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**“Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20th and 21st, 2023**

***Research Centre for BioSystems, Land Use and Nutrition (iFZ)***

***Justus Liebig University Giessen***

***Heinrich-Buff-Ring 26***

***35392 Giessen, Germany***

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**One Health drugs against parasitic vector borne diseases in Europe and beyond**

**OneHealth*drugs***

Event description used in the programme

In the development of new active substances, consideration of possible effects on non-target organisms is becoming increasingly important. What is already well established in pesticide development, namely the assessment of environmental effects, is still in its infancy in drug development. Here, the One Health approach often still falls short, as environmental health is an integral part of this concept, in addition to animal and human health. Efficient, innovative but also standardizable test methods and assays are needed to assess the behavior and effects of active ingredients released into the natural environment. The second workshop of WG4 will provide a platform to present and discuss different assays and test methods. Initial results of WG1-3 will be summarized and evaluated in an integrative manner with the goal of proposing a guideline paper to address ecotoxicological concerns in drug discovery and development in the anti-parasitic field. Visits to two research facilities focusing on drug discovery from bioresources or on risk assessment up to the mesocosm or field scale will round out the workshop.

**BOOK OF ABSTRACTS**

**Meeting Venue**

Research Centre for BioSystems, Land Use and Nutrition (iFZ)

Justus Liebig University Giessen

Heinrich-Buff-Ring 26

35392 Giessen, Germany

**Organizing committee**

Maria Paola Costi

Anabela Cordeiro

Federica Piccolo

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### Contents

------------------------------------------------------------------------------------------------------------------------------------------ **Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Fish embryos as tools for the assessment**

**of biological effects of water and sediment contamination**

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Given the dramatic worldwide decline in biodiversity and loss of habitats, the search for the underlying reasons has become a major concern. A common trait of pesticides and drugs is the fact that both are designed for biological effectiveness at lowest concentrations. Since pesticides are usually developed to control/kill organisms, effects on non-target organisms have always enjoyed great importance. In contrast, drug development is almost exclusively human-centered, and the assessment of environmental effects is still in its infancy. E.g., not a single drug has so far been banned for environmental concern, and examples for attempts to integrate aspects of environmental health into the risk assessment of drugs are scarce (e.g., no exemption for prescription of orally administered diclofenac in Sweden). In aquatic ecosystems, assessment of effects by drugs is extremely complex, since, due to the hydrophobic nature of at least part of the drugs, effects of not only water-borne, but also sediment-borne residues of both mother compounds and a multitude of metabolites and transformation products have to be considered. Given the multitude of drugs in human use (~ 100,000 in Germany), testing for drug effects in aquatic ecosystems has, therefore, become a major a major challenge, and there has for long been a quest for the development of high-throughput systems. Since, for historical reasons, fish are *the* prime test organism for effects in vertebrates, the trend towards animal-free testing in at least in the European Union presents an additional challenge. Apart from cell culture-based approaches, fish embryos so far represent the only legally acceptable model to meet all these requirements. After a brief historical outline of the development of the fish embryo tests, the presentation will present selected examples for the use of fish embryos for the testing of teratogenicity, genotoxicity, neurotoxicity, endocrine disruption, drug metabolization and development of cancer.

**Acknowledgment**

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**The adverse outcome pathway concept linking molecular interactions and**

**higher-level outcomes in ecotoxicology**

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The adverse outcome pathway (AOP) was proposed by Ankley and colleagues in 2010 as a conceptual framework serving to organize and evaluate (eco)toxicological knowledge about the successive progression of toxicity from lower to higher levels of biological organization [1]. AOPs typically start with a molecular initiating event and continue through a number of causally linked key events to finally reach an adverse outcome, which is defined as a higher-level key event relevant for risk assessment and regulatory purposes. Thus, AOPs help to establish confidence in the relationships between molecular-level changes and higher-level outcomes, with this ultimately enabling a broader use of Tox21-enabling methodology, such as *in vitro* assays or omics analyses, for next-generation hazard and risk assessment purposes [2, 3]. The AOPs’ focus on the molecular mechanisms of toxicity provides a useful avenue for cross-species extrapolations based on evaluation of sequence- and function-similarities. It also helps to bridge between human health and environmental risk assessment pipelines, in the sense of leveraging the molecular data generated for either purpose to inform on other outcomes of interest. For example, molecular data collected during pre-clinical phases of drug development pipelines could be re-used later on (or, alternatively, also early in the development process) to assess the potential environmental risks of a compound or compound groups. In my talk, I will briefly touch on the general aspects of AOP development and the role taken by the OECD to spearhead the adoption of this concept in the scientific and regulatory communities. I will then move to discuss several case studies illustrating the use of AOPs to support environmental risk assessment of bioactive substances.

