# Neuroactive psychotropic drugs in aquatic systems and the co-treatment of non-target organisms

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vorgelegt von

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*Datum* *Unterschrift*

# List of abbreviations

| | |
|---|---|
| °C | Degrees Celsius |
| µg | Microgram |
| ACh | Acetylcholine |
| AChE | Acetylcholinesterase |
| AMG | German Medicines Act |
| ANOVA | Analysis of variance |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dpf | Days post fertilization |
| DTNB | 5,5´-dithiobis(2-nitrobenzoic acid) |
| DMSO | Dimethylsulfoxide |
| EC50 | Median effect concentrations |
| EMA | European Medicines Agency |
| ERA | Environmental risk assessment |
| g | Gram |
| GLP | Good laboratory practice |
| h | Hour(s) |
| hpf | Hours post fertilization |
| ISO | International Organization for Standardization |
| L | Liter |
| LOEC | Lowest Observed Effect Concentration |
| min | Minute(s) |
| MLR | Multiple linear regression |
| NDRI | Selective reuptake inhibitors of dopamine and noradrenaline |
| NERI | Selective noradrenaline reuptake inhibitors |
| NOEC | No Observed Effect Concentration |
| OECD | Organisation for Economic Co-operation and Development |
| PCA | Principle Component Analysis |
| PEC | Predicted Environmental Concentration |
| PNEC | Predicted No-Effect Concentration |
| qRT-PCR | Quantitative real-time polymerase chain reaction |

| | |
|---|---|
| QSAR | Quantitative Struktur-Wirkungs-Beziehung |
| RNA | Ribonucleic acid |
| s | Second(s) |
| SAR | Struktur-Wirkungsbeziehung |
| SSRI | Serotonin reuptake inhibitor |
| SSNRI | Selective serotonin-noradrenaline reuptake inhibitors |
| UBA | Umweltbundesamt |
| WFD | Water Framework Directive |

# Abstract

The focus on the analysis of the major consequences of human impact on Earth for different ecosystems has been constantly changing over the last centuries. A brief overview of the current situation reveals the drastic consequences of climate change and the loss of different species for life on earth and shows how much humans are changing their environment. A serious but much less visible environmental problem is the contamination of freshwater systems by micropollutants, as the resulting consequences cause massive damage at aquatic species communities and have negative impacts on human health due to the closely connected circular systems. Micropollutants are an inhomogeneous group of various microparticles and chemicals that deteriorate the quality of drinking water and which can only be insufficiently eliminated by conventional purification methods. One group of micropollutants whose concentration in the environment has been steadily increasing are human psychotropic drugs. Increasing stress in different life areas is one reason for mental illnesses like depressions and burnout, which leads to an annual increase in the development and use of neuroactive pharmaceuticals. These substances finally enter the aquatic environment through excretion or incorrect disposal and cause ecological changes in the structure of the ecosystems. The European Medicines Agency has formulated a guideline to counteract these consequences, which clearly defines a two-stage procedure for testing the environmental hazard of active pharmaceutical ingredients. However, this system operates on the principle that not all pharmaceuticals need to pass through both stages and that only OECD and ISO-validated standard tests may be used for data generation in the selection of biological test methods. This approach has clear limitations in the effect analysis of neuroactive substances because they are designed to trigger a specific effect at a specific concentration range in the nervous system. The effects that occur at the molecular and physiological levels could not uniformly be measured by general standard test methods and many problems caused by these substances could not be quantified completely. This problem has not yet been sufficiently investigated for the mixtures of these substances that always occur in the environment. A tiered approach was used to investigate the pure substance mirtazapine, artificially prepared mixtures of various neuroactive substances, and native samples from hospital wastewater. The swimming behavior of zebrafish embryos, showed a decreased activity of more than 45 % for mirtazapine concentrations of more than 1 mg/L compared to the control groups. In comparison, the swimming activity of *Daphnia magna* increased concentration-dependently for the measured concentrations from 0.1 – 200 µg/L mirtazapine. Sedative effects on the swimming behavior of zebrafish embryos could be measured in the more complex samples that were artificially produced as well as in the native environmental samples. The activity decreases were also measurable for substance combinations with complementary modes of action. At gene regulatory level, increases of 8-fold were measured

compared to the control group for genes of the serotonin- and dopamine-systems (*slc17a6a, slc2a2, slc6a3a*). The analysis of native hospital wastewater samples was carried out in an interdisciplinary cooperation i.e. with the Psychiatry Department of the University Hospital Aachen and the Environmental Research Center in Leipzig. The results show a decreased activity for the swimming behavior of zebrafish embryos in relation to the concentration of the recovered neuropharmaceuticals. Activity decreases were measured at the beginning of the day and in the early afternoon. At the gene level, an increase in genes i.e. of the serotonin system could be measured. The behavioral effects can probably be attributed to an overload of the central neural system, which is responsible for the coordination of the movements.The central objective of this work was the development of a tiered test approach on different biological levels to improve the environmental risk assessment for neuroactive substances. Therefore, different quantification methods on physiological level and various ways of quantification these effects on cellular and molecular level are tested and improved. Finally, the results should help to set up recommendations on regulatory level for a better protection of aquatic non-target organisms against these substances in the future.

# Zusammenfassung

Die Frage nach den größten Folgen des menschlichen Einflusses auf Erden für Ökosysteme unterschiedlicher Art, hat sich im Laufe der letzten Jahrhunderte stetig verändert. Eine Momentaufnahme der aktuellen Situation legt vor allem drastische Folgen durch den Klimawandel und den Verlust von unterschiedlichen Arten für das Leben auf der Erde offen und zeigt dabei, wie sehr der Mensch seine Umgebung verändert.

Eine in diesem Zusammenhang schwerwiegende, jedoch auf den ersten Blick deutlich weniger sichtbare Gefahr geht von der Verunreinigung der Süßwassersysteme durch Mikroschadstoffe aus. Die daraus resultierenden Folgen führen zu massiven Schäden in aquatischen Artengemeinschaften und besitzen aufgrund der engen Verbindungen auch negative Auswirkungen auf die menschliche Gesundheit. Es handelt sich dabei um eine inhomogene Gruppe von Kleinstpartikeln und Chemikalien, die die Qualität des Trinkwassers massiv verschlechtern können und die bisher in vielen Fällen durch konventionelle Reinigungsmethoden nur unzureichend eliminiert werden konnten. Eine seit den vergangenen Jahrzehnten produktions- und verwendungstechnisch stetig steigende Gruppe von Mikroschadstoffen sind humane Psychopharmaka. Steigende Belastungen in unterschiedlichen Lebensbereichen lassen Krankheitsbilder wie Depression und Burnout stetig zunehmen, wodurch auch die Verabreichung von neuroaktiv wirksamen Pharmazeutika jährlich zunimmt. Diese Stoffe gelangen abschließend durch Exkretion oder falsche Entsorgung in die aquatische Umwelt und führen dort zu massiven Schäden. Um diesen Folgen entgegenzuwirken existiert eine von der European Medicines Agency formulierte Richtlinie, in der ein zweistufiges Verfahren zur Testung der Umweltgefahr von pharmazeutischen Wirkstoffen eindeutig festgelegt wird. Dieses System basiert jedoch auf einer Auswahl von biologischen Testverfahren die ausschließlich OECD und ISO-validiert sind. Dadurch besitzt dieser Ansatz deutliche Lücken in der Anwendung auf neuroaktiv wirksame Substanzen, da diese im Anwendungsprozess darauf ausgelegt sind bei sehr niedrigen Konzentrationen einen spezifischen Effekt im Nervensystem auszulösen. Die dabei auftretenden Effekte auf molekularer und physiologischer Ebene werden durch die allgemeinen Standardtests nicht einheitlich und detailliert genug aufgenommen, weshalb sie für die Bewertung nicht berücksichtigt werden.

Diese bereits in den vergangenen Jahren oftmals aufgezeigte Problematik, wurde bisher noch nicht für die in der Umwelt fast immer auftretenden Substanzmischungen untersucht. Basierend auf einem gestuften Ansatz, wurde dabei die Reinsubstanz Mirtazapin, künstlich hergestellte Mischungen verschiedener neuroaktiver Substanzen, sowie native Proben aus Krankenhausabwasser untersucht. Im Schwimmverhalten von Zebrafischembryonen konnte dabei für Mirtazapinkonzentrationen ab 1 mg/L eine Aktivitätsabnahme von mehr als 45 % im Vergleich zu den Kontrollgruppen gemessen werden. Im Vergleich dazu nahm die Schwimmaktivität von Daphnien konzentrationsabhängig zu. Bei

den komplexeren Proben, die künstlich hergestellt worden sind, als auch bei den nativen Umweltproben konnten sedierende Wirkungen auf das Schwimmverhalten von Zebrafischembryonen gemessen werden. Die Aktivitätsabnahmen waren dabei auch für Stoffkombinationen mit komplementären Wirkmechanismen messbar. Auf genregulatorischer Ebene konnten Steigerungen um das 8-fache im Vergleich zur Kontrollgruppe insbesondere bei Genen des Serotonin- und Dopaminsystems (*slc17a6a, slc2a2, slc6a3a)* gemessen werden. Die Analyse nativer Krankenhausabwasserproben wurde im interdisziplinären Umfeld unter anderem in Kooperation mit der Psychiatrie des Uniklinikums Aachen, sowie dem Umweltforschungszentrum in Leipzig durchgeführt. Die Ergebnisse zeigen eine Aktivitätsabnahme des Schwimmverhaltens von Zebrafischembryonen in Abhängigkeit von der Konzentration der wiedergefundenen Neuropsychopharmaka. Erhöhte Abnahmen konnten dabei zu Tagesbeginn und am frühen Nachmittag gemessen werden. Auf Geneebene konnte unter anderem eine Erhöhung von Genen des Serotoninsystems gemessen werden. Zurückgeführt werden können die Verhaltenseffekte voraussichtlich auf eine Überladung des zentralen neuronalen Systems, welches für die Koordination der Bewegungen verantwortlich ist. Das zentrale Ziel dieser Arbeit, die Entwicklung eines mehrstufigen Testansatzes auf unterschiedlichen biologischen Ebenen, zur Abschätzung der Umweltauswirkungen dieser Substanzen konnte mithilfe der Studien erreicht werden. Durch einen gestuften Ansatz von der Analyse der Auswirkungen der Reinsubstanz Mirtazapin bis zur finalen Untersuchung einer komplexen Krankenhausabwasserprobe wurden unterschiedliche Endpunkte auf Verhaltens-, zellulärer- und Genexpressionsebene analysiert und diskutiert. Abschließend konnten die Ergebnisse zur Formulierung von Handlungsempfehlungen für die zukünftige Gefahrenbeurteilung dieser Stoffe genutzt werden, um langfristig eine möglichst hohe Zahl von aquatischen Organismen gegenüber diesen Stoffen zu schützen.

