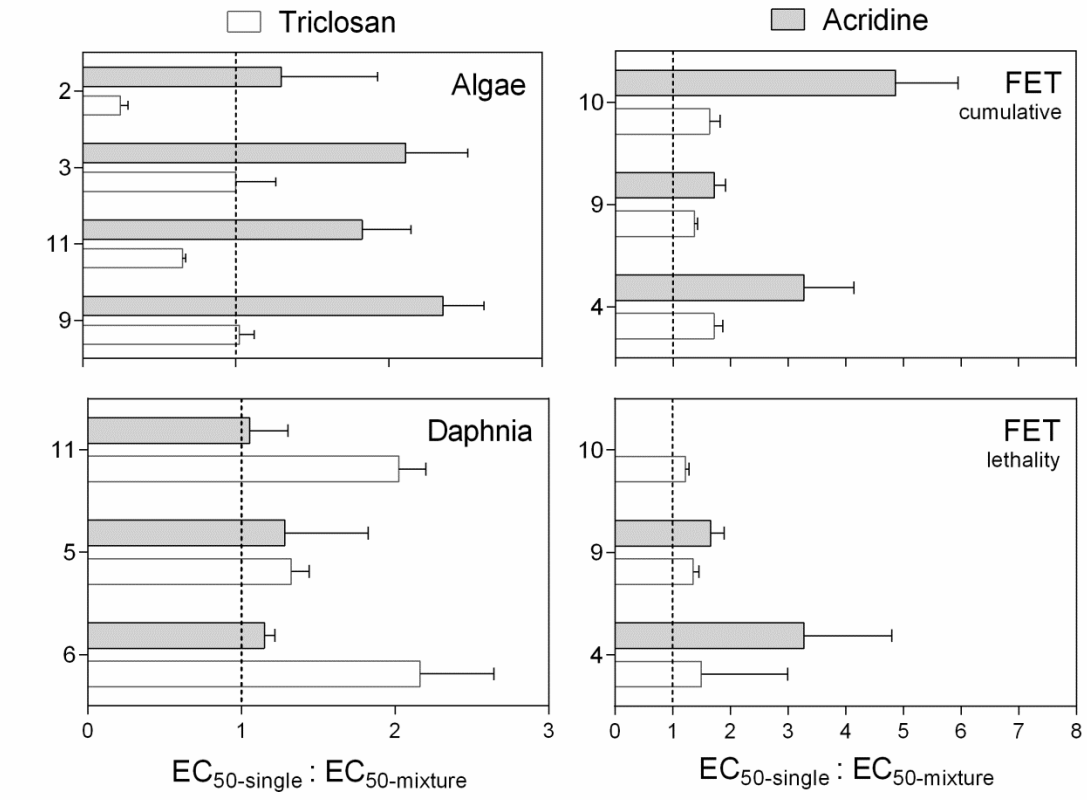


Figure 10.2: Ratios between EC_{50} values (µL/mL) for the single-chemical and mixture spikes containing a fixed ratio of respective single compounds ($EC_{50\text{-single}}$: $EC_{50\text{-mixture}}$) for the triclosan (white bars) and acridine (grey bars) spikes in the algae, Daphnia and FET (cumulative effects and lethality)

tests. Error bars correspond to the ratios between 95% C.I. for single chemicals and the $EC_{50\text{-mixture}}$ value. Numbers correspond to laboratory codes (Table 10.2).

Table 10.7: EC_{50} values for the triclosan (µg/L medium) and acridine (mg/L medium) spikes in the Algae, Daphnia and FET tests.

	Triclosan spiked extract (µg triclosan / L medium)			**Acridine spiked extract** (mg acridine / L medium)		
Algae test Growth inhibition	EC_{50}	95% C.I.		EC_{50}	95% C.I.	
72 h OECD / ISO						
Recetox	25.7	23.3	28.4	5.9	5.2	6.7
Waternet / Waterproef	14.8	14.2	15.3	4.1	3.4	5.0
24 h combined test						
Ecotox Centre	65.0	48.0	88.1	13.7	11.1	17.0
Entox	56.2	45.2	70.0	29.6	15.2	57.2
***Daphnia* test** 48 h immobilization	EC_{50}	95% C.I.		EC_{50}	95% C.I.	
ISSeP	478	372	584	3.8	3.6	4.0
INERIS	351	320	382	5.1	2.9	7.3
ITM	n.a.	n.a.	n.a.	3.0	2.6	3.6
Waterproef	516	471	560	4.0	3.1	5.0
FET test 96 h cumulative effects	EC_{50}	95% C.I.		EC_{50}	95% C.I.	
Ifremer	1,334	1,216	1,463	1.7	1.3	2.3
Recetox	1,440	1,380	1,500	1.2	1.1	1.4
RWTH	1,080	0,960	1,230	2.1	1.7	2.7
FET test 96 h survival	LC_{50}	95% C.I.		LC_{50}	95% C.I.	
Ifremer	1,316	n.a.	n.a.	0.7	0.4	1.3
Recetox	1,919	1,026	3,588	1.3	1.1	1.5
RWTH	1,565	1,486	1,647	1.3	1.1	1.5

n.a.: Values not available (“very wide” in regression).

(i) Algae test

The OECD/ISO Algae test was the most sensitive aquatic organism assay to triclosan, in agreement with freshwater algal growth being more sensitive than endpoints in bacteria, protozoa, macrophytes, daphnids, amphibians and fish (Orvos et al. 2002, Tatarazako et al. 2004, Harada et al. 2008, Tamura et al. 2013). Detected 72 h growth-inhibition $EC_{50\text{-}nom}$ (14.7 and 25.7 µg/L, n.s.) are in the same range as previous 72 and 96 h $EC_{50\text{-}nom}$ for *P. subcapitata* determined also in 96-well plates (Harada et al. 2008, Rosal et al. 2010). However, our values are 3-50 times higher than results obtained by incubation in 20-100 ml of medium (i.e. 100-500 times the present volume) (Orvos et al. 2002, Tatarazako et al. 2004, Yang et al. 2008, Tamura et al. 2013). Since triclosan is relatively hydrophobic, adsorption to the plate material could have occurred (Rojíčková et al. 1998). Triclosan is also prone to phototransformation (Tixier et al. 2002), which could be another source of variability. The OECD TG (2011) already discusses the interference of these aspects with single-chemicals, which can provide a basis for investigating the stability of water extracts components during exposure. Finally, the water extract matrix could have decreased triclosan bioavailability due to its high sorption capacity to organic matter (Reiss et al. 2002).

For acridine, even if our $EC_{50\text{-}nom}$ differed (5.9 and 4.1 mg/L, $p<0.01$), values were in good agreement with previous 72 h $EC_{50\text{-}nom}$ for *Desmodesmus subspicatus* following exposure in 24-well plates (Eisentraeger et al. 2008). However, values were circa one order of magnitude higher than 96 h $EC_{50\text{-}meas}$ for *Selenastrum capricornutum* (current *P. subcapitata*) exposed in 100-250 mL medium (Blaylock et al. 1985, Dijkman et al. 1997). Sensitivity differences are not known for acridine due to non-specific toxicity mechanism (Dijkman et al. 1997). Decrease in exposure concentration instead may be relevant, since 40-60% losses occurred already prior to exposure start, followed by additional circa 10% decrease during 72 h incubation in 24-well plates (Eisentraeger et al. 2008). Therefore for acridine chemical losses during sample shipping, handling and experiments could have interfered with effective test concentrations.

In the combined algae assay, 24 h growth inhibition $EC_{50\text{-}nom}$ values for triclosan (65.0 and 56.2 µg/L, n.s.) and acridine (13.7 and 29.6) spikes were 2-3 and 2-7 times higher than for the OECD tests, respectively. That indicates time-dependency of effects for both chemicals on algae growth. No tendency for specific photosynthesis inhibition was observed since the photosynthesis endpoint was equally or less sensitive than growth inhibition (results not

shown) (Escher et al. 2008, Tang and Escher 2014). Still, this is a very relevant endpoint since many current WFD priority and emerging compounds present this mode of action.

$EC_{50\text{-single}}$:$EC_{50\text{-mixture}}$ ratios for triclosan reached values near or less than1 and were lower than those for acridine, suggesting its effects were prevalent in the mixture. EE2 is not considered to have caused substantial growth inhibition, since the higher exposure concentration (0.1 mg/L) was seven to ten-fold lower than previous NOEC (0.71 mg/L) or LOEC (1.2 mg/L) (Maes et al. 2014).

(ii) Daphnia test

The OECD/ISO *Daphnia* immobilization test presented intermediate sensitivity to both triclosan- and acridine-spikes. Present triclosan 48 h immobilization $EC_{50\text{-nom}}$ (351 to 516 µg/L, n.s.) are in similar range as previous studies (Orvos et al. 2002, Harada et al. 2008, Peng et al. 2013). The compound was also found to cause effects in *D. magna* reproduction test lasting 21 days, with LOEC values for reduced number of neonates being circa half of respective 48 h immobilization EC_{50} (Orvos et al. 2002, Peng et al. 2013).

Also for acridine the obtained $EC_{50\text{-nom}}$ (3.0 to 5.1 mg/L, n.s.) agree with previous results (Blaylock et al. 1985, Feldmannová et al. 2006, Eisentraeger et al. 2008). Acridine caused also reduction in offspring number produced per brood in semi-static exposure during 14 d, with the LOEC being less than half of respective acute EC_{50} (Blaylock et al. 1985).

Considering $EC_{50\text{-single}}$:$EC_{50\text{-mixture}}$ ratios, acridine values were near 1 and lower than for triclosan, indicating that its effects were prevalent in the mixture. EE2 effects are considered to be negligible, since its highest exposure concentration (0.1 mg/L) was 50 times lower than previous NOEC (Goto and Hiromi 2003). Although no information for 3-NBA was found in the literature, acute effects are not considered relevant due to low concentrations.

(iii) FET test

The OECD FET test presented the lowest sensitivity to triclosan and the highest sensitivity to acridine among aquatic organism tests.

Triclosan 96 h $LC_{50\text{-nom}}$ (1.3 to 1.9 mg/L, n.s.) and $EC_{50\text{-nom}}$ (Table 10.7) are circa three times higher than previous 96 h $LC_{50\text{-nom}}$ for zebrafish embryos exposed in 24-well plates (Oliveira et al. 2009) or medaka in petri dishes under semi-static conditions (Ishibashi et al. 2004). This discrepancy could be related to differences in medium volumes and ratios surface area to volume of exposure vessels. However, triclosan concentrations decreased to circa half

even in 1 L of water after 24 h adult medaka exposure (Ishibashi et al. 2004). Therefore other factors could play a role such as phototransformation, which can be minimized by incubation in dark. Among sublethal effects, reduced growth and delayed development were prevalent, similarly to effects in *Xenopus laevis* embryos (Harada et al. 2008). Triclosan was also related to delayed swim-up behaviour initiation and reduced survival in rainbow trout early-life stages (Orvos et al. 2002); and to disrupted swimming and predator avoidance in fathead minnow larvae (Cherednichenko et al. 2012, Fritsch et al. 2013). We observed increased heartbeat rates at 96 h in zebrafish exposed to 1.0 and 1.3 mg/L (Fig. 10.3A), concentrations which caused none and circa 10% cumulative effects, respectively (Fig. 10.3B). Since triclosan can impair the excitation-contraction coupling of cardiac and skeletal muscle (Cherednichenko et al. 2012, Fritsch et al. 2013), increased compensatory heartbeat rate could have occurred. Therefore the assessment of sublethal endpoints can support the identification of toxic effects other than lethality (Di Paolo et al. 2015a, Jonas et al. 2015).