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none

**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Title: Omics-based responses to sexual endocrine active substances in zebrafish embryos**

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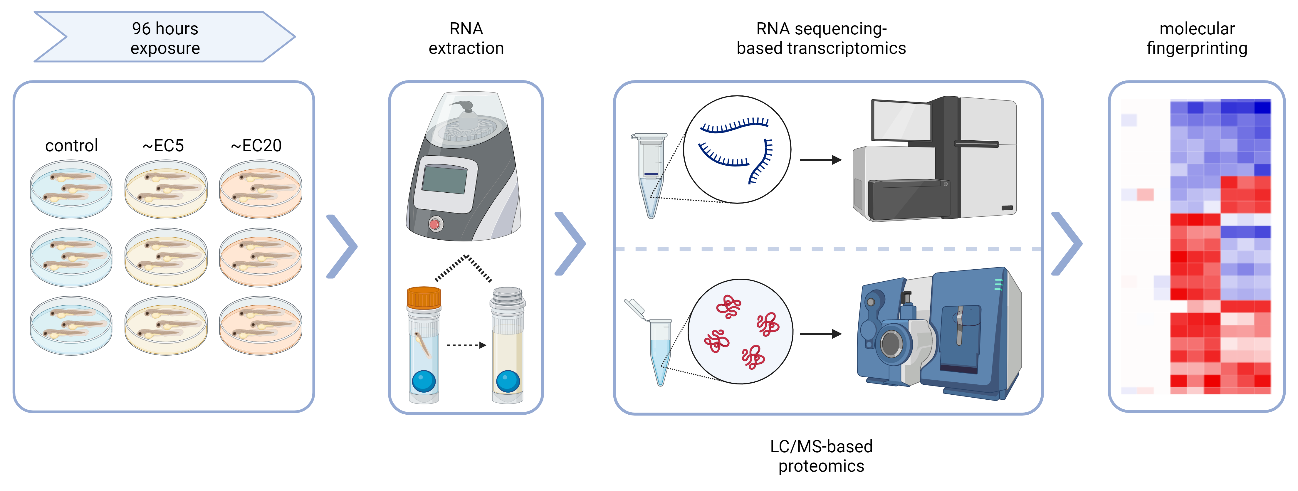
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The adverse effects triggered by active substances of biocides, pesticides, pharmaceuticals or by industrial chemicals can reverberate across ecosystems, posing a significant threat to environmental organisms. To avoid such harmful effects, chemical manufacturers are required to provide data for environmental hazard and risk assessment of these substances prior to their registration. However, conventional standardized assays employed to assess the impact of endocrine-active compounds on non-target environmental organisms are costly, both in terms of resources and animal use. To address this challenge, there is a need for alternative, cost-effective approaches, using animal replacement methods, with predictive capabilities in regard to toxicity mechanisms to support hazard assessment efforts. Omics-methodologies are attractive for collecting consistent high content data to discern chemicals modes of action (MoA) for hazard and risk assessment prioritization. In the context of identifying screening-compatible biomarkers for estrogen and androgen-mediated endocrine MoA, we analyzed and compared early molecular signatures induced by ethinylestradiol, tamoxifen, methyltestosterone and flutamide. While no significant phenotypic changes were observed for endpoints following a modified fish embryo toxicity test (zFET), results from transcriptomic and proteomic analysis revealed a concentration-dependent increase in the number of differentially expressed genes. Functional classification and overrepresentation analysis of the observed DEGs identified response to hormone, steroid metabolic process and cellular response to estrogen stimulus to be significantly perturbed. Our study demonstrates that omics-methodologies can pave the way for a deeper understanding of long-term ecological consequences, enhancing predictive models and guiding decision-making. Future screening approaches developed based on these data hold the promise of initiating the development of environmentally friendly substances right from the start, thereby minimizing adverse effects on aquatic ecosystems.