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# Chapter 1

# General Introduction

## 1.1 Neuroactive substances in aquatic systems – a problem in modern society?

An elevated human population density can be found along the watercourse of rivers which is indicative of the dependency humans hold towards such waterbodies. Since the establishment of the first settlements, rivers have been largely responsible for the survival of mankind and have undergone various changes of meaning over time (Borchardt et al., 2004; Nixdorf et al., 2004; Wöbbecke et al., 2003). In addition to functioning as a source of drinking water, they were also expended as natural sewage pipes for the disposal of used freshwater. In most cases, this had dramatic consequences for the environment (Gregory, 2006). In particular, the consequences of changes in species abundance are increasingly being elucidated by ecological field studies and in many cases show the negative effects of pollutions on the stability of natural ecosystems (Hartman, 1996). Therefore, the function of rivers as natural wastewater systems has been significantly criticized and replaced, due recreational and cultural practices (Akinsete et al., 2019). Though the pollution is no longer characterized by detergent residues or other directly noticeable contaminations, new problems have come into existence. In recent years, societal changes have contributed to the pollution of aquatic systems, which have led to the destruction of various landscapes and ultimately impacted the health of humans (Grizzetti et al., 2017; Petts et al., 1989). One of the most prominent problems are micropollutants, which in large numbers also consist of neuroactive substances such as human psychotropic drugs (Bláha et al., 2019; Meyer et al., 2019; Robson et al., 2020). Various (bio)monitoring studies have shown that the discharge of psychotropic drugs into European waters has increased severely in recent years as a result of increased disease incidence (Mossialos et al., 2004; Vernardet et al., 2005). Current studies speculate that various rivers worldwide are contaminated with the model substance Diclofenac (Scheurell et al., 2009; Wang et al., 2010). Concentrations of more than 100 µg/L have been detected in waterbodies and are shown to increase up to 1000 ng/L downstream thereof (Acuña et al., 2020). As a result, aquatic organisms are subjected to high levels of these substances. The increased production, as well as the prescription of these substances, explain the surge of psychotropic drugs found in aquatic systems. In 2002, the Water Framework Directive (WFD) was established in response to the increased risk of the stability of aquatic ecosystems. Their principal aim is to achieve a good ecological and chemical status of running waters. This is defined by a state-of-the-art with only minor deviations from an ideal system without disruptive human influences. Various quality characteristics (including (eco)toxicological, hydromorphological and chemical) are used to analyse this objective (Hollert et al., 2007). Unfortunately, only a minority of the European water systems were able to achieve this objective in the first phase of introduction (Richter et al., 2013). Reasons for failures are manifold and, in some cases, are unable to be identified. From an environmental science point of view, however, two

explanations for the inadequate formulation of protective laws for running waters can be brought to attention. On the one hand, the surveillance of substances in aquatic environments was - for a lengthy amount time-limited to small numbers of substances, as these were declared particularly harmful. Moreover, the availability of suitable measurement standards for the detection of these substances were restricted (Faust et al., 2019). On the other hand, the joint analysis of chemical-analytical data and scientific information gained from bioassay studies was disregarded. This meant that the actual environmental risk assessment could not be correctly assessed (Farré and Barceló, 2003; Schuetzle and Lewtas, 1986). Many biological tests have been further developed and optimised and now partly allow effect detection below the results of chemical analysis. Nevertheless, it is the combination of both methods that have shown significant progress in the assessment and definition of a suitable environmental risk assessment in various large-scale research projects such as SOLUTIONS (Brack et al., 2015).

In recent years, neuroactive substances have been of particular significance because of the ever-rising medicinal demand in both the occupational and private life of individuals. As a consequence of the rapidly developing world, humans are pressured to react and adapt to their environments in much shorter periods of time (Baum et al., 2019). In this regard, diseases associated with the cardiovascular system, or the development of carcinomas have become a part of everyday life. Additional illnesses include depression or a state of physical and mental exhaustion brought on by prolonged periods of stress, which professionals have termed “burn-out” (Burton et al., 2019). In most instances, a combination of neuroactive drugs are prescribed to patients to treat the symptoms linked to the illnesses such as headache and fatigue (Lianov et al., 2019). Comparative studies carried out since 2000 showed that in the Nordic countries of Europe, the amount of used neuroactive substances have more than doubled (WHO, 2017). Furthermore, the amount of Europeans in general that use neuroactive substances regularly has risen to more than 10 % compared to the total population (Schwabe and Paffrath, 2013). A possible reason for this increased use is the off-label prescription for diseases that do not represent psychological dysfunction. This off-label use is applied for different diseases such as chronic pain treatment, premenstrual symptom reduction and for the combat of behavioural disfunctions (Skånland and Cieślar-Pobuda, 2019).

The origin of pharmaceuticals in the environment can be traced back to two different pathways. So called ‘parent substances’ enter the environment primarily through the improper disposal of pharmaceuticals in (waste)water systems (Anwar et al., 2020; Bound and Voulvoulis, 2005). In addition to the disposal of drugs, which are no longer needed to treat an illness or have expired, large quantities can also enter the aquatic environment because of improper production. In most cases, this is caused by a lack of safety and environmental precautions (Fick et al., 2009; Götz and Walz, 2017). This poses

a particular problem for antibiotics, for example, since the formation of resistance towards these drugs starts directly at the production site, meaning that the drugs can no longer be used for treatment (Ghernaout and Elboughdiri, 2020). This path of entry can easily be managed through the education and sensitization of consumers. Large input volumes can be avoided by developing a general understanding of how to properly dispose and recycle medicinal products (Ebert et al., 2014). The second path of entry encompasses the human excretion of parent substances and metabolites into the environment. This pathways, in contrast the first input pathway, requires additional technical aids (Ternes, 2001). Hospitals can act as point sources, as complete removal of the substances cannot be achieved in many wastewater treatment plants (Nagarnaik et al., 2011). In spite of extensive further developments of the fourth purification stage in many wastewater treatment plants, the current results do not indicate whether a complete elimination of micro-pollutants could be realized (Oneby et al., 2010; Yeom et al., 2002). On top of this, unexpected flood events lead to an uncontrolled discharge of untreated wastewater into the downstream river systems, bypassing the treatment plants. A minor source for neuroactive substances in natural systems is the release of contaminated sewage sludge on agricultural land (Demeestere et al., 2010). This source is strictly controlled in Europe and poses a problem in countries with less stringent regulations.

Monitoring has been implemented to assess and regulate the pharmaceutical inputs into the environment. Hereby, the concentrations of selected chemicals are measured at one or more sampling points of a study site. An advantage of this approach is the simplification. The contamination of the environment is extremely complex, especially when regarding a myriad of different mixtures and evaluating their toxicity via means of bioassays (Boxall et al., 2014; Miarov et al., 2020; Vystavna et al., 2013). Information about the actual risk potential of a chemical (both as a pure substance and as a mixture) cannot be attained via sampling alone. Furthermore, the presence of a certain chemical does not necessarily mean that it has a harmful potential to the environment (Iyengar et al., 2001; Richards et al., 2004; Schoenfuss et al., 2016). Therefore, different approaches are implemented to assess the samples. In general, a tendency can be observed, wherein the increase of administration of neuroactive substances are linked to the increase of adverse effects in aquatic organisms (Daughton and Brooks, 2011; Kostich and Lazorchak, 2008).

Two important questions are posed with reference to the pollution of the environment: how are the effects characterized in aquatic organisms and how strongly do they burden the ecosystem?

Neuroactive substances pose a particular challenge in ecotoxicological research, as they are only able to be analysed to a certain extent, following the guidelines of the current OECD-validated bioassays. Effects of neuroactive substances introduced to aquatic organisms at high concentrations include genotoxicity, morphological and immunological changes. These concentrations are often much higher

than those detected in the environmental and therefore do not yield functional results in most biotests (Klüver et al., 2015; van den Brandhof and Montforts, 2010). Nevertheless, it has become apparent that the majority of neuroactive substances in aquatic systems lead to physiological changes in non-target organisms (Ogungbemi et al., 2021). This observation has been linked to and partially driven by a stagnant loss of biodiversity. Most interestingly, more sensitive characteristics such as behavioural changes as a result of contamination in organisms has been observed (Selderslaghs et al., 2010). These behavioural modifications have the potential of altering the structure of a population and ultimately shifting the dynamics of both aquatic and terrestrial ecosystems. This is of great ecological concern as little research has been conducted in the interspecies relationship network within an ecosystem, resulting in an incomplete assessment of the potential risk drugs can have on the environment (Gros et al., 2010).

The various effects of individual organisms, and in turn populations and ecosystems, as a result of environmental contamination have only been studied for a very small number of substances, the most popular being pesticides (Brühl and Zaller, 2019; Sánchez-Bayo et al., 2002). The mixtures of various substances can usually be found in the environment, which poses a particular challenge in the analysis of environmental effects. Mixtures of substances with opposing modes of action are particularly problematic, as they make it extremely difficult to determine the threshold values for the individual components (Ogungbemi et al., 2021).