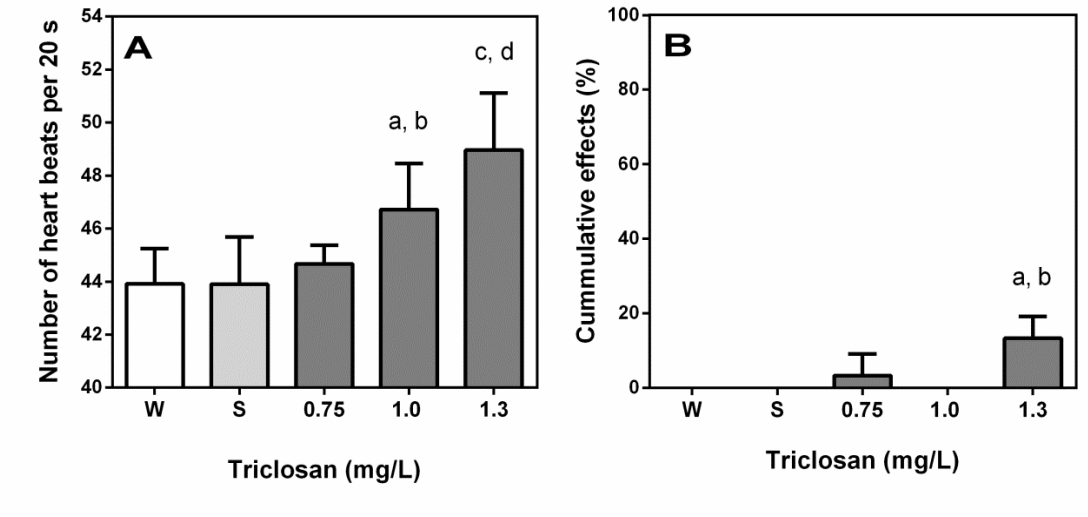


Figure 10.3: Number of heart beats in 20 seconds (A) and cumulative lethal and morphological effects (B) in 96 hpf zebrafish after exposure to triclosan concentrations of 0.75, 1.0 and 1.3 mg/L; and to water (W) and DMSO (S) controls. Average values and standard deviation for 3 experiments. Significant differences from water (a: $p<0.01$, c: $p<0.001$) and solvent (b: $p<0.05$, d: $p<0.001$) controls. One-way ANOVA followed by Tukey's multiple comparisons test.

For acridine, FET 96 h $LC_{50\text{-nom}}$ (0.71 to 1.28 mg/L, n.s.) were circa three times lower than those from *Daphnia* and algae tests. Present values are slightly higher than previous measured 48 h LC_{50} performed in 24-well plates (Peddinghaus et al. 2012). That can be related to possible acridine losses before and during experiments, since concentrations were shown to decrease to less than half of nominal values (Peddinghaus et al. 2012). Performance of semi-static exposure with solution renewal could be a possible solution to maintain exposure concentrations (OECD 2013b).

Considering the $EC_{50\text{-single}}$:$EC_{50\text{-mixture}}$, triclosan tended to present lower values when compared to acridine, indicating it was prevalent in the mixture toxicity. EE2 effects are considered to be negligible, since its highest exposure concentration (0.1 mg/L) was 50 times lower than previous NOEC (5 mg/L) (Goto and Hiromi 2003). For 3-NBA, although no information was found in the literature, acute effects are considered to be negligible.

10.3.2 Estrogenicity assessment

Although differences occurred between different estrogenicity assays and models, relative induction EC_{50} values were comparable to the literature, and obtained EEQ for the EE2-spike are in good agreement with previous values for ER-Luc and YES (Figure 10.4, Table 10.8).

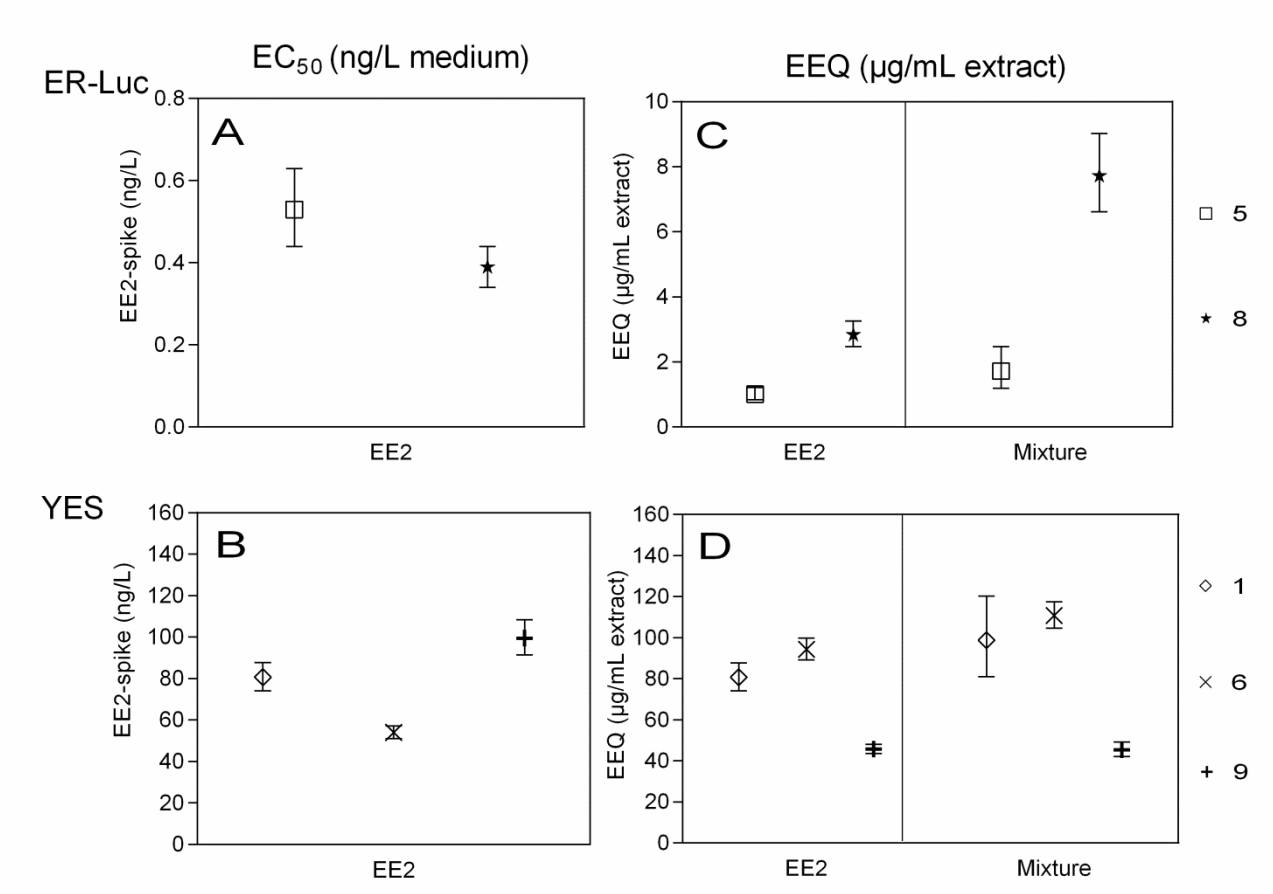


Figure 10.4: EC_{50} (ng/L) values for EE2 in the ER-Luc (A) and YES (B) assays, and EEQ values obtained for the EE2 and the mixture spikes in the ER-Luc (C) and the YES (D) assays. EC_{50} values (symbols) and 95% C.I. (error bars) for respective sample. Results are presented according to laboratory code numbers (Table 10.3). Biological models are: T47D-kbLuc (5) BG1Luc4E2 (8), β-galactosidase recombinant yeast by McDonnell et al. 1991 (1), β-galactosidase recombinant yeast by Routledge and Sumpter 1996 (6), and luciferase recombinant yeast by Leskinen et al. 2003 (9).

Table 10.8: EC_{50} (ng EE2 /L medium) and EEQ (µg E2 or EE2 / mL extract) values obtained in the ER-Luc assay and in the YES assays for the EE2 single-chemical extract.

	17-α-Ethinylestradiol spiked extract			
	EC_{50} (ng EE2 / L medium)			EEQ (µg/mL extract)
ER-Luc assay	EC_{50}	95% C.I.		
INERIS - T47DKB-Luc	0.53	0.44	0.63	1.0 (0.8 - 1.2)
IVM - BG1Luc4E2	0.38	0.33	0.43	2.8 (2.5 - 3.3)
YES assay	EC50	95% C.I.		EEQ (µg/mL extract)
BfG [a] - β-galactosidase yeast assay by McDonnell et al. (1991)	99.5	91.5	108.3	80.8 (74.2 - 87.8)
Recetox - bioluminescent yeast assay by Leskinen *et al.* (2005)	132.7	126.5	139.3	45.8 (43.6 - 48.0)
ISSeP - β-galactosidase yeast assay by Routledge and Sumpter (1996)	54.1	51.1	57.2	94.3 (89.1 - 99.8)

a: EEQ calculated in reference to EE2 (used as standard chemical in assay). All other EEQ values were calculated in reference to E2.

(i) ER-Luc assay

Among all assays performed by the coordinator (Table 10.4), the non-spiked water extract was active only in the ER-Luc (ER-CALUX), with an EEQ of 0.17±0.01 ng/L_{water} for the enrichment factor of 1. EE2-spike induction EC_{50} (0.53 and 0.39 ng/L_{medium}, n.s.) were within the range of previously reported values for EE2 (Legler et al. 2002, Murk et al. 2002, Wilson et al. 2004, Bermudez et al. 2012, OECD 2012d). Although EEQ values showed some variation (Fig. 10.4C), which could be related to differences in assay protocol or model sensitivity (Jarošová et al. 2014), EEQ determination showed to be a reliable measurement for sample content.

Considering the mixture-spikes, concentrations ≥0.5 µ$L_{extract}$/mL_{medium} caused cytotoxicity and were excluded from regression analysis. This effect is considered to be caused by triclosan concentrations (≥0.5 mg/L_{medium}) in the cytotoxic range for human cells (Henry and Fair 2013); while no acridine cytotoxicity is indicated (Brinkmann et al. 2014b). Tendency for higher EEQ values was observed for the mixture-spikes (Fig. 10.4C). It could be discussed that such response is related to estrogen receptor binding by other chemicals in mixture, since acridine induction in T47Dluc assay produced an estradiol equivalency factor (EEF) of $2.5.10^{-7}$ (Brinkmann et al. 2014b). However there is no evidence of triclosan agonism in estrogen-

receptor reporter gene cell-based assays (own results) (Ahn et al. 2008). More likely, non-specific effects on cellular membranes or metabolism (Ajao et al. 2015) could have interfered with induction.

(ii) YES assay

Our induction EC_{50} for the EE2-spike varied up to 2.5-fold (54.1 to 132.7 ng/L, $p<0.01$ to 0.0001), in similar range to literature data (Table 10.5). The lowest EE2-spike EC_{50} was produced by the Routledge/Sumpter strain (1996), in agreement with previous studies (Van den Belt et al. 2004, Balsiger et al. 2010), while the bioluminescent strain (Leskinen et al. 2005) produced the highest value. For the McDowell/ISO assay (ISO 2013), the EC_{50} of 99.5 ng/L was slightly higher than the EC_{50} obtained for the standard curve (80.4 ng/L), which also uses EE2 in this assay. EEQ values varied circa 2-fold (45.8 to 94.3 $\mu g/mL_{extract}$), which can be related to the fact that different yeast strains and protocols can produce different EEF values (Svobodová et al. 2009, Jarošová et al. 2014). Therefore for the application of estrogenicity assays in water quality, effect-concentrations for the standard chemical, main estrogens and investigated samples should be determined using the same model and protocol (Jarošová et al. 2014, Kunz et al. 2015).