**Keywords:** *Endocrine disruptors,**sexual impairment, biomarkers, ecotoxicogenomics, zebrafish embryo*

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**Acknowledgment**

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Nanotechnology May Eliminate Vector-Borne Parasitic Infections**

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Both humans and the animals with which they frequently come into contact are at risk of vector-borne parasitic diseases, which are widespread. Antiparasitic drugs are used to reduce or eliminate vector-borne parasitic diseases in humans and animals. The toxicity of these drugs can vary depending on the type of drug used and can be harmful. Prolonged or excessive use of antiparasitics can cause kidney failure, vision problems, and blindness. Antiparasitics can be almost as toxic as the parasites they are designed to eliminate. Common side effects of antiparasitics include diarrhea, nausea, vomiting, abdominal pain, and cramps. Other side effects may include loss of appetite, bloating, headache, dizziness, and behavioral disturbances. Promising results in reducing parasitic disease have been achieved through the use of nanoparticles. Liposomes are nanoparticles that can deliver antiparasitic drugs. They consist of a lipid bilayer that can encapsulate drugs and protect them from degradation in the body. These nanoparticles could improve antiparasitic drugs by reducing side effects and increasing targeted distribution. They encapsulate drugs and protect them from degradation in the body. They can be biodegraded in vivo, do not produce toxic substances, and reduce drug side effects. In particular, stearylamine liposomes have been studied as an antiparasitic agent with immunomodulatory activity. Liposomal delivery has the potential to control parasites with minimal side effects.

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Effectiveness of Syzygium aromaticum L. extracts against Hyalomma scupense, the most common vector tick species infesting cattle in Tunisia**

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In the present current study we evaluate the acaricidal and repellent properties of the ethanolic extract, essential oil, and primary component eugenol from Syzygium aromaticum against Hyalomma scupense cattle ticks, the most common vector tick species infesting cattle in Tunisia [1].

Clove essential oil was extracted using hydrodistillation technique. Gas chromatography-mass spectrometry (GC-MS) was performed to identify the chemical composition of clove. To evaluate the adulticidal, ovicidal, larvicidal and repellent proprieties of clove essential oil, eugenol and ethanolic extract on H. scupense, in vitro assays were performed using the adult immersion test (AIT), the ovicidal test, the larval packet test (LPT), the filter paper test and anti-acetylcholinesterase (AChE) activity.

the primary phytoconstituent of clove oil (eugenol), which accounts for 97.66% of the whole oil, had 99.22% acaricide activity and inhibited egg hatching at a concentration of 10 mg/mL. Eugenol and clove essential oil showed potent adulticidal effect at high concentrations (10 mg/mL), achieving 100 and 93.76% mortality, respectively. The ethanolic extract exhibited moderate activity. At high concentration, the larvicidal activity of S. aromaticum oil, eugenol, and ethanolic extract were 100, 100, and 77.18%, respectively. In filter paper experiments, when tested at the concentration 5 mg/mL; eugenol showed the longest repellent effect up to 6 h. We also found that eugenol was the most active AChE inhibitor (IC50 = 0.178 mg/ mL). Nevertheless, additional investigations are required to confirm the accurate mechanism and the relevance of clove in practical application for the control of H. scupense cattle ticks.

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Screening of bio resources to identify new antiviral compounds against influenza virus infections**

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Influenza viruses belong to the family of *Orthomyxoviridae* and cause contagious infections. Symptoms can range from irritable cough, fever, fatigue to severe complications like pneumonia or encephalomyelitis. According to the WHO, approximately 1 billion cases of seasonal influenza infections are reported worldwide each year. Of these, approximately 3-5 million cases result in severe infections, 10% of which lead to the death of the patient [1].

Approved drugs like M2 proton channel inhibitors and neuraminidase inhibitors suffer from poor efficacy if not applied early and are susceptible to the development of resistances. The lack of treatment options underlines the importance of identifying new antiviral strategies against influenza infections. Novel lead structures can be identified by screening natural resources such as bacterial and fungal extracts or compounds.

To identify new lead structures, we first established a screening assay with seasonal influenza virus strains based on the detection of a virus-induced cytopathic effect in MDCK II cells. Amongst the tested compounds are antimicrobial peptides of various arthropods, as well as extracts and pure compounds from bacteria and fungi. Extracts showing an antiviral effect were µ-fractionated and further analysed to detect the active compound, which is then purified by activity-guided purification. Subsequently, identified compounds were then further analysed regarding their antiviral properties.