Therefore, it is of utmost importance to first obtain an overview of the neuroactive substances present and bioavailable in the environmental samples (David et al., 2018). Subsequently, possible molecular target sites can be compared and transferred from selected aquatic non-target organisms onto humans. Such analyses are of great value and are based on the mostly intact evolutionary development of neuroactive pathways across species (Chowdhary et al., 1996; Zhu et al., 2003). Concurrently, more knowledge of higher-level neural structures, which play a decisive role in the processing of external stimuli and the subsequent physiological reactions, can be obtained (Maximino et al., 2010; Maximino et al., 2016). Based on the aforementioned information, it is beneficial to identify behavioural changes in individual organisms exposed to neuroactive test substances at various developmental stages. Consequently, the molecular foundations of the observed effects need to be elucidated to predict the mechanisms and adverse effect thresholds neuroactive substances have on endangered aquatic and terrestrial ecosystems. Finally, these information can be combined and used as the first building blocks for an improvement of a future risk assessment.

## 1.2 Micropollutants – characteristics and measurable effects

The number of micropollutants in the environment has increased considerably in recent years because of social changes and medical developments (Borrull et al., 2021; Rehrl et al., 2020; Sousa et al., 2020). Simultaneously, detection methods of micropollutants have been continuously improved (Danopoulos et al., 2020; Schwarzenbach et al., 2006; Zhang et al., 2018). A major problem in the analysis of complex sample mixtures with lack detailed composition information on the respective pollutant, makes the exact analytical determination and the measurement of effects at the organism level extremely difficult (Ogungbemi et al., 2021). According to information from the Federal Office for the Environment in Germany (UBA), micropollutants are residues of various substance classes. This encompasses pharmaceuticals, pesticides, biocides, and microplastic (Hillenbrand et al., 2015). These pollutants enter the aquatic environment via various pathways, for example during the production process of various materials, as well as through incomplete purification of wastewater (Kosjek et al., 2007). The lack of information on compositions and technical data on the actual management of wastewater treatment plants represent crucial informational gaps, which prevent a thorough assessment of these substances in the aquatic environment (Schwarzenbach et al., 2006). Nevertheless, despite the high level of inhomogeneity, different biological test methods exist to investigate the effects of these substances on non-target aquatic organisms, with a special focus on the respective problems. In principle, a broad repertoire of meanwhile more than 500 different standard tests allows us to consider different biological levels and assess effects on the individual, population, and ecosystem-level for chemicals of different groups (OECD, 1994). The test systems are divided into five subgroups to order them in a clear overview. A distinction is made between the analysis of physicochemical properties, the study of effects on biological systems, the fate and accumulation of chemicals in the environment, effect analysis on human health, and other test methods (OECD, 1994). The existing test methods are adapted to current developments and are continuously improved through regular review and control. Overall, developments in recent years have shown that for a complete risk assessment, information on the effect causes must be analysed in a standardised manner. This is based on the continuous development of tests at the protein- and gene level (De Oliveira et al., 2020b; Friedrichs et al., 2019). Nonetheless, it is apparent that despite the significant improvement in the quality of many ecosystems through a better understanding of the molecular causes for environmental pollution, a previously masked problem is becoming more prominent, namely exposure to micropollutants. This encloses a collective term for different substance classes that differ greatly in their modes of action and pose a danger to different organisms (Sehonova et al., 2019).

The pollution of aquatic freshwater systems poses a great danger for many humans, as this ever-scarcer commodity can only be cleaned of micropollutants inadequately up to now (Falås et al., 2016). Regardless, the detection of micropollutants and the elimination thereof makes up only a part of the greater issue. This is partially due to the dependency of large-scale application on cost-political aspects, which ensure that the cleaning process is as effective and inexpensive as possible (Schwarzenbach et al., 2006).

Two groups of substances that have gained considerable importance in recent years, especially in social-environmental policy issues, are microplastic particles and human pharmaceuticals (Baresel et al., 2019; Gautam and Anbumani, 2020). Various (inter)national funding programs have been established, which focus primarily on the analysis of microplastics in the environment (de Ruijter et al., 2020). The analyses range from defining microplastics particles to long-term removal approaches in both aquatic and terrestrial ecosystems. So far, the results show that the combination of microplastic particles with other substance classes such as pharmaceuticals pose an increased threat to the environment and ultimately humans. By facilitating the transport of these substances past organismic barriers, effects can be triggered or enhanced that would not have been possible without this "Trojan Horse"-effect (Chen et al., 2017; Syberg et al., 2015).

For this reason, pharmaceuticals should be considered as a second vital branch of micropollutant research. The analysis of human pharmaceuticals is extremely challenging because of the large number of substances found in the environment (Ford and Fong, 2016; Sehonova et al., 2018). An appropriate approach encloses a reduction, or rather simplification of the possible physiological effects and molecular origins, which are induced by the pharmaceutical products (Ogungbemi et al., 2021). Hence, this thesis focusses specifically on the effects and evaluation of neuroactive psychotropic drugs.

## 1.3 The occurrence and problems caused by pharmaceuticals in wastewaters

Steadily increasing volumes of micropollutants, such as pharmaceuticals, can be found in wastewaters and pose a potential risk for humans and aquatic non-target organisms of different species (Koopaei and Abdollahi, 2017). This is a result of patients being treated with medicinal substances, which have increasingly been developed and prescribed to treat illnesses in the last decades (Pacher et al., 2001). For this reason, the effects of micropollutants have become an important discussion topic and scientific analyses, focussing on the systematic determination of the actual environmental concentrations of

various classes of psychotropic drugs. Such evaluations are often conducted in parallel to the routine purification regimes of sewage treatment plants (Meza et al., 2020).

Various methods are available for the detection of these substances, whereby the measurement via solid-phase extraction, coupled with an LC-MS / MS or tandem mass spectrometry method have proven to be efficient tools (Beccaria and Cabooter, 2020). However, most methods require an internal standard for each predicted substance. In some instances, this can be challenging to perform with sufficient accuracy. Many metabolites, for example, have neuroactive properties themselves, which interfere with the quantification of the exact concentration. The effect strength often corresponds to the activity of the parent substance (Kosjek and Heath, 2010). This is a consequence of the metabolization steps primarily in the liver before they show psychological modifying properties. (Gomez et al., 2010). In this context, the clearance rates are usually higher in the liver than in the kidneys (Kusuhara and Sugiyama, 2009). While renal clearance in humans averages less than 12 %, the hepatic clearance rate is more than 20 % (Lajeunesse et al., 2008). The clearance rates depend strongly on the species and on the substance class and can still be below 5 %, especially in early developmental stages (Kantae et al., 2016). The resolution limits of these methods vary depending on the testing laboratory but in current measuring procedures, they range between 10 ng/L and 50 ng/L (Mousel et al., 2020). This restriction is particularly important when discussing possible risk quotients since many neuroactive substances detected in the environment are usually almost equivalent to these detection limits (Pivetta et al., 2020). The actual measured environmental concentrations of selected psychotropic drugs does not depend only on the sampling location but also on several other factors such as seasonal fluctuations, system composition, and chemical properties of the substances (Thiebault et al., 2017; Zuccato et al., 2008). In a recent study by Pivetta and colleagues from 2020, it was shown that psychotropic drugs of different classes of active substances enter the sewage treatment plants at concentrations ranging from 90 - 450 ng/L and exit the treatment plants at concentrations between 60 - 450 ng/L (Oneby et al., 2010; Pivetta et al., 2020). The authors point out that the purification rates vary according to the type of treatment plant as well as the chemical composition of the effluents (Pivetta et al., 2020). Post treatment, the wastewater is discharged into larger aquatic systems, resulting in a strong dilution of the micropollutants up to 10 times that of the original concentration (Keller et al., 2014; Link et al., 2017). Particularly problematic here are the constantly increasing ‘knockdown’ values of wastewater treatment plants of different dimensions, which lead the unfiltered wastewater past the treatment plant into the aquatic systems. In addition to the constantly increasing pressure on the existing infrastructure due to new residential areas, climate change is an important factor for the overloading of many systems (Schlüsener et al., 2015). In this regard, heavy rainstorms lead to periodic fluctuations of the large wastewater volumes, pushing many – smaller waste treatment plants – past their capacity limits (Reznik et al., 2020; Vo et al., 2014;

Zouboulis and Tolkou, 2015). Therefore, the quantities of pharmaceuticals recovered in the environment fluctuate continuously. Even the annual figure dependent on various factors such as geographical area of an aquatic system or time of year. Nevertheless, existing data compilations for various periods allow a determination of active substance classes. These have increased in recent years, mostly in terms of administration quantity and recovery rate in the environment (Ebert et al., 2014). One of these compilations - published by the Federal Environment Agency in 2020 - shows that neuroactive substances were of increasing importance and considered micropollutants in the environment, even over a decade ago (Aubrecht et al., 2021). Additionally, other neuroactive substances that do not belong to the classic psychotropic drug family have increased in pharmacological use (Bergmann et al., 2011).

Previous studies have focussed on the efficiency of wastewater treatment plants with regard to neuroactive drugs, development of alternative treatment methods, as well as additional purification methods (Comber et al., 2019). Based on the findings it can be concluded that investigations need to be repeated and in turn modifications made (Mousel et al., 2020).

Compared to classical wastewater treatment, these processes have the advantage that they can reduce the micropollutant load on a more sensitive scale. At the same time, however, it has not yet been conclusively investigated to what extent the metabolites formed from the drugs pose an additional pollution risk to the environment (Leclercq et al., 2009).