The highest mixture-spike test concentrations (≥ 0.1 $\mu L_{extract}/mL_{medium}$) caused cytoxicity to the yeast cells and were excluded from regression analysis. This is attributed mostly to triclosan (≥ 0.1 mg/L_{medium}), since acridine concentrations are not expected to be toxic to the yeast cells (Brinkmann et al. 2014b). No differences occurred between respective EEQ values for single and mixture spikes (Fig. 10.4D). Previously, acridine was not identified as estrogenic by the lyticase YES assay (Brinkmann et al. 2014b). Although triclosan was active in the Routledge/Sumpter strain, the compound was not identified as estrogenic in the bioluminescent YES (Svobodová et al. 2009).

10.3.3 Mutagenicity assessment by the Ames fluctuation assay

Strong revertant induction occurred following 3-NBA-spike incubation with the TA98 strain in the absence of S9 fraction (-S9) (Fig. 10.5A), which was of lower magnitude after metabolic transformation and for TA100 -S9 (Fig. 10.5B and 10.5C). 3-NBA-spike revertant induction EC_{50} values (Table 10.9) were 0.21 and 1.56 µg/L ($p<0.01$) for TA98-S9; and 5.73 µg/L for TA100-S9. Such results are in agreement with previous studies describing 3-NBA as a strong direct-acting mutagen in the TA98 strain, and the fact that it is less active in TA100 suggests that it causes frameshift-type mutations (Enya et al. 1997, IARC 2014). Further,

there are indications that 3-NBA is also genotoxic *in vitro* and in *in vivo* (Watanabe et al. 2005b). 3-NBA is a major mutagen in diesel particles, sediments, and surface soils (Enya et al. 1997, Watanabe et al. 2005a, Lübcke-von Varel et al. 2012); and concentrations up to 2.6 ng/L were identified in rainwater (Murahashi et al. 2003).

For the mixture-spike, test concentrations ≥0.5 $\mu L_{extract}/mL_{medium}$ caused toxic effects in –S9 exposures (attributed to triclosan 50 ng/mL medium), which were excluded from regression analysis (Fig. 10.5B). Cytotoxic effects were reduced by the S9 mix incubation, suggesting that resulting triclosan metabolites present less toxic effects than the parent compound. Our results showed that neither triclosan nor acridine caused increase in the number of revertants, in agreement with previous studies investigating their mutagenicity through the Ames plate incorporation method (Eisentraeger et al. 2008, SCCP 2009).

Table 10.9: EC_{50} (µg/L) values for the 3-NBA single-chemical extract obtained in the Ames fluctuation assay.

	3-NBA spiked extract		
Ames fluctuation assay Revertant induction	EC_{50} (µg 3-NBA /L medium)	95% C.I.	
TA98 –S9			
BfG	0.21	0.19	0.23
IVM	1.56	1.33	1.82
TA100 –S9			
Entox	5.73	3.89	8.51

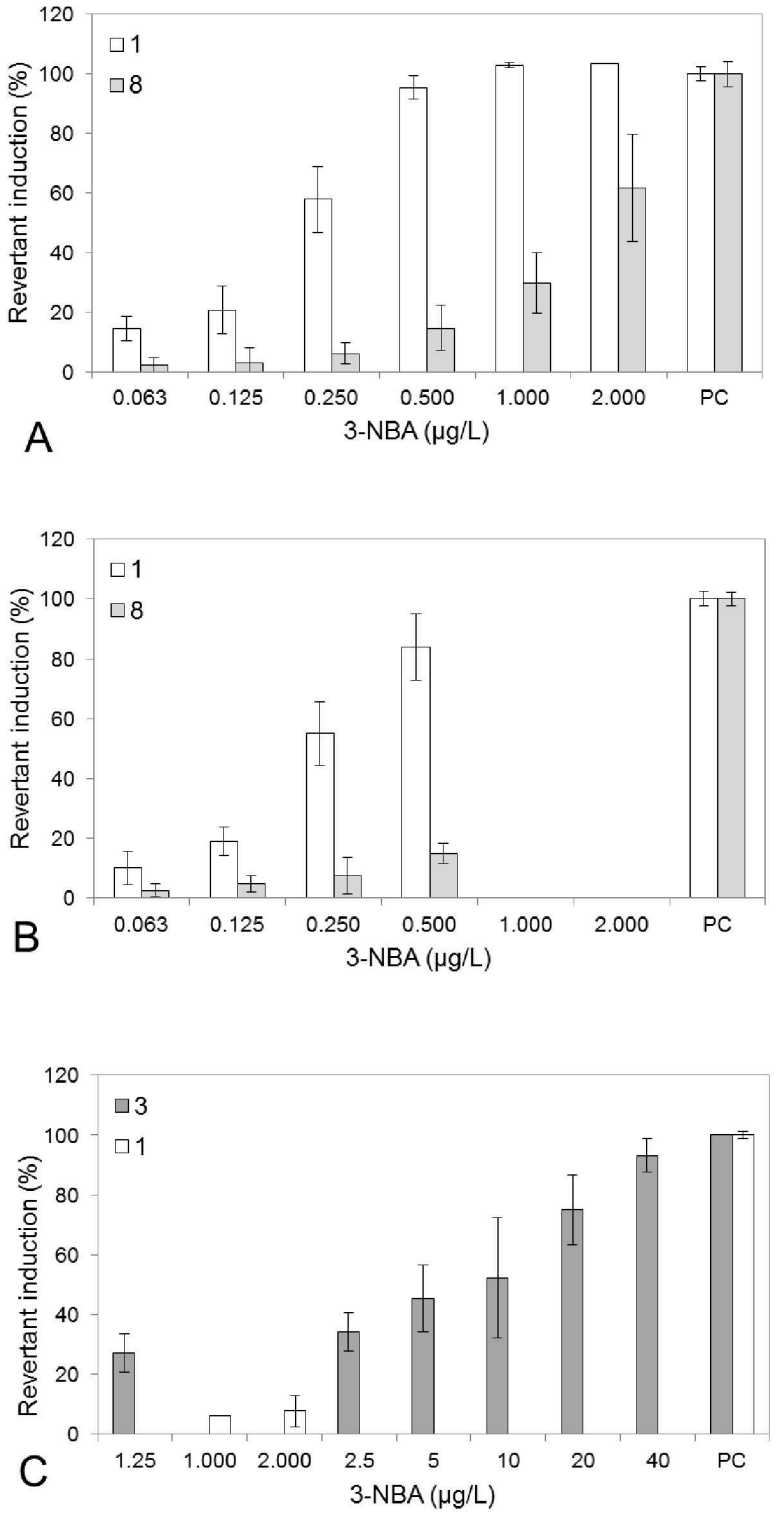


Figure 10.5: Revertant induction versus 3-NBA concentrations (µg/L) contained in (A) 3-NBA-spike in TA98-S9, (B) mixture-spike in TA98-S9, and (C) 3-NBA-spike in TA100-S9; plus respective positive control (PC) conditions. Average values (bars) and standard deviations (error bars) for two to three experiments. Results are presented using laboratory code numbers (Table 10.2).

10.3.4 Bioassay battery strategy

Bioassay battery assessment of water quality is based on the consideration that one single bioassay does not provide an overview on potential effects on different organisms and toxicity mechanisms. Since sensitivity to different toxicants varies between organisms, multi-taxa assessment supports the comprehension of toxicant effects on aquatic communities (Guillen et al. 2012). While the algae test investigates population-level effects in primary producers, the *Daphnia* and FET tests provide information on the toxicity to filter-feeder invertebrate and fish individuals, respectively. Multi-taxa toxicity assessment is applied for EQS derivation within the WFD, which requires evaluation of acute and chronic data for (i) alga/macrophyte, (ii) *Daphnia*/another invertebrate, and (iii) fish (Weigt et al. 2011). Similar strategy is applied in REACH to evaluate aquatic pelagic toxicity (ECHA 2014). The suitability of the algae, *Daphnia* and FET assays to compose a basic (eco)toxicity test battery was evaluated for hazard waste, wastewater effluent, freshwater and drinking water assessment (Keddy et al. 1995, Diaz-Baez et al. 2002, Manusadžianas et al. 2003, Pandard et al. 2006, Gartiser et al. 2009, Römbke 2009); and for effect-directed analysis (Brack et al. 2013, Di Paolo et al. 2015b, Brack et al. 2016). Therefore the assays are expected to be already established in diverse laboratories worldwide. Finally, miniaturized assay performance in comparison with higher-volume methods (and with adult fish for the FET) has already been investigated (Eisentraeger et al. 2003, Knobel et al. 2012, Baumann et al. 2014).

Complementary, mechanism-specific bioassays can provide information on modes-of-action that are intrinsically of concern for ecosystems and health. For example, the photosynthesis inhibition endpoint of the combined algae test covers many current WFD priority compounds and emerging compounds. Furthermore, endocrine disruption and mutagenicity are of particular relevance for population-level effects and humans (European_Union 2000, Weigt et al. 2011, ECHA 2014). For estrogens, regulatory strategies involving bioassays are reinforced after the recent inclusion of estrogenic pharmaceuticals in the WFD watch list (Hecker and Hollert 2011, EC 2013). In fact, both ER-Luc and YES assays have been recommended for estrogen monitoring in water bodies (Loos 2012). Regarding mutagenicity, the Ames fluctuation assay round-robin study was the first step towards its regulatory implementation in water legislation (Wolz et al. 2010, Reifferscheid et al. 2012). Moreover, the Ames and umu tests are recommended as mutagenicity and genotoxicity methods for the waste ecotoxicological characterization (Römbke 2009). Due to their environmental and health relevance, estrogenicity and mutagenicity assays are established in many laboratories.

10.3.5 Stepping-stones towards the establishment of bioassays in water quality monitoring

Currently there are diverse European initiatives towards bioassay application in water quality assessment, such as the Technical Report on effect-based tools in the context of the WFD (Wernersson et al. 2015) and activities towards the validation of low volume, high-throughput bioassay batteries (Brack et al. 2013, Altenburger et al. 2015, Brack et al. 2015, Neale et al. 2015, Schulze et al. 2015). Such applied studies will be of high relevance for the decision on a basic battery for water monitoring.

After the setup of such basic battery, its composition can certainly be expanded according to regional requirements or specific investigation. For instance, when freshwater sediments present a concern, whole-sediment toxicity assays with different organisms are available. Ring tests have demonstrated the good performance of tests evaluating macrophyte growth impairment (Feiler et al. 2014); and growth and reproduction effects on interstitial water nematodes (Hoss et al. 2012). Recent studies include also a methodological investigation of a freshwater ostracod sub-chronic test (Casado-Martinez et al. 2016); and a tiered strategy for sediment risk assessment integrating different toxicity tests (Diepens et al. 2016). Additionally, when chronic fish toxicity is suspected, the decision on whether to perform chronic tests can be supported by toxicity assays with fish early-life stages (OECD 2013a, Di Paolo et al. 2015a).