For example, the peptaibiotic tolypin, isolated from *Tolypocladium niveum* showed an antiviral activity against two tested influenza B viruses. We observed a 4-log unit reduction in the virus titers of both Malaysia/B and Massachusetts/B after tolypin treatment compared to untreated cells.

Besides the antiviral properties, the toxicity of the compounds was analysed. Therefore, the cytotoxicity was determined in MDCK II cells and in Calu-3 cells. Compounds showing an antiviral effect, like tolypin, were further analysed in the model insect *Galleria mellonella* (greater wax moth) to obtain first *in vivo* data.

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Discovery of a novel therapeutic candidate against animal trypanosomiasis**

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Animal trypanosomiasis (AT) is a widespread disease caused by *Trypanosoma spp.* and has a devastating effect on animal husbandry all over the world due to the scarcity of efficient drugs and development of drug resistance, hence emphasizing the need for novel treatment options. Following previous identification of 3’-deoxytubercidin as a highly potent trypanocide with curative activity in mouse models of both stage-1 and stage-2 Human African Trypanosomiasis (HAT), we now present a comprehensive preclinical evaluation of new 6-amino substituted tubercidin analogues with promising activity against a broad range of AT species. Potent hits were identified *in vitro* across all important AT species, *i.e. Trypanosoma brucei brucei*, sensitive and isometamidium (ISM)-resistant *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma evansi* (type A and B) and *Trypanosoma equiperdum*. Selected ‘hits’ were further tested for *in vitro* metabolic stability (using bovine, horse and piglet liver microsomes), *in vivo* mouse models for each AT species, genotoxicity assays and mode-of-action studies (*i.e.* genome-wide RNA interference library screening, metabolomics). Analogue **3** was highly active in *T. vivax*, *T. congolense*, *T. equiperdum*, *T. evansi* and *T. brucei* curative mouse models. Furthermore, there was no indication of *in vitro* genotoxicity as confirmed by Vitotox®, the micronucleus and the comet assays. Mode-of-action studies for **3** revealed that the P1 nucleoside transporter and adenosine kinase are involved in drug uptake and activation, respectively. Ecotoxicological assessments on *Daphnia* and green algae revealed that the compound has a relatively low ecotoxicological footprint. Given the preferred target product profile for a broad-spectrum drug against AT, analogue **3** represents a promising ‘lead’ candidate for treatment of animal trypanosomiasis, regardless of the causative species.

**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Analysis and detection of antibiotic resistance genes in waste waters in Albania, in the "One Health" approach**

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Antibiotics are one of the most important groups for the treatment of many infectious diseases in humans and animals, including some of the parasitic infectious diseases caused by vectorial transmission. Some of the infectious diseases caused by protozoa, transmitted from humans to animals, are treated through the use of different classes of antibiotics such as sulfonamides, fluoroquinolones, lyncosamides, macrolides, nitromidazoles, tetracyclines, etc. According to the statistical data in Albania for the use of these antibiotics in the last 5 years 2019-2023, it results that the classes of sulfonamides, fluoroquinolones, macrolides and beta lactams are used in a comparable way in humans and animals as part of therapeutic protocols of infectious diseases. The amount of antibiotic use, the characteristics of their biotransformation and elimination by humans and animals in the environment, as well as their circulation in a vicious way from the environment back to animals and humans through ground water constitutes a fundamental threat to the growth of antimicrobial resistance that threatens improve public health. For this purpose, in this study, water samples were analyzed to identify the presence of antibiotic resistance genes in the environment as an ecotoxicological evaluation parameter of the environmental microbiome resistant to antibiotics. Water samples were taken from sewage and analyzed by PCR test.

In these samples were identified: sul1, blaTEM, bla OXA 48, bla CMy-2, tel C, mcr-1.