## 1.4 Classes of neuroactive pharmaceuticals

Antidepressants can be divided into different drug classes based on their molecular modes of action. The largest groups are made up of the following classes:

- selective serotonin-noradrenaline reuptake inhibitors (SSNRI) (e.g. venlafaxine)
- atypical neuroleptics (e.g. clozapine)
- selective noradrenaline reuptake inhibitors (NERI) (e.g. reboxetine)
- selective reuptake inhibitors of dopamine and noradrenaline (NDRI) (e.g. bupropion)
- serotonin reuptake inhibitor (SSRI) (e.g. sertraline)

Since the 1960s, the production of antidepressants and the quantitative measurement thereof have been rising steadily. Yet, no data sets have been published on the actual number of pharmaceuticals consumed in Europe (Kümmerer, 2009). The side effects of the first neuroactive substances are manifold and must be taken into consideration as they often bring up undesired effects in treated

patients. These have the capacity to effect various biological human systems such as the cardiovascular system and gastrointestinal tract. In addition to these side effects in humans, pharmaceuticals have several negative effects on the environment, which have become more popular to the public in recent years (Sagy et al., 2014). With this problem in mind, the development of new psychotropic drugs, which harbour fewer or no side effects, have become a topic of focus (Chaki, 2017). Hereby, the active ingredients in each tablet are reduced. For most drugs, the basic molecular target sites in humans and the resulting physiological reactions are known. However, the situation is different for non-target organisms such as aquatic vertebrates (Lopes et al., 2020; Sehonova et al., 2018). These organisms encounter the neuroactive substances because of incorrect disposal or incomplete wastewater treatment and are usually negatively affected in their physiology (Fuertes et al., 2020; Shore et al., 2014). In the following section, the molecular modes of action of the different pharmaceutical classes in humans and zebrafish will be characterized.

Selective serotonin-noradrenaline reuptake inhibitors are a group of antidepressants developed in the 1990s and act on the serotonin and noradrenaline transporters (Baldwin, 2006). With regard to the central nervous system (CNS), these bind to the transporters and inhibit the reuptake of neurotransmitters from the synaptic cleft into the presynaptic cell (Sangkuhl et al., 2009). Thus, the neurotransmitter can bind increasingly to the receptor of the presynapse and trigger an action potential. Due to the specificity of the antidepressants in this group (which include venlafaxine and duloxetine), they have significantly fewer side effects than more dated drug classes such as acyclic antidepressants (Baldwin, 2006). A possible reason for this is the low influence of other neurotransmitter systems such as the dopamine, histamine, and cholinergic system (Kostev et al., 2019).

Atypical neuroleptics are a non-uniform group of drugs with all similar effects but different molecular causes of action. Originally developed in the early 1950s (with clozapine as the first drug in the group), this group is initially characterized by a very broad spectrum of active ingredients (Toren et al., 1998). Associated with this, however, are various side effects such as extrapyramidal motor disorders, which occurred at a very early stage of treatment. The drugs have a high affinity to dopamine receptors (especially D2), serotonin (5-HT2A), and the histamine system. Due to a lack of alternatives, these side effects were accepted for an extensive period. Since the beginning of the 1990s, however, rapid advances in development, especially because of the application of molecular methods have led to the development of much more selective drugs that act specifically on a particular neurotransmitter system (Baldwin, 2006).

In addition to SSRIs, these would include selective norepinephrine reuptake inhibitors (NERI). These are selectively acting psychotropic drugs, which primarily alter the function of norepinephrine

transporters in the CNS and prevent the reabsorption of the neurotransmitter from the presynapse (Baldwin, 2006; Hajós et al., 2004; Versiani et al., 2002). Like SSRIs, there is also an increased concentration of neurotransmitters in the synaptic cleft, which inevitably leads to an increased frequency of signal formation at the presynapse (Hajós et al., 2004). Examples from the NERI group include reboxetine and viloxazine.

Closely associated with the norepinephrine reuptake inhibitors, bupropion from the group of amphetamines, is considered a selective reuptake inhibitor of dopamine and norepinephrine (SNDRI). This group encompasses activity-enhancing drugs that are mainly used to treat patients with depression (Stahl, 2013; Stahl et al., 2004). Their molecular effect is based in particular on the selective blocking of the transporters for the reuptake of norepinephrine and dopamine, whereby these two messengers remain longer in the synaptic cleft and lead to a longer-lasting reaction (Stahl, 2013). The recovery rate of around 100 ng/L for bupropion in the environment is high compared to the other antidepressants (Schultz and Furlong, 2008). Potential reasons are a higher stability towards degradation processes, a higher dosage and administration quantity, as well as a higher use in the treatment of off-labeled dysfunctions (OECD, 1994).

The classes of selective reuptake inhibitors described above act solely on a comparatively small circle of targets and tricyclic neuroleptics, which have a large spectrum of action with many associated side effects. Furthermore, there is the group of general reuptake inhibitors, which additionally code for a specific type of neurotransmitter, but whose presence covers a much larger area in the CNS (Hiemke and Härtter, 2000). Nevertheless, their selectivity differs significantly from tricyclic antidepressants, as they only inhibit the reuptake of the neurotransmitter serotonin and do not act on different systems (Hiemke and Härtter, 2000). Examples for each group will be given in the following.

Bupropion is one of the most frequently prescribed neuroactive substances in the world (Fava et al., 2005). As an amphetamine, it belongs to the class of selective reuptake inhibitors of dopamine and norepinephrine (SNDRI). Nonetheless, its dopaminergic effect has been questioned in recent studies, and a predominant blockade of serotonin receptors is assumed to be the cause of its action (Foley et al., 2006). In zebrafish embryos, the morphological and physiological effects, as well as the effects at protein level have been investigated and show a response to the psychotropic drug at different concentrations in the µg/L range (Franco et al., 2019). Lethal effects occur at a concentration of 44800 µg/L, which is well below the average active ingredient concentration of 900 mg per tablet. Morphological effects occur at a concentration of 7300 µg/L and physiological behavioural effects at a concentration of 8.8 µg/L. At the same time, the Acetylcholine Esterase (AChE) assay can be used to determine effects at the protein level at a concentration of 158 µg/L (Franco et al., 2019). Besides,

bupropion is metabolized seven days post fertilization by the class of cytochrome P450 1A (*cyp1A*) enzymes (Alderton et al., 2010).

Mirtazapine belongs to the new generation of tricyclic antidepressants and, as a member of the group of noradrenergic and specifically serotonergic substances (NaSSA), has clear advantages over less selective psychotropic drugs such as clozapine in terms of greatly reduced side effects (Masand and Gupta, 2002). Previous environmental studies on mirtazapine have mainly focused on the recovery rate of the substance at different sampling points in the environment and less on the ecotoxicological effects on aquatic ecosystems (Golovko et al., 2014; Golovko et al., 2020). Toxicological studies have been carried out mainly with rodents and have provided detailed insights into the foreseeable consequences for humans (de Oliveira et al., 2020a). Moreover, the further development of the drug for sustainable environmental protection has been assessed (Barbosa-Méndez et al., 2020; Dekeyne and Millan, 2009; Nowakowska et al., 1999). Behavioural studies using the pure substance mirtazapine have been conducted to elucidate the physiologically observable behavioural changes in zebrafish, as well as the elucidation of the underlying molecular modes of action and protein changes. These are analysed in the following sections.

In order to interpret the results of the present study at a molecular level correctly, additional medical data is required to assess the observed effects of the antidepressants on a molecular level. The results will then be evaluated and the homologues structures between humans and zebrafish presented in the discussion. The limit of quantification (LOQ) for this drug is currently 0.002 µg/L, which is slightly below other studied antidepressants (Golovko et al., 2020). For mirtazapine, there is a comparatively large database on the occurrence at the inlet and outlet of a wastewater treatment plants.

The minimum value measured was 0.023 µg/L, while the maximum value determined was 0.17 µg/L. The elimination rate within the wastewater treatment plant, excluding the fourth treatment stage, was 32 % of the originally measured concentration at the entry to the treatment plant (Golovko et al., 2020).

Clozapine, initially the only member of the group of atypical neuroleptics, has been used to treat psychosis since the 1970s (Hippius, 1989). Previous studies have described altered physiological effects of zebrafish after exposure to the drug. The effects have been characterised as ‘sedative’ and influence the swimming behaviour of the test organisms as well as the total distance moved over a certain period of time (Boehmler et al., 2007). Functional changes in various neurotransmitter systems can be detected at gene regulatory level (Viana et al., 2020).

## 1.5 Further bioassays for testing neurotoxicity at various biological levels

Compared to other substance classes, neuroactive substances show biological effects on a molecular and physiological level in aquatic non-target organisms. The effects can even be observed at concentrations in the ng/L range (Bossus et al., 2014; De Castro-Català et al., 2017). Here, physiological effects occur at much lower concentrations than observable morphological changes. Changes in natural behaviour are closely linked to the individual fitness of the organism. The more individual organisms are exposed towards external stress factors, the higher the probability of a negative influence through contact with neuroactive pharmaceuticals are expected (Smith and Blumstein, 2008). For the quantification of acute and chronic effects, different OECD and ISO validated standard test methods have been developed for the aquatic triad, which is made up of algae, *Daphnia*, and fish. However, these do not include standardised methods for behavioural studies (Cunha et al., 2019).

In the ecotoxicological assessment of substances of different molecular groups, several model organisms with high sensitivity have proved to be particularly suitable for analysis (Faimali et al., 2017). A variety of effects in aquatic systems can be studied using the zebrafish *Danio rerio* and the arthropod *Daphnia magna*. Zebrafish embryos have proven very advantageous in exposure studies. One of the reasons being that it is not considered animal testing up to 120 h post fertilization (Strähle et al., 2012). The uncomplicated husbandry of the fish provides a high number of fertilised eggs, which allow a quantitative analysis of morphological, physiological, endocrine, and neurotoxicological effects. Additionally, the effects of psychotropic drugs in aquatic systems, and in turn the test organisms, have corresponded to the molecular reactions observed in humans (d'Amora and Giordani, 2018). This has proven a useful quantifiable endpoint at the physiological level. Differently, studies with *Daphnia magna* can detect various population-relevant endpoints that also affect organisms at higher ecological levels. Overall, it can already be seen from the analysis of a few organisms of different trophic levels that the interaction within a species and between organisms of different species is altered by neuroactive substances. It is difficult to predict which species in this context has a higher adverse effect due to exposure to the neuroactive substance or the substance mix (Ogungbemi et al., 2021). In an ecological context an increase in the feeding rate of secondary consumers through the influence of neuroactive psychotropic drugs lead to a reduction of the total amount of primary consumers (Brodin et al., 2013). These studies represent only a part of the complex ecological interrelationships and do not consider any compensation and reinforcement effects resulting from shifts in the equilibrium.