Importantly, the investigation of additional mechanism-specific toxicities can rely on diverse reporter-gene assays, for which effect-based trigger values to support decisions on water quality assessment are being established (Loos 2012, Brand et al. 2013, Escher et al. 2015). In parallel to these tests, it is necessary to investigate the occurrence of non-specific toxicity caused by sample components, which can interfere with the performance of assays and even mask mechanism-specific effects (Brack et al. 2016). That was demonstrated in our study for the ubiquitous contaminant triclosan, which was cytotoxic to human cells, yeast and bacteria at concentrations representative of water samples or extracts (von der Ohe et al. 2012).

10.4 Conclusions and outcomes

The battery of miniaturized bioassays presented complementary sensitivity and specificity to the water extract spikes containing four emerging pollutants as single-chemicals or mixtures. Aquatic organism sensitivity varied following exposure to different chemicals, confirming the complementary role of the tests with the three taxa for water quality assessment. Estrogenicity and mutagenicity assays identified with high precision the respective mechanism-specific effects of spikes, even though non-specific toxicity of mixture compounds affected the evaluation of higher test concentrations. Since differences in experimental protocols, model organisms, and data analysis can affect the determination of effect-concentrations, respective standard methods and harmonized procedures should be followed when implementing bioassays in water monitoring. Together with other ongoing activities for the validation of a basic battery of bioassays, the present study is an important step towards the implementation of bioanalytical monitoring tools in water quality assessment and monitoring.

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Chapter 10 - Annex - Plots for the bioassays
Algae test 72 h

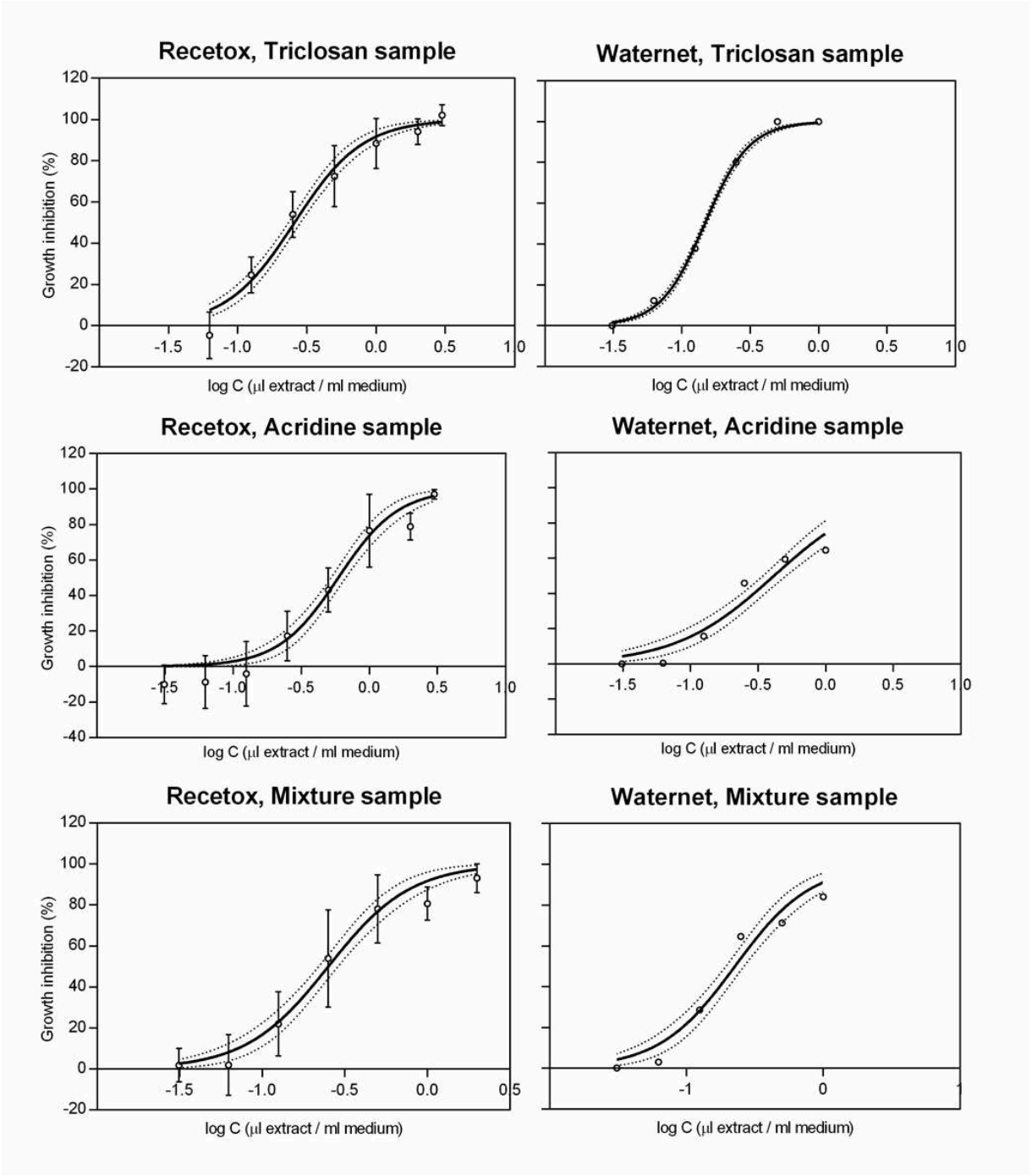

Figure S1: 72 h algae growth inhibition versus log concentrations values (µl extract / ml medium) for Recetox (left, pooled data for 3 experiments for each sample) and Waternet / Waterproef (right, data for one experiment for each sample). Mean values (○), standard deviations (error bars) and 95% confidence bands.

Algae test 24 h

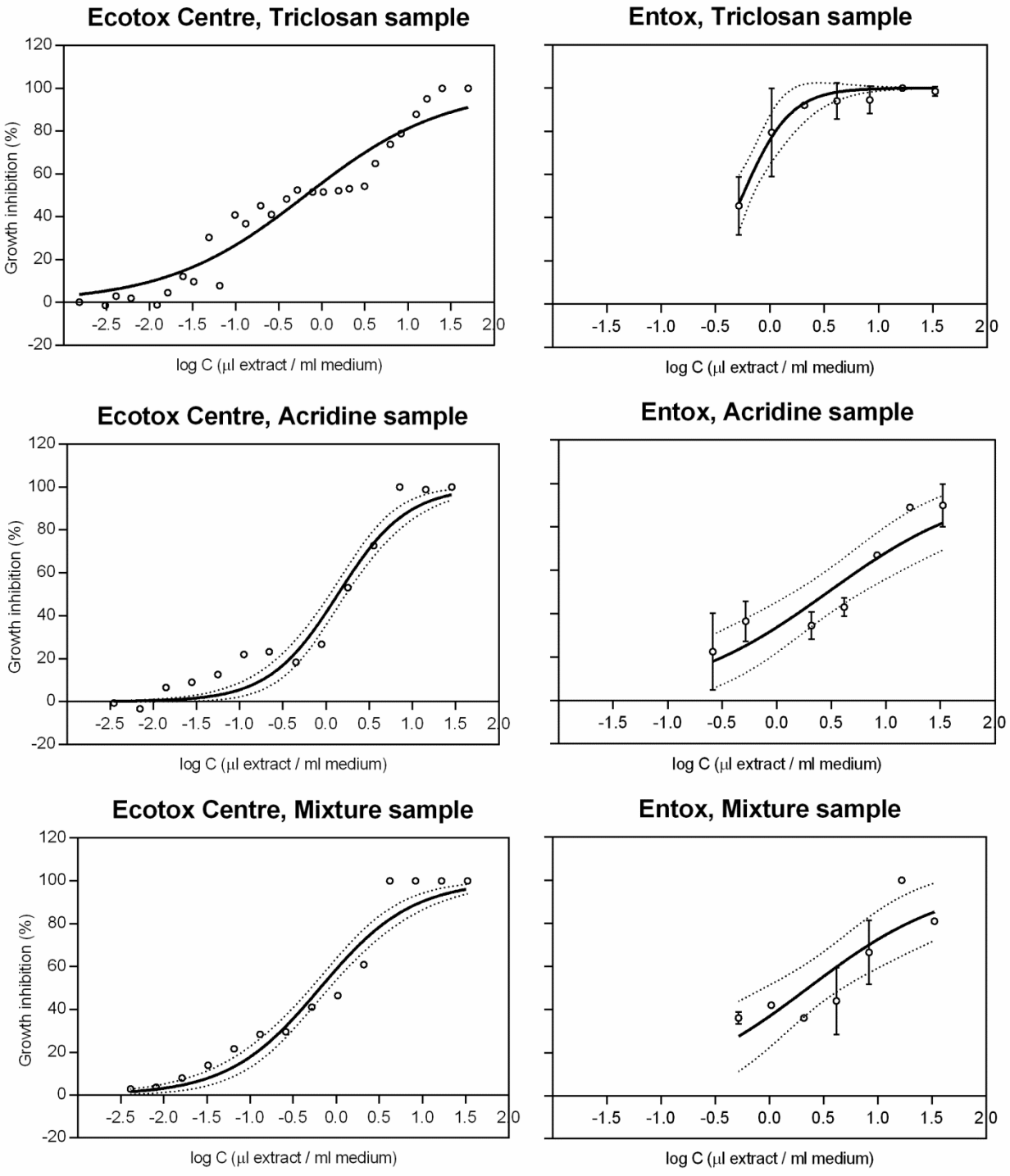

Figure S2: 24 h algae growth inhibition versus log concentrations values (µl extract / ml medium) for Ecotox Centre (left, pooled data for 1 experiment for each sample) and Entox (right, pooled data for 2 experiments for each sample). Mean values (○), standard deviations (error bars) and 95% confidence bands.