**Key words:** antibiotics, resistance genes, infective disease, sul1, blaTEM, , tel C, mcr-1.

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Assessment of pharmaceuticals in Ishmi Basin, Albania**

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Availability, toxicity, and general degradation of surface water quality in Albania are issues of serious concern especially as the country aims to join the European Union (EU). Albania has to assess and report the levels of pharmaceuticals according to the EU requirements such as water framework and marine strategy directives. Pharmaceuticals are recognized for the adverse effects on ecosystems and organisms, especially on aquatic species. The highest concentrations of pharmaceuticals in source waters have been reported in low- to middle- income countries having poor water treatment/management infrastructure [[1](#_ENREF_1)]. Ishmi basin is located in the central-west part of Albania and discharges to the Adriatic Sea. In this area, Tirana, Kamza, Kruja and Durres municipalities with more than one million people are located. Thus, the assessment of these pharmaceuticals such as anti-inflammatories (ibuprofen, naproxen and diclofenac), the commonly used antiepileptic (carbamazepine) and antibiotics (sulfamethoxazole, trimethoprim, erythromycin, anhydro-erythromycin, azithromycin, ciprofloxacin and clindamycin) is of high importance. In this study six sampling points were selected. Samples were collected in three different seasons and glass bottles were used. Samples were extracted within 48 h. Anti-inflammatories and carbamazepine were extracted with solid phase extraction (SPE) and determined with HPLC-UV/FLD [[2](#_ENREF_2)]. Meanwhile, antibiotics were determined directly with HPLC-MS/MS. This study demonstrated that anti-inflammatories such as ibuprofen and naproxen are predominant and detected almost at all sampling points (except point one). Also, the most detected antibiotic was ciprofloxacin. Sampling point two, which is impacted from Tirana municipality shows the presence of almost all pharmaceuticals. Diclofenac was also detected but not at all sampling points.

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**“Eprinomectin: fate and effects in a mesocosm system”**

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With the surge in environmental chemical exposure, it is becoming impractical and costly to conduct mesocosm tests for every substance. Predictive simulation models are increasingly vital for efficient yet thorough risk assessments, although they cannot entirely replace existing methods. Our project aims to support these models by creating the MeMo-Environmental DatabasePlus. This comprehensive database will feature water chemistry, physical parameters, and seasonal population trends of key freshwater organisms. Utilizing mesocosm experiments, we are also focusing on the environmental fate of the endectocide eprinomectin in water, sediment, and aquatic plants. The data collected will be valuable in enhancing the accuracy, optimization, and validation of future predictive models for aquatic ecosystems. Eprinomectin and other endectocides are of particular importance because they can pose environmental risks to nontarget species [1] while also being vital for One Health initiatives, particularly in disease and vector control [2].

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Do microplastics influence the partitioning of ivermectin in soil?**

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Plastics are now found in all natural environments, including soil. The fate and effects of microplastics in terrestrial ecosystems remain largely unexplored. It is discussed that microplastics interact with organic pollutants due to their hydrophobic properties, possibly affecting their distribution and transport in the soil [1]. This research addressed the antiparasitic agent ivermectin, which is excreted largely unmetabolized after treatment of livestock and can potentially introduced into the soil [2]. Sorption experiments were carried out to investigate the partitioning and fate of ivermectin in a microplastic-contaminated reference soil. Subsequently, the influence of microplastics on the bioaccumulation of IVM in earthworms was investigated. The first preliminary results of this study will be subject of the short communication.

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

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**Zebrafish embryo vs mouse – An alternative method in assessing neurotoxic potencies of pharmaceuticals?**

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Therapeutic drugs represent one of the greatest benefits, but also challenges for modern society. Through continuous increase in consumption, wastewater treatment plants and improper disposal, pharmaceuticals such as psychoactive drugs are increasingly released into natural ecosystems, which already at low concentrations can affect non-target organisms. To protect the environment and human health, hazard and risk assessments of these chemicals are now mandatory as part of the authorisation process and ecotoxicological studies. However, the rapid increase in toxicity testing, mostly using mammalian model organisms, has led to ethical concerns in the scientific community and the public. Therefore, alternative methods for screening as well as of toxicity and ecotoxicity testing need to be developed and applied.

In accordance with this, zebrafish (*Danio rerio*) embryos were investigated for their potential to rapidly identify neurotoxicity induced by the antiepileptic drug valproic acid and up to 7 analogues in mammals. For the identification of neurotoxic potencies, the compounds were tested using the Fish Embryo Acute Toxicity (FET) assay (OECD TG 236, OECD (2013)). Adjustment of the evaluation by a subsequent comparison of selected FET endpoints with *in vivo* mouse data expressing exencephaly revealed a good correlation between zebrafish embryos and mice. By taking into account the pH of the test medium in a second step, the comparability could be further improved.