This is particularly problematic when investigating psychotropic drugs as these have been developed specifically to alter a particular type of receptor and influence the physiological reactions, especially at

low concentrations. This poses a risk to non-target aquatic organisms, which have thus far been addressed insufficiently in regulatory assessments (Cunha et al., 2019). Nonetheless, further methods, which do not pertain to the standardised tests, have been developed to quantify these effects. The swimming distance of the test organisms over a given period of time or the swimming speed have been utilized to assess the behavioural changes after drug exposure, for example (Audira et al., 2018). Various recording devices and software can be obtained commercially or be developed individualistically for studying the movement patterns. Based on applications from the aquatic Triassic, analyses with the zebrafish *Danio rerio* and the invertebrate *Daphnia magna* are particularly suitable because of their exposure sensitivity. Validated test systems exist for both organisms (OECD, 2012; OECD, 2013). Nevertheless, current neurotoxic analyses focus primarily on test systems that represent exposure on a physiological level rather than the methods stated in the preceding guidelines. In recent studies, the cross-species analysis of these effects is acknowledged as the "read-across" hypothesis and provides important insights, especially for highly conserved receptors, that can be used in medical applications in humans (Huggett et al., 2003; Rand-Weaver et al., 2013). In the following sections the physiological reactions and molecular responses for different non-target organisms will be presented in more detail.

### 1.5.1 Cell level analysis and advanced gene expression analysis

The study of physiological changes in various organisms and the interpretation of the underlying modes of action are important steps in the assessment of the risk. Nevertheless, the complexity of the neuronal links, which ultimately lead to a measurable behavioural response, poses a challenge to the elucidation of the mechanistic background (Maximino et al., 2013a; Maximino et al., 2013b; Theodoridi et al., 2017). They can be significantly simplified by including further organisms and experimental methods. According to Gunnarsson and colleagues (2008) around 86 % of the zebrafish genome and 61 % of the *Daphnia* genome have proven identical to that of humans (Gunnarsson et al., 2008). A greater likeness has even been established between the genes of mice and humans. Hence, medical substances are tested on rodents prior to the final step of the three-step evaluation process, namely human testing. The use of these organisms is correspondingly suitable for the retrospective examination of psychotropic drugs already present in the environment and the elucidation of possible changes at the cellular level (Marancik et al., 2020). This quantification can be carried out either at the gene-regulatory level or through specific histological slices. Due to strict animal welfare regulations, such analyses can only take place under rigid conditions. For this reason, cell cultures retrieved from

the brains of mice were used exclusively in the present study to evaluate gene expression. Moreover, histological sections of developing zebrafish embryos up to 120 h after fertilization were prepared.

The determination of both structural and functional neurological networks in developing zebrafish embryos is very challenging. Standardised methods for the assessment have been developed, wherein fixation, staining, and microscopy are defined (Copper et al., 2018). However, these steps are commonly documented for endocrine systems such as the ovary or relatively large organs like the pancreas (An and Martin, 2003; Gurr, 1958). Through these methods have been applied to neuroactive areas of the brain in recent studies, the quantitative evaluation is still, to a large extent, in the early stages of actual standardization in environmental research (Bownik and Wlodkowic, 2021). Therefore, the present study relied once more on medical expertise. The histological examination of tissue sections has been used in various disciplines of medical research for many years and provides essential information for many modern imaging methods (Ralph et al., 2007).

Standardised histological protocols, which are commonly carried out in medical research, have been applied to zebrafish. These are adjusted to accommodate the small size of the organism and the various organs. Certain systems can be prioritized, such as the specific neuroactive brain regions focussing mainly on structures in the CNS, which are homologous in humans and zebrafish (Sabaliauskas et al., 2006). Due to the similarities in the neurotransmitters, and thus the neurotransmitter systems, a prioritization to central and superordinate systems is useful. Especially when considering the cost-intensive staining, which uses both primary and secondary antibodies (Shams et al., 2018).

The recorded imaging data consequently close an important interface between the physiological reaction, i.e., changes in swimming behaviour, and the molecular elucidation of the effects at the gene level (see also chapter 2). As already mentioned in the description of the protein assays, the currently available detection methods at protein level are extremely cost-intensive or do not provide reliable information on possible changes at this biological level (Link et al., 2006). The visualization makes it possible to localize the changes in the CNS and a quantitative interpretation of possible effects can, at least to some extent, be conducted.

Any evaluation of recorded images is purely subjective because of the lack of standard methods, which can already be seen, for example, in the orientation of the zebrafish embryos during incision. This error can be minimized by ensuring that all evaluations are carried out and documented consistently by the same person. The added value for the overall interpretation of the data lies particularly in the highly cross-cutting nature of the test methodology, as it detects morphological changes and relates the

causes of these changes in reduced or increased concentrations of the selected neurotransmitter (Schiebler et al., 2013).

### 1.5.2 Behavioural and gene expression studies with *Daphnia magna*

A predominant number of neurotoxicological studies of aquatic non-target organisms have been performed on standard model organisms of ecotoxicology such as zebrafish embryos (Ton et al., 2006). The results led to the development of various test systems, which provided different effect data.

A group of primary consumers of the first order, with central importance for the stability of the ecosystem, is the group of Cladocera, which also includes *Daphnia magna* (Miner et al., 2012). Due to their food intake as filter feeders, they are in direct contact with the contaminants and bioaccumulation over several trophic levels. While *Daphnia* as model organisms are already used for acute and chronic bioassays, their application in neurotoxicological studies is not yet widely spread and developed (Wollenberger et al., 2000). Particularly because of their position in the ecosystem daphnids occupy an important ecological niche in natural systems. They control algal density and serve as an important food source for consumers of higher orders (Hooper et al., 2008). Population density shifts due to physiological changes have not yet been studied in sufficient detail and it is not possible to provide detailed regulatory conclusions.

Therefore, various endpoint studies with daphnids are important for the assessment of neurotoxic effects. Mortality and the total amount of offspring's are indirect measurable parameters of neurotoxicity for a wide range of different test concentrations (OECD, 2012). The effect strengths of neuroactive substances depend on the surrounding environmental factors, such as temperature or pH-value (Nys et al., 2017). Previous studies with other drug classes have shown a general increase in the stress level of the individual organisms (Heckmann et al., 2008a). It can be deduced that comparable effects on the number of offspring occur above a substance-specific threshold concentration. A cross-species comparison with the morphological effects in zebrafish embryos showed at a concentration as low ng/L a statistically significant decrease in body length during development (Yang et al., 2014). Behavioural effects could be observed in the majority of studies even at lower exposure concentrations (Legradi et al., 2018). Again, various methods that have been developed and validated using zebrafish embryos can also be used to study behaviour in daphnids.

Compared to previous studies on zebrafish embryos, specific patterns in the swimming behaviour of daphnids can be analysed and considered for a possible effect assessment (Bownik, 2020). Thus, the distance covered, and the swimming speed can be analysed quantitatively. Compared to zebrafish,

however, the actual sequence of movements is not only a horizontal movement, but rather a vertical "hopping" movement on different levels (Bownik, 2020; Bownik and Pawlik-Skowrońska, 2019). An influence of neuroactive substances on movement sequences have already been shown for different pharmaceutical classes. Pery and colleagues (2008) demonstrated that fluoxetine, a member of the group of selective serotonin reuptake inhibitors, influences the life cycle of *Daphnia magna* in the ng/L range (Pery et al., 2008).

These physiological reactions are of great importance for the survival of the individual as well as for the stability of the entire population. Although complex movement sequences do not occur for example during mating, a changed behaviour leads to an increased feeding rate by higher-order predators. This in turn endangers the stability of the entire community over a medium-term period (Liu et al., 2019).

The causes of these physiological reactions can be found at the molecular level. A predominant part of the superior neurotransmitter systems already presented in this thesis is also found in daphnids, at least in simplified form. They play a central key role at the interface between absorbed stimuli and the corresponding physiological reaction (Vesela et al., 2008). Changes caused by neuroactive psychotropic drugs, which lead to an altered concentration of neurotransmitters in the synaptic cleft or to an altered number of receptors can be harmful for daphnids (Gómez-Canela et al., 2019).

Adult daphnids with an average size of 2 - 3 mm are much smaller than zebrafish embryos with a body length of 4 mm after 120 h development time. This poses special challenges for the recording of any kind of locomotion data. Also, the special vertical kind of locomotion requires some technical skills which can be used as qualitative characteristics for the evaluation (Bownik and Pawlik-Skowrońska, 2019). As there are no standardised protocols for these test procedures, like those used in zebrafish behaviour research, the development of test methods is based on existing literature recommendations and experience from testing other groups of organisms (Bitton et al., 1995).

The analysis of changes at the gene level by quantitative real-time PCR is an organism independent procedure. The extraction of the RNA and the subsequent transcription into more stable cDNA are the basic steps for an analysis. Therefore, it is possible to determine the underlying molecular causes of physiological behavioural changes in daphnids. The primer sequences for many general oxidative stress genes, including glutathione S-transferase, different catalases, and various genes of heat shock proteins are already known and validated in various studies. This allows the effect analysis of different substances and provides detailed insights into the molecular causes.

In terms of the analysis of neuroactive substances, only very few transmitter systems have been studied already. Despite the sequenced genome, there is usually a lack of knowledge about the actual

function of the respective sections (Dominguez et al., 2015; Schwarzenberger et al., 2009; Soetaert et al., 2007). This makes the primer selection complex and difficult, even for higher-level systems, and usually only provide results with a low efficiency that can no longer detect minor changes. Therefore, it makes sense to use information from other species that are closely related to the daphnids (Sturm et al., 2009). *Drosophila melanogaster* is one organism that belongs to this group of related species that could be used for a comparative gene expression analysis. This model organism provides important insights especially in the clarification of neuronal structures in basic zoological research. A comparison of the genes and especially the protein sequences of both organisms show great similarities.