Daphnia test - A

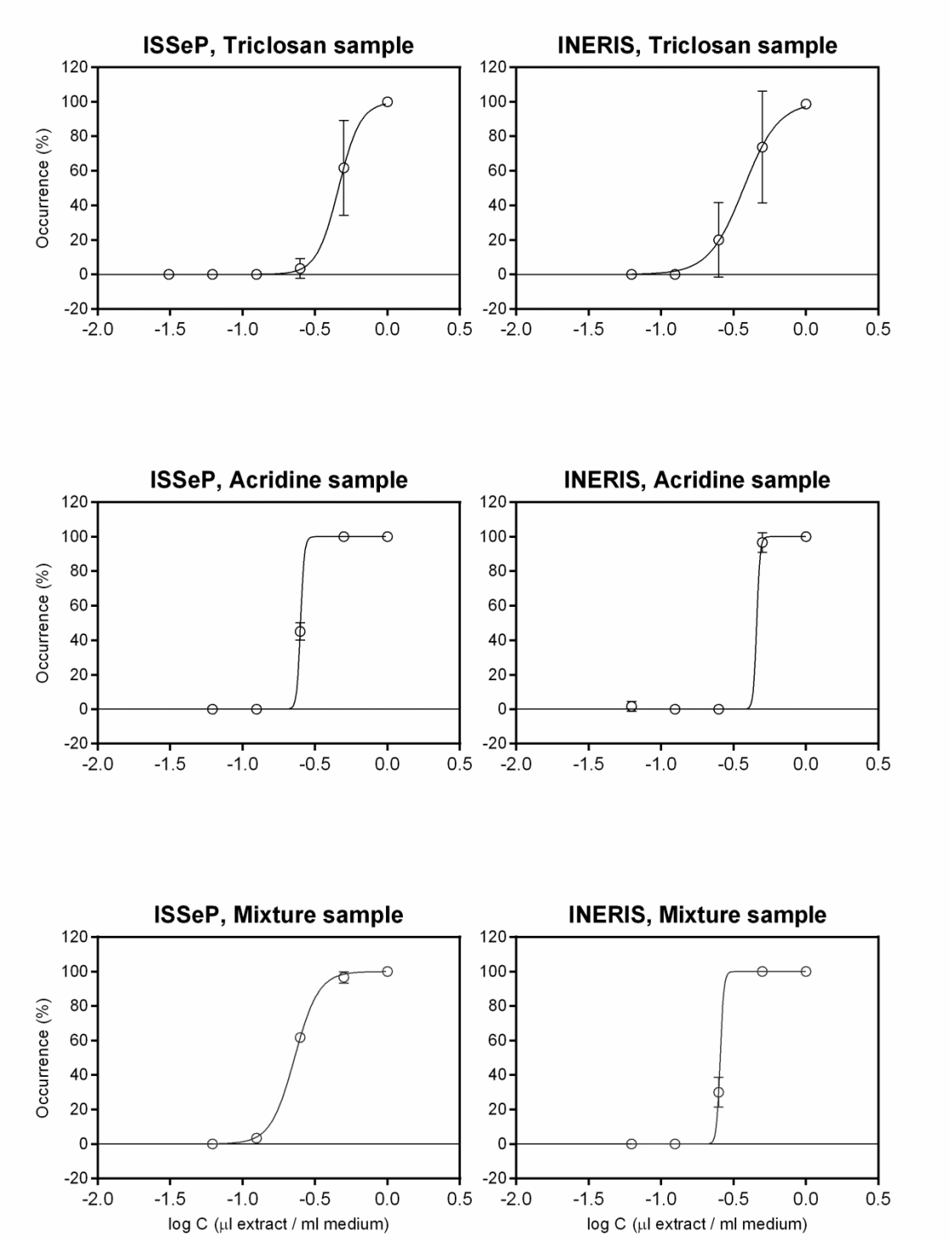

Figure S3 A: 48 h *Daphnia* immobilization versus log concentrations values (µl extract / ml medium) for ISSeP (left, pooled data for 3 experiments for each sample) and INERIS (right, pooled data for 3-4 experiments for each sample). Mean values (○) and standard deviations (error bars).

Daphnia test - B

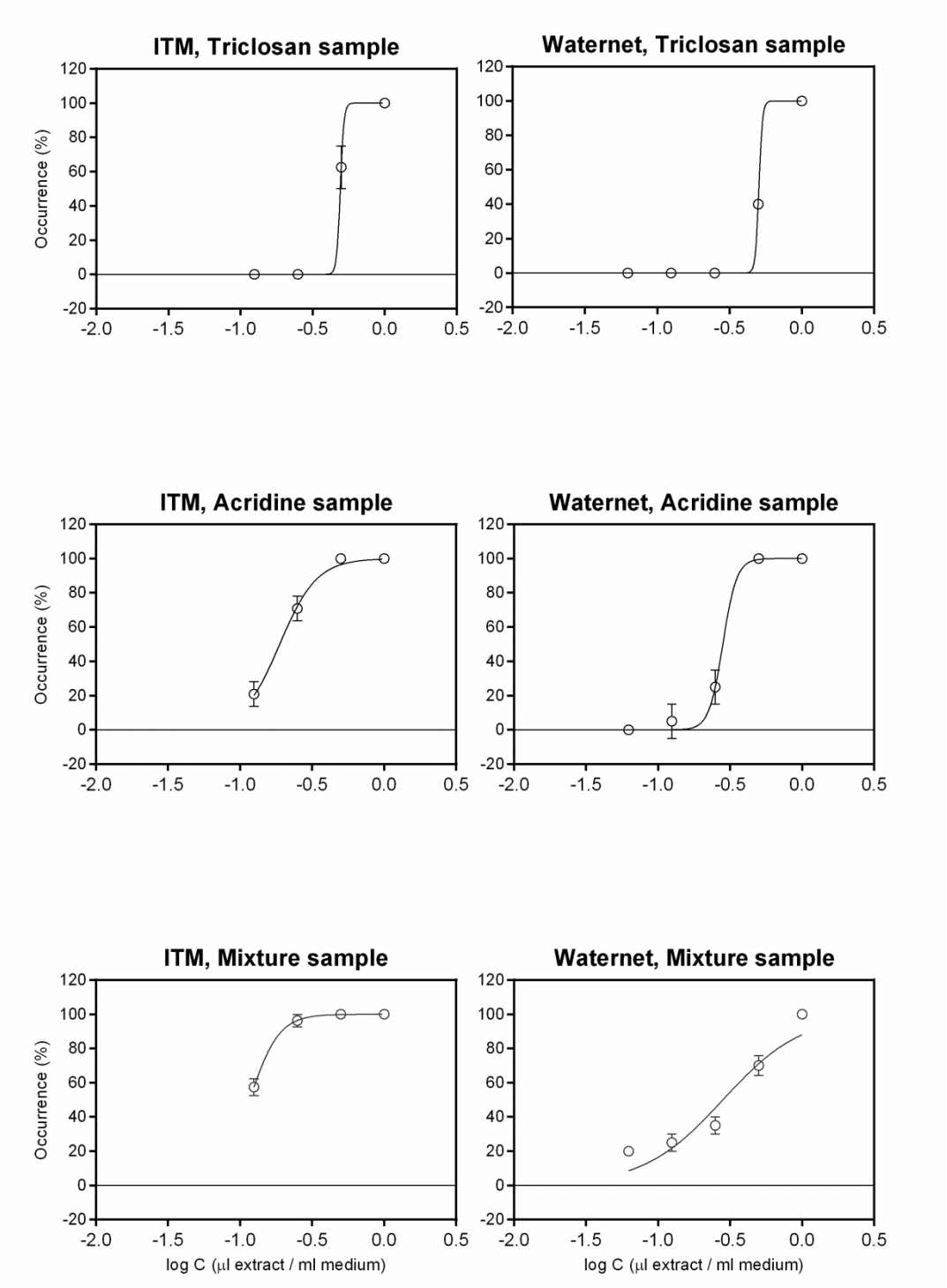

Figure S3 B: 48 h *Daphnia* immobilization versus log concentrations values (µl extract / ml medium) for ITM (left, pooled data for 3 experiments for each sample) and Waternet (right, pooled data for 3-4 experiments for each sample). Mean values (○) and standard deviations (error bars).

FET test

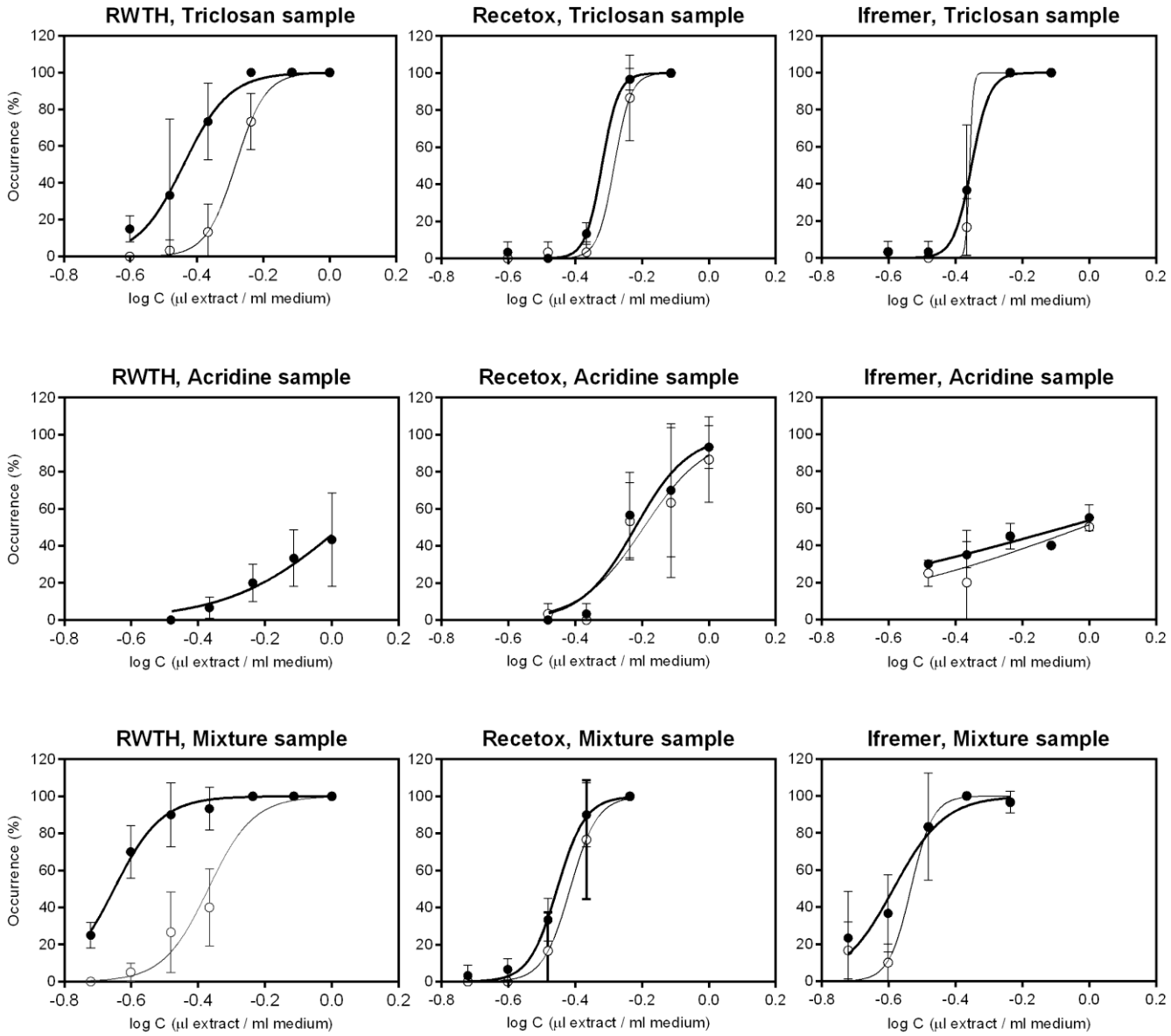

Figure S4: 96 h FET occurrence of lethal (-○-) of cumulative (-●-) effects versus log concentrations (µl extract / ml medium) for RWTH (left), Recetox (middle) and Ifremer (righ). Pooled data for 3 experiments for each sample. Mean values and standard deviations (error bars).