In summary, a good correlation between zebrafish and mammals could be observed for the neurotoxic effects of valproic acid. Considering the variety of therapeutic drugs, the FET assay can be used as a rapid and simple first screening for neurotoxicity in zebrafish, which may highlight substances in screening that need to be further investigated for their neurotoxic potential with more time-consuming methods such as the neuromast assay or behavioural studies.

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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

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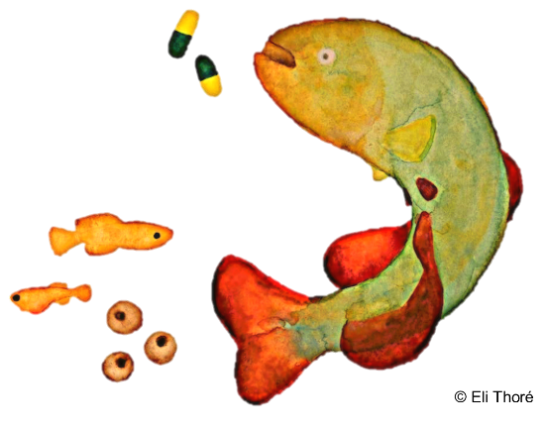
**Unveiling challenges of ecotoxicological testing in One Health drug discovery and development: Insights from fluoxetine and turquoise killifish**

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Chemical pollution is an urgent and escalating global concern, as numerous chemicals continue to enter the environment. A significant challenge is to evaluate how these chemicals affect ecosystems and set safe concentration limits to safeguard the environment. To tackle this, standardized ecotoxicological tests using model organisms have been pivotal in assessing the toxicity of chemicals, especially for substances like pesticides and heavy metals. However, these tests are not optimally designed to address the prolonged and low-dose exposure scenarios that are typical of pharmaceutical pollutants. In this presentation, I contend that refining ecotoxicological testing for pharmaceuticals could benefit from incorporating more nuanced biological effects, like changes in behavior, and adopting chronic assessments across different life stages and generations. I will spotlight a series of recent case studies that focus on the impact of the antidepressant fluoxetine on a model fish species that is still relatively new to science: the short-lived turquoise killifish (*Nothobranchius furzeri*). I will show that even extremely low concentrations of fluoxetine can trigger noteworthy changes in ecologically important fish behaviors. Furthermore, I will illustrate that these effects may differ across various life stages and generations and could be influenced by simultaneous exposure to other chemicals. Finally, I advocate for injecting more real-world complexity into ecotoxicological research. This is crucial if we are to fully understand, predict, and mitigate the ecological impact of pharmaceuticals.



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**Workshop: “Biomarkers, screening, standardized and non-standardized ecotoxicological testing in One Health drug discovery and development”**

**September 20 and 21, 2023**

**Revealing potentially harmful effects of sunscreen**

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The occurrence of skin cancer correlates positively with the amount of ultraviolet (UV) radiation [1]. Sunscreen protects due to UV filters from the dangerous radiation. Some of these organic molecules are structurally similar to hormones and act as endocrine disruptors [2]. Additionally, oils contain genotoxins, which may also be present in fatty creams [3]. Thus, sunscreen can have a negative impact on humans and the ecosystem. Through effect-directed analysis using biological test systems, a toxicological assessment of unknown samples can be performed. High- performance thin-layer chromatography with planar bioassays contribute to the detection of active compounds in complex mixtures [4]. The endocrine effects of several UV filters were confirmed, such as the potential endocrine disruptor benzophenone-3, which is no longer used in cosmetic products [5]. Ethylhexyl salicylate (EHS) was already under investigation as endocrine disruptor by European Chemicals Agency (ECHA), but continues to be used (withdrawn in 2022 due to lack of information). It also exhibited estrogen-like and antiandrogenic effects, although 10 times less than benzophenone-3. Screening different sunscreens revealed various estrogen-like and antiandrogenic substances. Focusing on the effects of EHS in one selected sunscreen, the half maximal effective concentration (EC50) was

2.5 µg for estrogen-like and 2.7 µg for antiandrogenic EHS effects. The screen for genotoxic effects showed up to six active zones (partly unknown) and the EC50s ranged between 105 µg and 303 µg sunscreen. Skin layer depth profiles show that EHS remained on the uppermost skin layer and only possibly penetrated into deeper layers via hair follicles. However, genotoxic effects were detected in the dermis and could enter the circulatory system. In addition, antimicrobial activity against marine Gram-negative Aliivibrio fischeri bacteria was detected in sunscreen.

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