### 1.5.3 Behavioural and gene expression studies with *Danio rerio* embryos

Pisces and birds are the top predators in most aquatic systems (Steinmetz et al., 2003). Pollutants that accumulate along the food chain are mostly found in the tissues of fish and are finally absorbed by humans (Fu et al., 2020; Zhao et al., 2015). This effect has already been demonstrated for various substances from the subgroup of selective serotonin reuptake inhibitors (Daughton and Brooks, 2011). But even if there is no accumulation of pollutants directly, changes in species density could lead to dramatic changes in the ecosystem. These changes can be caused by physiological changes of the individual organisms.

Various single-substance studies with neuroactive pharmaceuticals have already shown, that fish are influenced on physiological, morphological and teratogenic level (Boehmler et al., 2007). With a special focus on neuroactive pharmaceuticals changes can occur on different biological level. A powerful indicator of changes in development is the total body length. Changes of this factor are indicators for major molecular disfunctions. Studies by Yang and colleagues (2014) have shown that a reduction of the body length found in zebrafish embryos after an exposure to amitriptyline already occurs at concentrations in the low µg/L range (Yang et al., 2014). These concentration ranges correspond to the effect thresholds for physiological changes which occur at much lower concentrations than morphological changes. More in-depth studies on learning processes in fish have shown that changes in cognitive performance occur in cuttle fish for an exposure with fluoxetine at a comparable concentration range as in Di Poi and colleagues (Di Poi et al., 2014). Altogether, these cognitive traits are important preconditions for a successful mating (Parkos III et al., 2011).

The swimming behaviour of fish play a decisive role in the interaction of the individuals for example in the context of mating behaviour, as well as between different species during fight and escape reactions

(Dreosti et al., 2015). Therefore, highly specialized behavioural patterns have developed, which are of great importance for the success of the intended action (Paull et al., 2010; Pradhan and Olsson, 2015).

In aquatic organisms such as vertebrates, behavioural effects are undesirable and are caused by the interaction of the neuronal receptors with the target substance. As a result, there are changes in swimming behaviour, which can manifest itself in different endpoints. In addition to a classical increase or decrease of the covered swimming distance, the frequency of changes of direction, swimming speed, residence times in the peripheral or central area, or the sensitivity of the organisms to a light source can be analysed (Bilotta and Saszik, 2001; Drapeau et al., 2002). The quantification of the behavioural effects has become much more popular in recent years, as new computer-based evaluation protocols allow the detailed evaluation of complex databases (Colwill and Creton, 2011). This testing approach is known as "intelligent testing" because it incorporates as much information as possible for planning (Gunnarsson et al., 2008).

The neuronal processing of the visual stimuli takes place from the brain stem into the spinal cord via approx. 200 neurons, while the subsequent muscle reaction is switched from the brain to the various executing organs via a total of approx. 150 neurons, whose cell bodies can be found in both the midbrain and the hindbrain (Knafo and Wyart, 2018). Mauthner cells (M-cells) and their segmental homologs MiD2 and MD3 play a special role in this interconnection between information uptake and final execution by the muscles (Takahashi et al., 2002). However, it should already be mentioned that not all the movements described below are controlled by these cells and that there is control by other cells, especially during food intake.

The already described set of different basic movements consists of rotations of different complexity as well as movements at different speeds. Thus, the C-start, the J-bent, the O-bent can be seen in the rotations, while the forward movements are both slow scoot and burst swims (Roberts et al., 2016). The movements differ both in their usefulness for the organism and in their neuronal origin.

Changes in movement patterns can be triggered at the neural level by shifts in neurotransmitter balance. Specifically designed psychotropic drugs influence the release of neurotransmitters into the synapse, the concentration of neurotransmitters in the synaptic cleft, or the reabsorption of the signal substance into the presynapse (Tarleton et al., 2016; Vazzana et al., 2015). An additional intensification of the effects can be caused by an accumulation of substances in the organism even at low drug concentrations. The concentrations at which these effects occur are in the µg/L range and thus well below the effect thresholds for morphological effects that are effective in the mg/L range. Fish have been identified as particularly sensitive organisms to psychotropic drugs and possess a particularly high number of homologous structures to humans at the molecular level where psychotropic drugs can

cause reactions (Furuhagen et al., 2014). Compared to other aquatic organisms, fish are not necessarily the most sensitive organisms because although they are the highest level predators at the end of a bioaccumulation process, lower level organisms such as daphnids have much higher exposure times to psychotropic drugs due to their filtering lifestyles (Rand-Weaver et al., 2013).

For the analysis of changes at the molecular level, standardised methods are independently available to the organism and have already been used in various studies on single substance testing of psychotropic drugs. Nevertheless, it is necessary to prioritize higher-level control systems that it is possible to couple the evaluation of physiological behaviour data with the mechanistic modes of action. Again, the highly selective mode of action of the psychopharmaceutical groups is advantageous in this context that a specific analysis of individual neurotransmitter systems is possible.

## 1.6 Regulatory approval and evaluation of human pharmaceuticals

Human medicinal products pass through different testing stages during and at the end of their developmental phases, where their effects on human health as well as on various environmental organisms are assessed (Bundesministerium der Justiz und für Verbraucherschutz). The requirements for testing the effect in humans are staggered into different levels and follow strict legal guidelines. This system is intended to provide the most effective protection against unknown side effects in humans, hence medicines may also only be developed by specially trained pharmacists and applied and tested by medical professionals. The effect analysis of the environmental risk assessment is carried out after the human impact assessment and is less strictly regulated compared to the previous stages. It is a compilation of various acute and chronic effect tests on different biological levels (Rand-Weaver et al., 2013). However, this test procedure has proven to be incomplete, especially when applied to neuroactive and endocrine-disrupting substances, and therefore intensive efforts have been made for some years to apply new and more specific test systems for the assessment of environmental hazards.

### 1.6.1 Regulatory framework for the handling of human pharmaceuticals

- Requirements for medicinal products

Human pharmaceuticals go through a long and multi-stage development process with various tests to exclude possible side effects for humans until they are approved (Bundesinstitut für Arzneimittel und Medizinprodukte, 2020). However, most of these toxicological tests only include effects that pose a potential risk to humans (Bundesministerium der Justiz und für Verbraucherschutz). The

ecotoxicological tests to analyse the effects on the environment are less stringent and are based on OECD standardised studies with individuals of different biological levels such as algae, *Daphnia*, and fish (Wei et al., 2006). Ultimately, however, the results of the ecotoxicological analyses do not lead to rejection by the regulatory authority if there are no alternative drugs that could be approved instead (Ebert et al., 2014). Since this is usually not the case because the drugs are already subject to the idea in their planning to fill a gap in the therapy of a certain disease, contamination of the environment in favour of humans is permitted by law. In Germany, the community codes 2001/83/EC and 2001/82/EC, together with the EU Regulation 726/2004/EC (European Parliament and the Council, 2007), provide the legal basis for the environmental assessment of medicinal products. A distinction must be made between the different approval procedures for human and veterinary medicinal products, whereby in both cases there has been a significant strengthening of environmental controls because of the EU directive. Above all, the German Medicines Act (AMG), which came into force in December 2005, regulates the requirements, manufacture, approval, registration, and supply of human pharmaceuticals in Europe with its latest amendment of 2009.

- Definition of medical products

According to the AMG, drugs are substances or preparations intended for use in or on the human body. They serve to cure, alleviate, or prevent diseases and complaints. At the same time, however, substances that restore physiological functions and that can be used in medicine to establish a diagnosis are described as drugs. The highest priority is to improve the health of the patient, so there is a total ban on questionable drugs. These are substances which, according to the state of the art in science, if used as intended, would lead to a harmful effect in humans which would not be within the scope of medical justification.

- Production and approval of drugs

To produce medicinal products, a permission by the authority is required. Persons who produce drugs must have sufficient expertise, which must be proven either by a license to practice as a pharmacist or by at least four years of completed studies in selected fields of study with the subsequent examination, including at least two years of practical work (Kügel et al., 2011). Possible changes, for example over the required length of university studies, can be found in §15 of the AMG.

After a drug has been developed, it must be approved in the Federal Republic of Germany by the appropriate higher federal authority. The approval is based on the requirements of the German

Medicines Act and contains data from a three-stage review process in which stricter requirements are imposed on the effect of the drug in each research phase (Kügel et al., 2011). The exact contents of the approval documents can be found in §22 of the AMG. The approval review by the authority can take place within a maximum period of seven months and the subsequent decision on the recognition of the approval by the producer must be made and communicated to the approval authority within three months after receipt of the decision (§27 AMG). Overall, attention must be paid to whether the approval of the drug is a new approval or a renewed approval of an already submitted drug.

After the final approval, the distribution of the drugs is carried out by approved bodies, usually pharmacies (both inside and outside hospitals). Prescription drugs are dispensed exclusively upon presentation of a doctor's prescription to the consumer and through dispensing in a pharmacy. This approach is based on the idea that even with proper use of these substances a potential risk for the consumer could not be excluded and that only medical supervision can control the correct application. Even after placing pharmaceuticals on the market, they must be monitored regularly, which is regulated by §64 and §65 of the AMG.

### 1.6.2 Environmental risk assessment (ERA)

The determination of the Environmental Risk Assessment (ERA) for human pharmaceuticals is regulated by the Guideline on the ERA of Medicinal Products for Human use, issued by the European Medicines Agency (EMA) since 2006.