ER-Luc assay

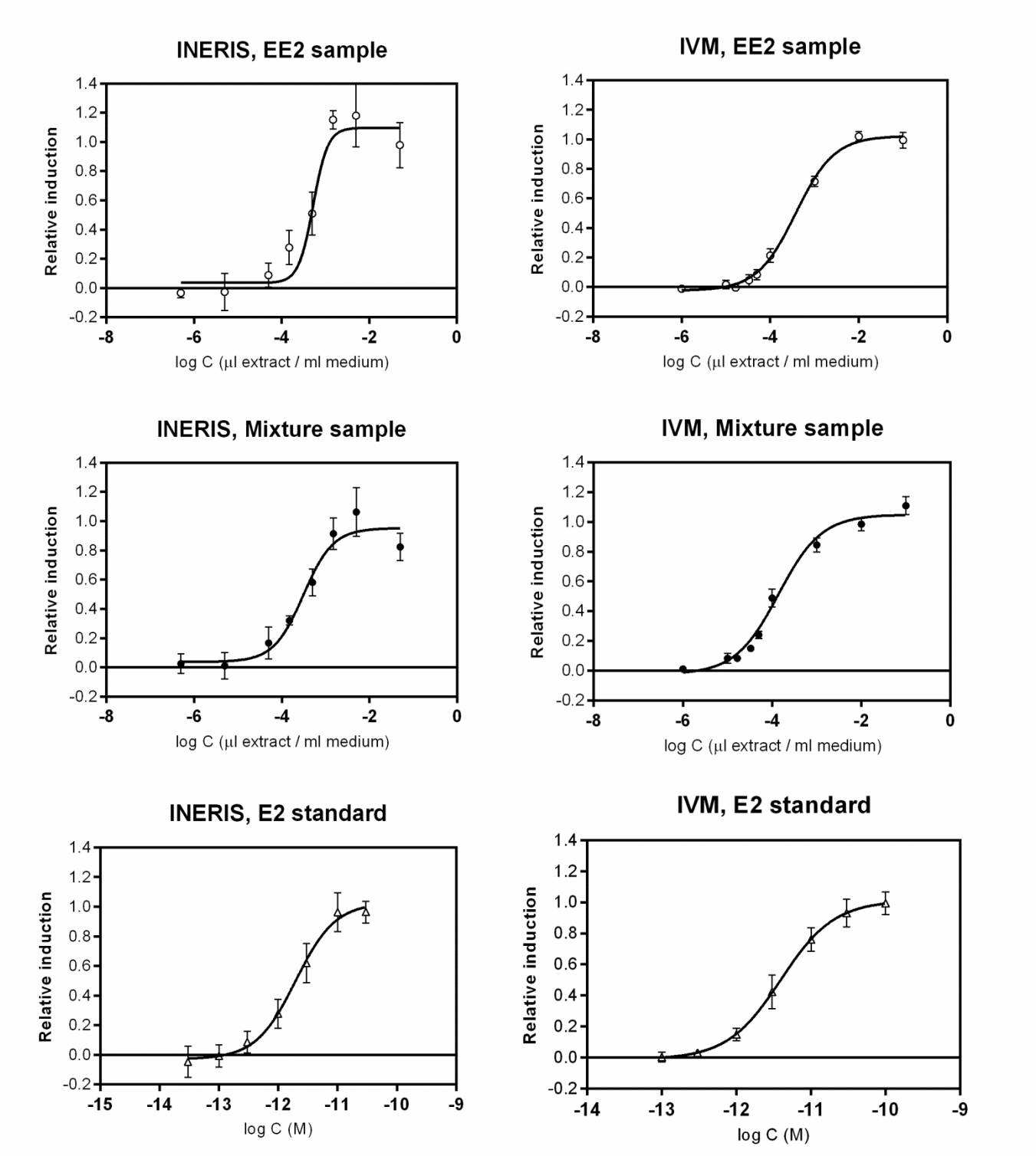

Figure S5: ER-Luc relative induction versus log concentrations (µl extract / ml medium) for INERIS (left) and IVM (right). Pooled data for 3 experiments for each sample. Mean values and standard deviations (error bars).

YES assay

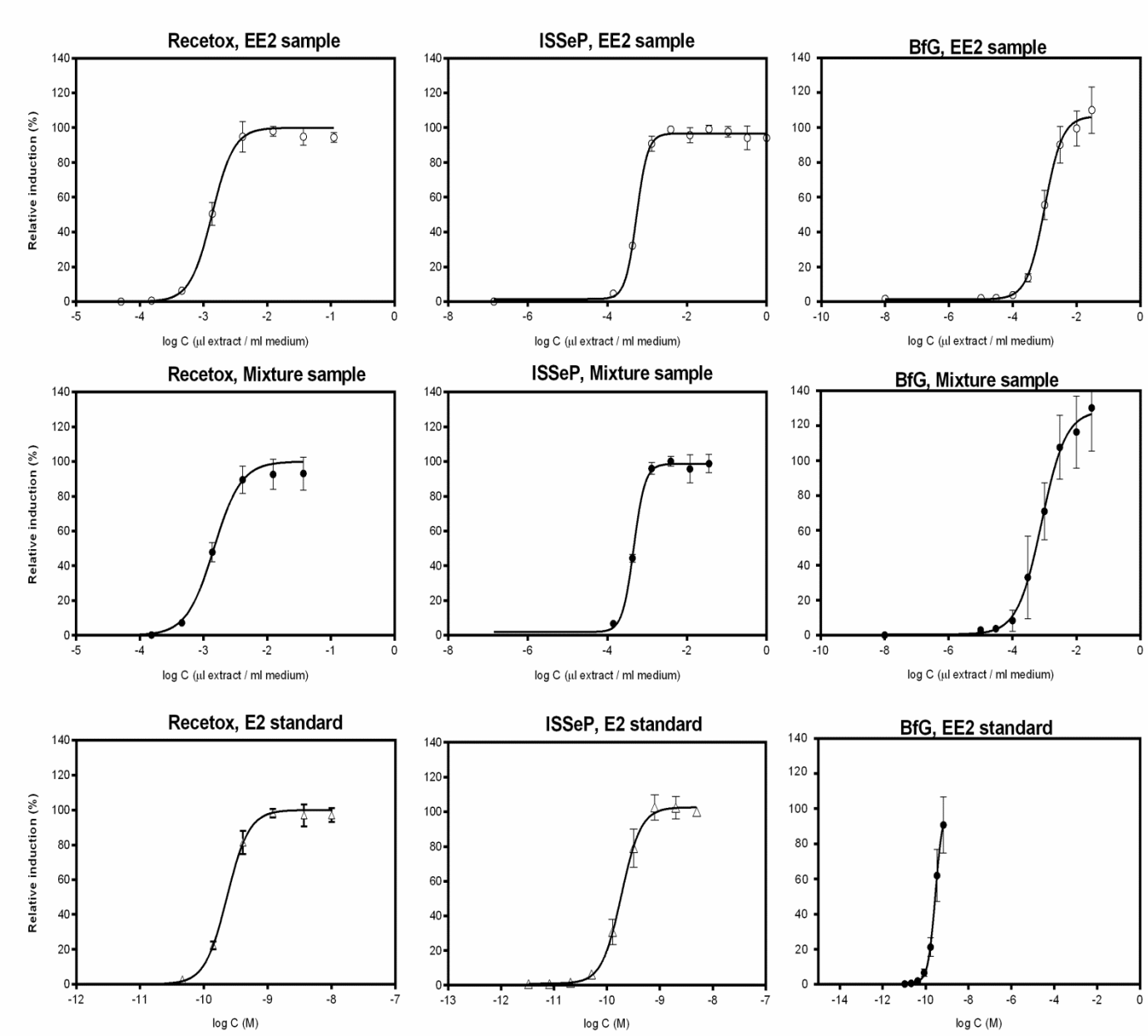

Figure S6: YES relative induction (%) versus log concentrations (µl extract / ml medium) for Recetox (left), ISSeP (middle), and BfG (right). Pooled data for 3 experiments for each sample. Mean values and standard deviations (error bars).

Ames assay

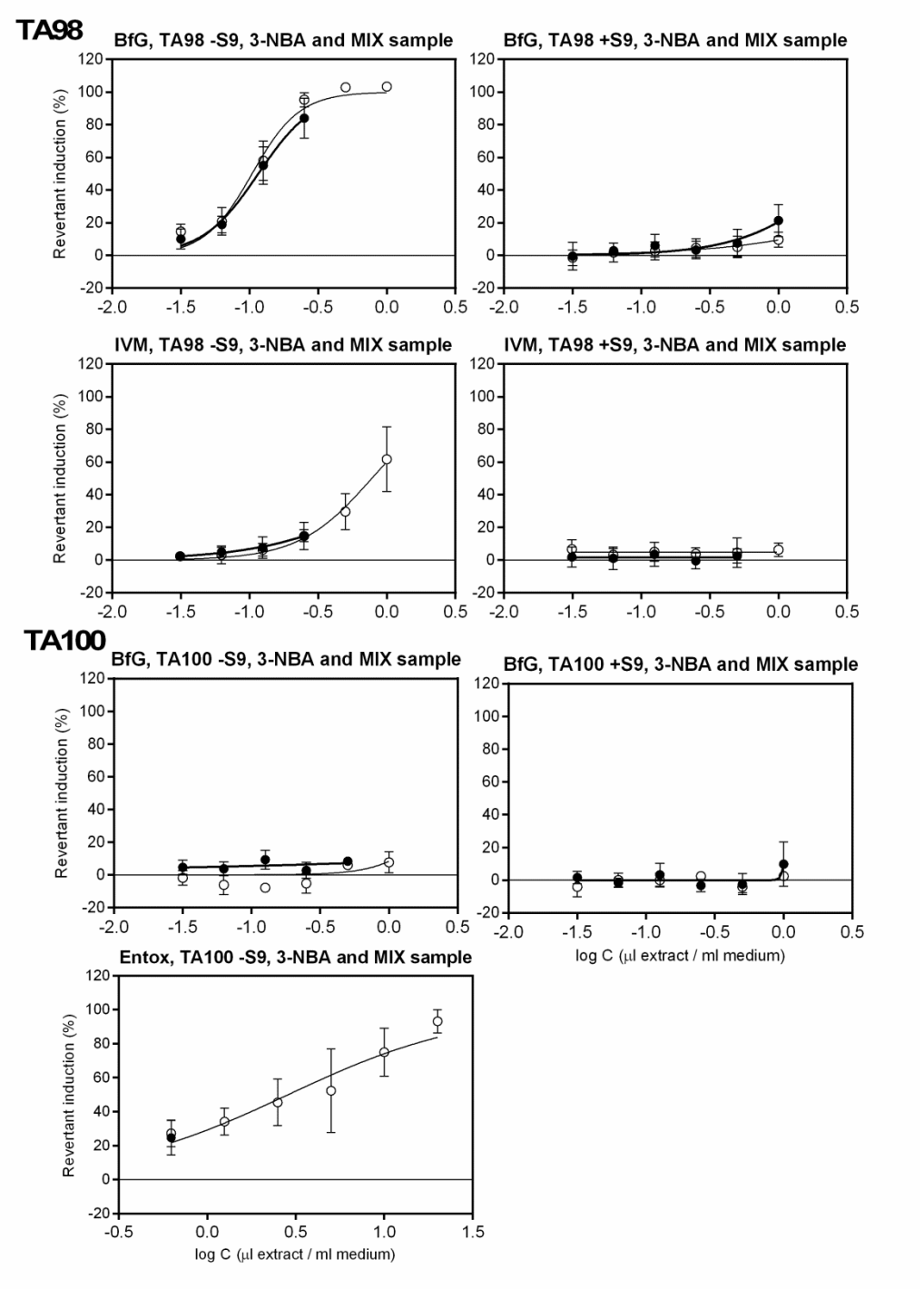

Figure S7: Ames assay revertant induction (%) in the 3-NBA (-○-) and mixture (-●-) spikes versus log concentrations (µl extract / ml medium) for BfG (pooled data for 2 experiments for each sample), IVM (pooled data for 2 experiments for each sample), and Entox (data for 1 experiment for each sample). Missing values for the mixture were excluded due to cytotoxicity. Mean values and standard deviations (error bars).