The approval of drugs follows a two-stage system of phase I and phase II studies, which is not necessary for all drugs. As part of an exposure assessment, the first step is to determine the predicted environmental concentration (PEC) of the pharmaceutical. This calculation based on the maximum daily dose, a market penetration factor, the daily water consumption of a person, and a dilution factor based on the knowledge from the treatment plant. If this value is above a trigger value of 0.01 µg/L, an in-depth environmental assessment becomes necessary. Excluded from this test are natural substances such as amino acids or peptides, while hormones and special lipophilic substances must in any case undergo a second testing stage (Ebert et al., 2014). As part of the studies of this in-depth environmental review, different data on degradability, bioconcentration, toxicity to micro-organisms for the protection of the sewage treatment plant, and some tests on aquatic organisms will be carried out based on GLP and various OECD or ISO-validated guidelines. Only the results of already validated tests are considered in this assessment which means that molecular and physiological data are not included in the evaluation. If the final risk ratio is determined from the improved PEC-value and the

no-observed effect concentration value determining risk to the aquatic ecosystem, the authority can only react with additional instructions on disposal or with a regulation on the maximum package size (Ebert et al., 2014). The directive does not provide a refusal of authorization based on these data.

This approach shows that the risk assessment for neuroactive substances is complicated by most OECD standard tests. They are not suitable for the analysis of neurotoxicological effects, as they do not show any effects at exposure concentrations for behavioural alterations (de Farias et al., 2019). This is particularly evident for most neuroactive substances which show physiological effects already in the range of ng/L to µg/L, while morphological effects are usually only visible in mg/L (Franco et al., 2019; Zon and Peterson, 2005).

For most neuroactive substances, no clear no-observed effect concentration (NOEC) can be measured. The determination of these values requires a concentration with no effects on the test organism. These values cannot be established for physiological changes due to the high individual characteristics of each test organism. Morphological effects usually occur at much higher concentrations in comparison to physiological alterations (Huang et al., 2014; Lammer et al., 2009). Furthermore, quantification of physiological effects is difficult, and the definition of a threshold value depends on many factors that do not necessarily have to be effects of the exposure substance (Clements and Rohr, 2009; Jager et al., 2011).

The current procedure for determining the risk potential for the environment must be reconsidered and adapted for neuroactive substances concerning their modes of action. The determination of classical parameters such as the NOEC or lowest observed effect concentration (LOEC) is difficult or even impossible for most substances. The quantification of physiological reactions is difficult (Green et al., 2013). Single-substance studies with different drugs have shown that there are not only different reactions in juvenile and adult aquatic organisms but also differences in the respective developmental phases (Kimmel et al., 1974). Exposure in the first 72 h after fertilization, can lead to different developmental reactions.

Effects that have an impact on the composition of the aquatic system community are excluded in the environmental risk assessment. Although many interactions have already been described and investigated, there is a lack of analysis of neuroactive effects. It should be considered that no ecosystem can be considered self-contained and on its own, but that there is a lively cross-border exchange between aquatic and terrestrial systems (Smith and Blumstein, 2008). In the context of these interactions, new effects occur in the analyses of the complete system and could not be estimated as the sum of single effects. This principle has been used in various other scientific disciplines for many years and should be considered in the complex ecosystem contexts.

Therefore, the combination of chemical analysis with bioassay investigations must lead to a rethinking of the respective combination of the different results.

## 1.7 Framework, background and aims of the PhD thesis

The PhD project focus on a specific part of the current discussion of the effects of micropollutants on aquatic systems. The chosen acronym NAP (neurotoxicological analysis of psychotropic drugs) highlights the focus on clinically used substances that target especially behavioural changes which are triggered by changes of major regulatory systems.

The idea behind NAP is to evaluate current regulatory methods for the preparation of an improvement of the actual environmental risk assessment for pharmaceuticals and the use of bioassays to record physiological movement data and molecular modes of action. The chosen methods are mostly neither OECD nor ISO validated but have a high informative value concerning the effect analysis of substances that cause behavioural changes.

Thus, different methods will be used for the analysis of neuroactive drugs and the concentration ranges will be compared to classical endpoints like morphological changes. This should allow recommendations for furthers steps in the development of a future risk assessment of this substances and a final recommendation how to deal with them in the future. However, this includes on the one hand the identification and quantification of behavioural changes but on the other hand also implicit the elucidation of the underlying molecular mechanisms of action. This is in line with the constantly increasing knowledge that the effects of pharmaceuticals produced with very high specificity for a certain type of receptor and a specific behavioural response have not been sufficiently reflected in the validated test strategies for assessing the actual environmental effects. A particular challenge is posed by the definition of concentration thresholds for molecular and physiological reactions, which are lower than acute and chronic standard bioassays. For the investigation of these new test approaches, however, it is imperative to bring together a close link between ecotoxicological test data, chemical-analytical data, and medical patient knowledge. This holistic testing approach implies that considerably more information is already considered in the planning stage, which is generally referred to as "intelligent testing" in current development studies.

The retrospective application of this concept to neuroactive substances has not yet been carried out on a broad scale using realistic mixtures. With a special focus on psychotropic drugs, as well as on the further development of existing neurotoxicological bioassays, the studies within the framework of the PhD project are intended to create new approaches for future improvements in risk assessment.

This creates the prerequisites for detecting and classifying the effects that are below the threshold values of classical bioassays that are used in risk assessment for human pharmaceuticals. In addition, to the analytical composition of the samples, the information from the medical application provides important information for a cross-species consideration. A cross-species analysis approach has become one of the most important methods for the risk potential assessment.

This approach serves as an information tank for the effect analysis for related species that could not be considered for testing. Altogether, this information could be used for the development of a future regulatory framework of these substances which deliver much more specific information than the classical bioassays. At the lowest biological level, but at the highest mechanistic level, the elucidation of the molecular modes of action is a decisive step to substantiate the findings and demonstrate the molecular causes of the investigated pharmaceuticals. A particular advantage is that the molecular modes of action are known for all drugs in human and that due to various homologies at physiological and molecular level, these findings can be extrapolated to other species like fish. The test design at the modular building of psychiatry supports this approach because it allows the combination of data from different fields in one concept.

Figure 1.1 gives an overview about the different test designs on various biological level that are combined within the PhD studies. The test design is structured into different sections with increasing complexity of the sample compositions. First, a pure substance study and different samples of artificially produced mixtures will be used for the composition of a new biotest battery protocol for the detailed testing of neuroactive pharmaceuticals. Finally, this protocol will be used for the analysis of native hospital wastewater samples.

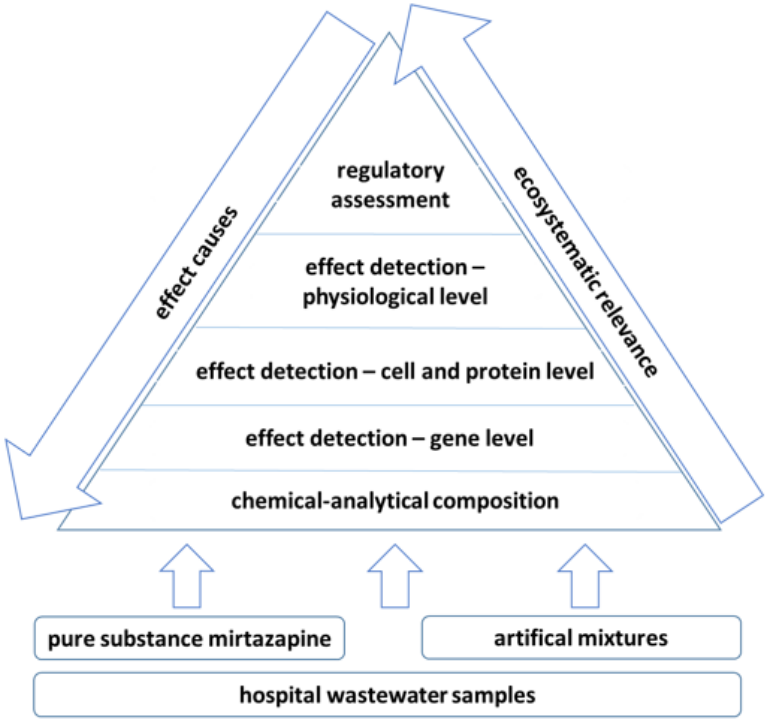



**Figure 1.1: Graphical abstract of the PhD thesis** with various effect levels and different sample types

Thus, this approach is intended to support two different approaches. One approach is the combination of different test systems for the quantification of different movement sequences as one physiological endpoint and the identification of the underlying modes of action. Another approach is the analysis of previous methods for risk management and the development of alternative ways for future assessment.

However, the aim is not to further develop and assess each method in detail in all aspects, but rather improve the application for a future assessment of environmental effects in the context of drug approval. Neuroactive substances pose an increasing risk to the quality of aquatic systems and yet important chemical and bioanalytical data are not detailed enough to show a complete picture of these effects. This does not evaluate the method accuracies but the connection or interconnection of the different test systems to a higher-level test protocol which helps to detect the effects and to combine the analysis.

This test protocol is a staged testing approach which is also used in the sub-chapter structure of the thesis. The analysis of behavioural effects of zebrafish embryos and *Daphnia* after exposure to the pure substance mirtazapine should provide insights into the optimisation of the test design for the analysis of more complex mixture samples. Parallel linkage with a section of the modes of action of higher-level neurotransmitter systems should establish cause-effect relationships that are necessary for interpreting the effects of more complex mixture samples. The examination of artificially produced

mixtures of psychotropic drugs will be used to analyse whether sedative or enhancing effects predominate at the behavioural level and which molecular effects are detectable. The final analysis of native hospital wastewater samples is a worst-case assessment of the effects on aquatic non-target organisms.