Chapter 11: Conclusions and recommendations

11.1 Thesis outcomes, conclusions and recommendations

Aquatic pollution is a recognized urgent issue worldwide that requires improvement and prioritization of monitoring efforts in order to properly understand and consequently promote good water quality. In this thesis, the contribution of bioassays to water quality assessment has been explored under different perspectives, including extensive training, systematic literature review, method development and adaptation, application of assays for the evaluation of emerging pollutants and water samples, and as guiding tools in effect-directed analysis (EDA).

As main outcomes, this thesis presents an overview on the value of zebrafish bioassays for EDA investigations (Chapter 3); provides new and improved mechanism-specific bioassays for application in water quality assessment and EDA (Chapters 4, 6, 8 and 9); and demonstrates the usefulness of different methods to evaluate mechanism-specific toxicity of emerging pollutants in single exposures or as mixtures (Chapter 4, 6, 7, 8, 9, 10), of water sample extracts or fractions (Chapter 2, 5, 10), and as guiding tools in EDA case studies (Chapters 5 and 6). Further, bioassay battery testing approaches were incorporated, including the coordination of a respective interlaboratory study (Chapter 10). Another important outcome of the context of the thesis development was the participation of extensive training courses (Chapter 2) on different topics of relevance for EDA and water quality monitoring.

11.2 Development and adaptation of mechanism-specific bioassays and testing strategies for application in EDA studies

As presented and discussed in Chapter 3, although zebrafish bioassays have successfully guided different EDA studies further method developments are still needed. In fact, the main findings of the review can be extrapolated for the application of bioassays in general as guiding tools in EDA. For instance, it was proposed that process blank preparation and bioltesting should be standardized. In this thesis, testing of method blanks was incorporated in different studies for the evaluation of water samples (Chapter 5) and fractions (Chapter 2 and 6) regarding non-specific and mechanism specific toxicity. This step was concluded to be particularly important for reporter gene antagonistic assays such as the method applied for antiandrogenicity assessment, since non-specific toxicity also leads to reduction of assay response that can cause false positive occurrence. Further, alternative dosing procedures were also discussed as a need, being in fact required to develop the EDA case study investigated in this thesis (Chapter 6). Finally, a main conclusion of the review was that endpoint- and

mechanism-specific bioassays are the most promising methods for EDA of environmental samples. When integrated into broader environmental assessment strategies, EDA guided by specific bioassays can support not only the identification of compounds causing bioassay and ecological effects, but ultimately contribute for environmental quality improvement.

An important outcome of this thesis was obtained through the downscaling of bioassay procedures for the antiandrogenicity assay that allowed the development of an EDA case study investigating a surface water sample of limited volume (Chapters 5 and 6). Through the evaluation of more than 30 surface water samples extracts using an antiandrogenicity testing strategy combined with chemical analysis, it was possible to identify a water sample that presented particularly high bioactivity in a dose-response manner (Chapter 5). The newly developed low-volume procedures for the steps of dosing and exposure in the (anti)androgen receptor reporter gene cell-based assay produced results that were very similar to those obtained with higher volume procedures and the literature. The comparability of results was demonstrated for the assay in the agonistic and antagonistic formats and for different compounds. An important aspect is that the acceptance criteria of the assays were met, indicating that the downscaling procedures did not reduce the reliability of results. Additionally, the low-volume procedures were shown to also efficiently evaluate the water sample fractions and respective method blanks regarding antiandrogenicity or cytotoxicity in the EDA case study. The downscaling procedures developed in this thesis can provide a simple and effective solution for studies that need to minimize sample use while maintaining reliable, reproducible and quantitative bioassay results. The miniaturized methods are expected to be efficient also in other bioassays; therefore in future studies the procedures can be extended to additional methods for use in screening and EDA studies.

11.3 Aquatic relevant mechanism-specific bioassays with zebrafish early life stages and zebrafish liver cell lines

In this thesis, zebrafish embryos and larvae were applied in bioassays for delayed toxicity (Chapter 4), genotoxicity (Chapter 8) and behavioral effects assessment (Chapter 9); while the zebrafish liver ZFL cell line was applied in a genotoxicity assessment method. The Fish Embryo Toxicity (FET) was also integrated into bioassay batteries (Chapter 2 and 10) however endpoint assessment was limited to lethality and morphological sublethal effects.

A new method using zebrafish early life stages for the investigation of delayed toxicity and respective early predictive endpoints was developed in this thesis (Chapter 4). Effects on

swim bladder inflation and cartilaginous tissues appeared to be the most promising early endpoints for prediction of delayed mortality caused by the model dioxin-like compound PCB126. That could be related at least to a certain extent to the fact that these effects can impair the fish swimming and feeding capacity, but also the possibility of a shared toxicity mechanism with later effects is considered. Therefore, these endpoints are recommended to be carefully evaluated in toxicity tests with early larvae as early indicators of more severe later effects. Further, the observed zebrafish patterns of delayed mortality and sublethal effects on growth and development were similar between zebrafish and previous studies with sole. The two most critical periods for lethality occurrence were the transition into the free-feeding stage following yolk resorption and the time point when the energy obtained from the yolk-sac is completely depleted, which in zebrafish occur at around 7 and 12 days post fertilization (dpf) respectively. Also, the 12-14 dpf time point is of relevance for zebrafish larval development and metamorphosis, which was also affected in exposed fish. Consequently, when delayed toxicity is suspected it is recommended to extend observation of effects until at least 14 dpf. Additionally, since PCB126 residues did not reach steady state within the five days of exposure, studies that attempt to use zebrafish early stages for bioaccumulation studies should carefully consider toxicokinetic processes and temporal pattern for bioaccumulation. The developed method is recommended to be applied for the evaluation of other chemicals or environmental samples suspect of causing delayed toxicity.

Zebrafish-based genotoxicity methods were developed in this thesis, with the establishment of protocols to assess micronucleus (MN) formation in ZFL cell lines and early larvae (Chapter 8). The ZFL MN test was successful in identifying increased MN formation in cells exposed to candidate positive control chemicals and one environmental contaminant, which included both direct as indirect acting agents. Since 4-Nitroquinoline 1-oxide (NQO) caused the highest MN formation among tested compounds and presented dose-response trend, it is therefore recommended as a direct-acting positive control chemical for the test. MN formation after cyclophosphamide (CPP) and 3-nitrobenzanthrone (3-NBA) was relatively lower, indicating limited but present metabolic capacity of the ZFL cell line. Still, both compounds induced significantly MN formation and could be considered as indirect acting positive control chemicals, particularly CPP for being recommended by the OECD TG 487. The MN assay with primary cells of 5 dpf zebrafish larvae was also established, however the observation and scoring of MN was not as easily achieved as for the ZFL cells. The cells were observed to be rather small and their cytoplasm was often not clearly visible, which

restricted MN scoring. Although exposure to NQO and CPP led to tendency for increased MN formation, there was high variation between experimental replicates and differences were not significant. A reason for that can be the fact that total larval suspension included very diverse cell types, which would contribute to high variability between replicates. Still, additional investigations can be performed in the future to further improve the presently developed protocol.

Zebrafish 5 to 6 dpf early larvae were also applied to investigate the behavioral effects of neuroactive and neurotoxic compounds following a light-dark transition test for experiments performed at RWTH Aachen and at IVM Amsterdam (Chapter 9). The established experimental protocol was able to identify alterations of behavioral responses following exposure to the candidate positive controls ethanol, nicotine and caffeine; and to the insecticides, α-cypermethrin, chlorpyrifos and its metabolite chlorpyrifos-oxon. The new parameter peak:basal ratio, together with distance moved and burst activity values, supported the interpretation of responses following exposure to the different chemicals. The RWTH self-built system presented in general consistent results with those obtained with the IVM commercial system. Even though some differences occurred, it is considered that factors other than the test system, such as different used zebrafish strains, could have played a role. Therefore in order to properly investigate interlaboratory differences a specific study should be designed and performed in the future. Considering the performance of different aged larvae for behavioural assessment, 5 dpf larvae presented more consistent behavioural responses to chemical exposure, supporting the recommendation that behavioural assessment should always that possible be performed with zebrafish up to 5 dpf of age. Among tested chemicals, ethanol produced the more robust responses, causing stimulatory or inhibitory responses depending on concentration exposure period. Caffeine and nicotine instead presented very brief stimulatory effects, followed by inhibitory effects only. Therefore short exposures to ethanol 1-3% are recommended to be applied in positive control conditions for light-dark transition tests with zebrafish 5 dpf larvae. The tested insecticides presented less evident pattern of behavioural responses, which can be related to inadequate exposure scenarios. Since respective mixtures caused much more severe effects than respective single chemicals, only the EC_{10}-based mixture was evaluated regarding behavioral effects, which was identified to clearly affect larval behavior. Particularly, it is recommended that future studies investigate the temporal profiles of effects of α-cypermethrin, chlorpyrifos and respective mixtures on the behaviour of fish early life stages.

11.4 Effects of emerging pollutants as single chemicals and as mixtures on aquatic organisms in vitro bioassays.

Emerging pollutants are a current priority in water quality, presenting challenges for water quality monitoring and EDA investigations. In this thesis, the effects of emerging pollutants as single exposures or mixtures were evaluated using a cell-based reporter gene assay for the induction of the p53 tumour suppression protein pathway, a main driver of cell fate following DNA damage (Chapter 7); and using a bioassay battery (Chapter 10).

The reporter gene cell-based p53 induction assay was able to identify p53 induction following exposure to chemicals with different mechanisms of toxicity both in the presence and absence of S9 metabolic activation system (Chapter 7). Test chemicals included the compounds evaluated also in the MN test described in Chapter 8, plus actinomycin as a direct-acting p53 inducer. The p53 assay was also efficient in evaluating binary and a tertiary mixture of the compounds; however while binary mixtures presented additivity or even synergism at the lowest test concentrations, the tertiary mixture presented instead infra-additivity tendency. That suggests that, when testing complex mixtures of chemicals such as those contained in environmental samples, the p53 induction factor can be lower than the response that would be caused by the most active chemicals in mixture. Still, the predicted LOECs were always in agreement with measured values, and in general the peaks of induction occurred at concentrations lower than predicted. That indicates that the assay sensitivity was maintained even after the exposure to mixtures, which is an important aspect for the test of water samples. The fact that peaks of induction differ between mixtures and respective single chemicals is a factor of relevance for EDA, since test concentrations of fractions and candidate chemicals should be adjusted accordingly. Importantly, cytotoxicity assessment showed to be a very important aspect of p53 induction assessment. Particularly for the single chemical exposures, the peaks of induction often occurred at concentrations that caused less than 80% of cell viability. Therefore concentration ranges investigating p53 induction should include also cytotoxic ranges, even if results obtained with cell viability lower than 50% should be interpreted with caution due to risks of non-specific effects.

Mixtures of emerging pollutants were also investigated through a bioassay battery (Chapter 10), which will be discussed in details in the next topic. The most relevant conclusion for the test of mixtures is that, although non-specific toxicity of some mixture components such as triclosan occurred at higher test concentrations, estrogenicity and mutagenicity assays identified with high precision the respective mechanism-specific effects of spikes. However,

that might not be the case when investigating complex environmental samples. Consequently, when evaluating water samples for mechanism-specific toxicity it is of high importance to always investigate the interference of non-specific toxicity as well in order to select test concentration range and also to support result interpretation. Unrecognized cytotoxic effects of mixture components could lead to the occurrence of false negatives (for agonistic assays) or false positive (for antagonistic assays) results in mechanism-specific tests.

11.5 Bioassay battery approaches to investigate water samples and emerging aquatic pollutants

Bioassay battery testing strategies for water quality assessment consider that a comprehensive toxicity evaluation of environmental samples is not provided by one or few methods only. Even if bioassay batteries have been applied in different scientific investigations, a basic battery for the assessment of water quality on the regulatory level is not available yet. Therefore studies that advance and support the application of bioassay batteries for water quality are needed. One test battery was applied in the European Demonstration Program to evaluate water samples collected in different European river basis. It was developed as common activity by all the project partners, involving organism-level and mechanism specific assays. Since this was a collaborative initiative involving many different partners, only a brief overview of the study is presented in Chapter 2.

As part of this thesis, there was the organization of the bioassay battery interlaboratory study in the context of the NORMAN network (Chapter 10). The study investigated whether a battery of miniaturized assays, performed by different laboratories following their own protocols, would produce consistent results for the evaluation of blinded samples containing pristine water extract spiked with representative emerging pollutants as single-chemicals or mixtures. The battery composition was set to investigate effects on aquatic organisms from different taxa and trophic levels (algae, daphnids, zebrafish embryos), and mechanism-specific assays for estrogenicity and mutagenicity. Investigated emerging pollutants included compounds presenting different mechanisms of toxicity, i.e. triclosan, 17α-ethinylestradiol, acridine and 3-NBA; plus the water extract was included as a matrix to improve the relevance of the study for water quality assessment. The outcomes of the study confirmed that the battery of miniaturized bioassays presented complementary sensitivity and specificity to the water extract spikes containing the test chemicals as single exposures or mixtures. The sensitivity of the aquatic organism assays varied following exposure to different chemicals, supporting the complementary role of the tests with the three taxa for water quality

assessment. Mechanism-specific assays for estrogenicity and mutagenicity were able to identify with high precision the respective activity of spikes. However, as discussed above the non-specific toxicity of mixture components interfered with the tests of higher concentrations. Therefore tests evaluating also non-specific effects such as cytotoxicity should be integrated in bioassay batteries. The study outcomes include the consideration that differences in sample handling, experimental protocols, model organisms, and data analysis can affect the determination of effect-concentrations, with the development of respective standard methods and harmonized procedures being recommended for the implementation of bioassays in water monitoring. In fact, since for the different assays there are respective or comparable test guidelines (OECD, ISO), diverse relevant aspects have already been discussed for single chemicals and can provide the basis for water sample evaluation. As discussed in Chapter 10, there are other ongoing activities for the establishment of low volume, high-throughput bioassay batteries for water quality assessment. In this context, the outcomes of the present study contribute to demonstrate the performance and usefulness of a basic bioassay battery for water quality assessment, ultimately promoting the inclusion of bioassays in water quality monitoring.

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ZIRC Zebrafish International Resource Center - ZIRC. *https://zebrafish.org*.

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Curriculum Vitae

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Education

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2009	MSc Environmental Sciences with specialization in Water Quality and Aquatic Ecology at Wageningen University, the Netherlands Thesis: "Black-Carbon Inclusive Modelling of PBDEs and PCBs in estuarine food webs" Supervisor: Prof. Dr. A.A. (Bart) Koelmans
2006	MSc Biological Oceanography at University of São Paulo, Brazil Thesis: "Comet Assay applied to DNA damage study in fat snook, *Centropomus parallelus* (Poey, 1860), exposed to β-naphthoflavone" Supervisor: Prof. Dr. Phan Van Ngan
2003	BSc Veterinary Medicine at University of São Paulo, Brazil Thesis and internship: "Methods for the assessment of toxic effects of pollutants in marine organisms" Supervisors: Prof. Dr. Phan Van Ngan and Prof. Dr. Luiz Carlos de Sá-Rocha

Work Experience

Since July 2012	Research Assistant at the Institute for Environmental Research at RWTH Aachen University, Aachen, Germany.
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Languages

Portuguese and Italian: Native or bilingual proficiency; English: Full professional proficiency; Spanish: Limited working proficiency; German, French, Dutch: Elementary proficiency.

Teaching Experience

2004 – 2016 (Short periods)	Student assistant RWTH Aachen University (2012-2016): "Toxicology seminar", "Ecotoxicology"

	Wageningen University (2008-2009): “Processes in Aquatic Systems”, “Principles of Environmental Sciences”, “Marine Systems”
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2003	Biology teacher, ACEPUSP. São Paulo, Brazil
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Jonas Nelles. BSc Thesis “Diuron and diazinon effects on behavior of zebrafish embryos and larvae”. 2016.

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Scientific contributions

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Research articles published in international peer-reviewed journals

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Research articles submitted for publication in international peer-reviewed journals

Di Paolo, C., Müller, M., Thalmann, T., Hollert, H. and Seiler, T.B. (submitted to Environmental Science and Pollution Research) p53 induction and cell viability modulation by genotoxic single chemicals and mixtures.

Velki, M., Di Paolo, C., Nelles, J., Seiler, T.B. and Hollert, H. (submitted to Aquatic Toxicology) Diuron and diazinon alter the behavior of zebrafish embryos and larvae in the absence of acute toxicity.

Research articles in preparation

Schulze, T., Ahel, M., Ahlheim, J., Brion, F., Di Paolo, C., Hollender, J., Hollert, H., Kloß, A., Koprivica, S., Krauss, M., Schollee, J., Shao, Y., Slobodnik, J., Sonavane, M., Tousova, Z., Walz, K.H. and Brack, W. (in preparation) A novel device for on-site large volume solid phase extraction for the targeted and nontargeted chemical as well as effect-based screening of water resources. Planned submission: Chemosphere.

Tousova Z., Oswald, P., Schulze, S., Muz, M., Hu, M., Brack, W., Krauss, M., Koprivica, S., Ahel, M., Schollee, J., Hidasi, A., Hollender, J., Suter, M., Sonavane, M., Ait-Aissa, S., Creusot, N., Brion, F., Di Paolo, C., Hollert, H., Froment, J., Almeida, A.C., Thomas, K., Tollefsen, K.E., Tufi, S., Ouyang, X., Leonards, P., Lamoree, M., Torrens, V.O., Schriks, M., Špírhanzlová, P., Tindall, P., Blaha, L., and Slobodnik, J. (in preparation) European Demonstration Program on the effect-based and chemical identification and monitoring of emerging organic pollutants with adverse effect potential in European surface waters using large volume sampling tool in combination with analysis by battery of bioassays and chemical screening. Planned submission: Science of the Total Environment.

Lam, M.M., Di Paolo, C., Maletz, S., Kuckelkorn, J., Seiler, T.B., Hilscherová, K., Bloch, R., Hug, C., Krauss, M., Schulze, T., Brack, W. and Hollert, H. (in preparation) Screening for cytotoxic, anti-androgenic and estrogenic effects of surface water of the Saale-Mulde Catchment by in vitro assays. Planned submission: Water Research.

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Di Paolo, C., Graf, K., Legradi, J., Legler, J., Seiler, T.B., Werner, I., Fenske, M. and Hollert, H. (in preparation) Zebrafish larvae behavioural responses following exposure to neuroactive and neurotoxic compounds - part A: Evaluation of candidate positive control conditions and chemicals. Planned submission: Neurotoxicology and Teratology.

Di Paolo, C., Legradi, J., Cousin, X. and Hollert, H. (in preparation) Zebrafish larvae behavioural responses following exposure to neuroactive and neurotoxic compounds - part B: Interlaboratory evaluation of ethanol effects on the behaviour of zebrafish larvae from different strains. Planned submission: Neurotoxicology and Teratology.

Platform presentations

Di Paolo, C., Wüster, S., Hamman, R., Krauss, M., Fenske, M., Werner, I. and Hollert, H. Oral presentation: Effects of neuroactive pharmaceuticals on gene expression profiles in zebrafish embryos and larvae. 21st SETAC GLB, September 2016, Tübingen, Germany.

Di Paolo, C., Graf, K., Legradi, J., Fenske, M, Ottermans, R., Hausen, J., Legler, J., Werner, I., Seiler, T.B. and Hollert, H. Oral presentation: Alcohol, coffee and cigarettes: interlaboratory evaluation of candidate positive control chemicals for behavioural assessment in zebrafish larvae. 20th SETAC GLB, September 2015, Zürich, Switzerland.

Di Paolo, C., Ottermanns, R., Keiter, S., Ait-Aissa, S., Bluhm, K., Brack, W., Breitholtz, M., Buchinger, S., Carere, M., Chalon, C., Cousin, X., Dulio, V., Escher, B.I., Hamers, T., Hilscherová, K., Jarque, S., Jonas, A., Kase, R., Maillot-Marechal, E., Marneffe, Y., Nguyen, M.T., Pandard, P., Schifferli, A., Schulze, T., Seidensticker, T., Seiler, T.B., Tang, J., van der Oost, R., Vermeirssen, E., Zounková, R., Zwart, N. and Hollert, H. Oral presentation: The NORMAN interlaboratory study on biotesting of spiked water extracts. 20th SETAC GLB, September 2015, Zürich, Switzerland.

Di Paolo, C., Seiler, T.B., Keiter, S., Brack, W. and Hollert, H. Oral presentation: Zebrafish embryos as an integrative model to evaluate mechanism-specific toxicity of surface waters in Effect-Directed analysis. 18th SETAC GLB, September 2013, Essen, Germany.

Di Paolo, C., Groh, K.; Zennegg, M., Christoffel, A., Sigrist, N., Vermeirssen, E., Murk, A. J., Eggen, R.I.L. ,Hollert, H., Schirmer, K. and Werner, I. Oral presentation: Can the sensitivity and predictive potential of zebrafish early life stage (ELS) tests be improved by additional endpoints and chemical analysis? 3rd Young Environmental Scientists Meeting YES Meeting – SETAC, February 2013, Krakow, Poland.

Di Paolo, C., Groh, K., Kienle, C., Kunz, P., Vermeirssen, E., Schirmer, K. and Werner, I. Oral presentation: Molecular and behavioral endpoints in zebrafish early life stage tests: increased sensitivity and link to delayed effects. 6th SETAC World Congress / SETAC Europe 22nd Annual Meeting, May 2012, Berlin, Germany.

Di Paolo, C., Groh, K., Schirmer, K. and Werner, I. Oral presentation: Additional morphological, molecular and behavioral endpoints in zebrafish early life stage tests: increased sensitivity and prediction of delayed effects. Swiss and Upper Rhine Valley (SURV) Zebrafish Meeting 2012 at the University of Basel, April 2012, Basel, Switzerland.

Di Paolo, C., Groh, K., Kienle, C., Kunz, P., Vermeirssen, E., Schirmer, K. and Werner, I. Oral presentation: Molecular and behavioral endpoints in the zebrafish early life stage (ELS) test: identifying modes of action and increasing sensitivity. 12th Annual XeRR Meeting at the University of Zürich-Irchel, December 2011, Zürich, Switzerland.

Poster presentations

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Di Paolo, C., Laura S., Krauss, M., Fenske, M., Werner, I. and Hollert, H. Effects of neuroactive pharmaceuticals on gene expression profiles in zebrafish embryos and larvae. 26th SETAC Europe, May 2105, Nantes, France.

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Graf, K., Di Paolo, C., Fenske, M., Legradi, J., Werner, I., Seiler, T.B. and Hollert, H. Behavioural effects of Chlorpyrifos, α-Cypermethrin and their mixture on zebrafish larvae. 19th SETAC GLB, September 2014, Gießen, Germany.

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