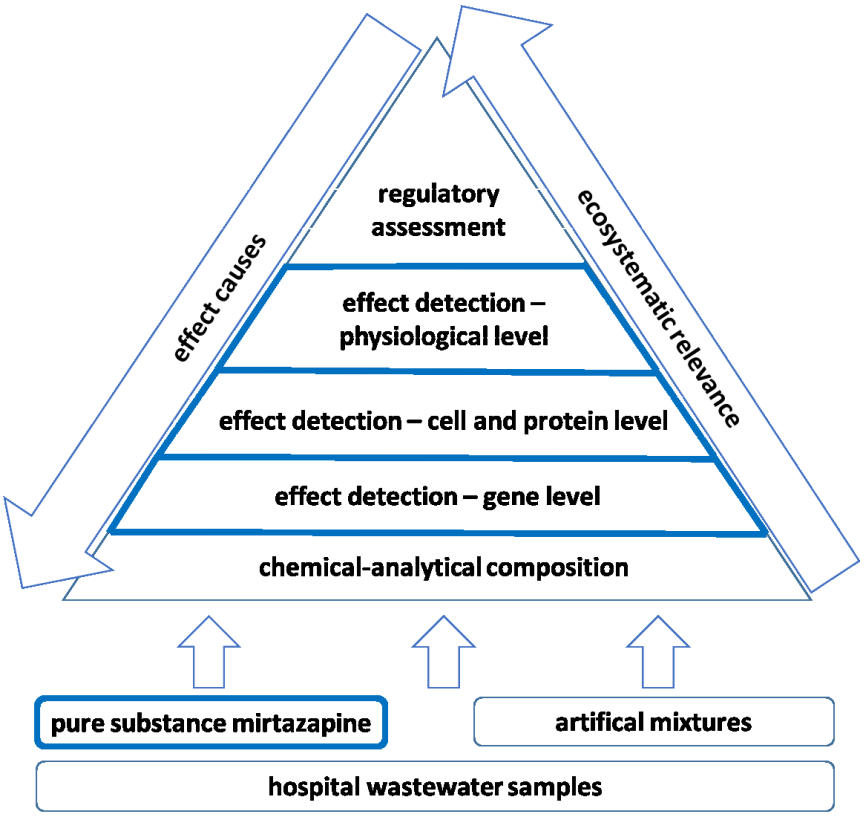
This thesis is intended to provide a partial building block for a better understanding of the molecular causes and the physiological consequences of this special group of pharmaceuticals in different aquatic non-target organisms. These include the following objectives which will be examined in detail.

- Pathway analysis at the **molecular level** and identification of changes in superordinate neurotransmitter systems
- Quantification of the neurotoxicological potential of psychotropic drugs on the **physiological responses** of selected non-target aquatic organisms
- Assessment of the **consequences** of the effects of neuroactive substances contained in complex hospital wastewater samples for the future assessment of the risk potential of psychotropic drugs in the environment.

The test design is an application-oriented approach with less focus on the improvement of the individual methods but with an improvement of the cross-test interpretation of the data. This should help to close the existing knowledge gap of the effect analysis of neuroactive substances which has not been considered comprehensively enough in regulatory terms.

# Chapter 2

## Effects of the antidepressant mirtazapine on the swimming behaviour and gene expression rate of *Danio rerio* embryos – Is the sedating effect seen in humans also evident for fish?




This chapter is based on a publication in an international journal:

Gundlach, M., Augustin, M., Smith, E.C., Kämpfer, D., Paulzen, M., Hollert, H. (2021). Effects of the antidepressant mirtazapine on the swimming behaviour and gene expression rate of *Danio rerio* embryos – Is the sedating effect seen in humans also evident for fish? Science of the Total Environment, Volume 792, 20 October 2021, 148368: https://doi.org/10.1016/j.scitotenv.2021.148368

## Abstract

In the last decade, mirtazapine has become an important antidepressant in clinical use and has also been found at many different environmental sampling sites. Several homologies between the zebrafish *Danio rerio* and humans, combined with a number of advantages for behavioural and gene expression research using zebrafish embryos, make their use for the analysis of mirtazapine appropriate. The sedative effect of mirtazapine in humans was also found for a specific concentration range in zebrafish embryos (1333.4 µg/L – 2666.9 µg/L). Specifically, 116 hpf old zebrafish embryos showed a reduced swimming distance when exposed to 1334.4 µg/L mirtazapine. Furthermore, changes at the gene regulatory level could be measured (1333.4 µg/L), in particular in the superordinate regulatory systems. For selected transporters of all regulatory systems, an up regulation of the genes by a factor of more than five times could be measured at the highest mirtazapine exposure concentration that was tested. Finally, studies on the protein levels demonstrated an increase in acetylcholinesterase activity for several exposure concentrations (83.3 µg/L – 666.7 µg/L). The physiological changes in zebrafish embryos caused by mirtazapine demonstrate the relevance of these types of studies in aquatic non-target organisms. Such neuroactive substances could pose a potential risk for aquatic organisms below the previously considered concentration threshold for morphological effects.


## 2.1 Introduction

In the field of ecotoxicology, awareness of the impact of residues of pharmaceutical drugs in wastewater and aquatic environments are increasing. Among the group of modern antidepressants, mirtazapine is the most important compound in the subgroup of the noradrenergic and specific serotonergic antidepressants (NaSSAs). According to the Medical Expenditure Panel Survey, there were more than 6.2 million prescriptions of mirtazapine in the U.S. in 2017 (M.E.P.S., 2017). As a result of the high number of mirtazapine prescriptions, inputs into the environment is high and have even increased over the last years (Li et al., 2019a; Li et al., 2019b). The actual concentrations depend on the sampling site but have been shown to be around 0.4 µg/L in the surface waters of different rivers and it is expected that concentrations of around 1.5 mg/L are possible at polluted point sources for comparable neuroactive substance classes (Kosma et al., 2019; Li, 2014; Li et al., 2019b; Pivetta et al., 2020). Psychotropic drugs such as antidepressants are of particular interest as they could have negative impacts on the nervous system of organisms such as fish (Galus et al., 2013b). Antidepressants have been detected in wastewater effluent-impacted streams in the U.S. and have been shown to

affect the brain tissue composition of *Catostomus commersoni*. Here, samples were taken from stream water as well as from the sediment (Schultz et al., 2010). Furthermore, physiological effects could also be demonstrated for other antidepressants such fluoxetine in the mosquitofish as an example of another aquatic non-target organism (Martin et al., 2019). Previous studies measuring antidepressants in wastewater have also indicated a seasonal pattern, with concentration peaks in the range of µg/L occurring in autumn and winter (Golovko et al., 2014; Huerta-Fontela et al., 2011; Jelić et al., 2012). This all highlights the importance of confirming the actual test concentrations, which can be determined by a chemical-analytical methods (König et al., 2017). This step provides important information about the possible metabolic rate in the fish and also offers details about sorptive losses to the test vessels (Shao et al., 2019b). In general, a detailed elucidation of the test concentrations provides important information for the design of ecotoxicological tests as well as for the subsequent data interpretation and risk assessment (Tousova et al., 2018).

In humans, mirtazapine is extensively metabolized via the cytochrome P450 isoenzymes in the liver. A total of 80 to 94 % of the administered dose is excreted; with more than 75 % found in the urine and the rest in the faeces (Delbressine et al., 1998; Timmer et al., 2000). A reported removal efficiency level of only 32 % in a wastewater treatment plant suggests that a large amount of this excreted mirtazapine could enter the environment (Golovko et al., 2014; Kumar et al., 2019). Mirtazapine was found in surface water, affected by effluents from a sewage treatment plant at concentrations ranging from ng/L to µg/L (Breitholtz et al., 2012; Grabicova et al., 2017). The metabolism of mirtazapine takes place in human and probably also in vertebrate liver and leads to the formation from the parent substance of the neuroactive metabolite N-desmethyl mirtazapine (Anttila and Leinonen, 2001). This metabolite can also be detected in wastewater samples, and sometimes exceeds the concentration of the original substance (Gurke et al., 2015). The metabolite itself is a neuroactive drug that is used for medical treatment (Lavasani et al., 2014).

In humans, mirtazapine blocks adrenergic alpha2-receptors, as well as serotonin receptors such as the 5-HT2A, 5-HT2C, 5-HT3 and histamine H1-receptors (Anttila and Leinonen, 2001; Sitsen and Zivkov, 1995). Accordingly, mirtazapine affects several neurotransmitter systems including the noradrenaline, serotonin, and histamine systems. Antagonism at the histamine H1-receptor is associated with sedation and weight gain (Kroeze et al., 2003; Nicholson et al., 1991), and mirtazapine stands out among the group of modern antidepressants by additionally exerting sedative properties. As one of several antidepressants, mirtazapine is also used off-label to treat insomnia thus expanding the number of possible patients that receive mirtazapine (Kamphuis et al., 2015; Lai et al., 2011).

The zebrafish *Danio rerio* has been established as an important aquatic model organism for the detection of neuroactive substances (Johann et al., 2020; Legradi et al., 2018; Nüßer et al., 2016; Parng

et al., 2007; Selderslaghs et al., 2010). In addition to the advantages of having a high number of transparent eggs and low husbandry requirements, links between physiological reactions such as changes in the swimming distance covered and the molecular causes such as gene alterations are important for the analysis of the effects of antidepressants (Legradi et al., 2015; Strähle et al., 2012). Zebrafish also possess superior (neuro-endocrine) control systems, such as the serotonergic- and dopaminergic-systems, in order to process external stimuli and develop a physiological reaction (Maximino et al., 2013a; Panula et al., 2010; Panula et al., 2014). The close homologies in anatomy and functioning of specific regions of the central nervous system (CNS) in zebrafish and humans would suggest comparable effects between both organisms (Barbazuk et al., 2000). Previous studies have shown homologies at central CNS interfaces in zebrafish and humans, and this leads to the hypothesis that mirtazapine also has a sedative effect on zebrafish behaviour (Khan et al., 2017; Panula et al., 2010). Based on current studies, the steadily increasing importance of physiological changes within environmental risk assessment can be observed for different substances (Ågerstrand et al., 2020). However, little is known about the effects of mirtazapine on different species and their behaviour in aquatic environments. A potential bioaccumulation of mirtazapine has been suggested, due to the high concentrations detected in brown trout kidney and liver (Grabicova et al., 2017). Therefore, mirtazapine is a compound that can be detected in aquatic environments and its psychoactive effects may also extend to any species that is exposed. Effects at the ecosystem level have already been demonstrated for different neuroactive substances, and the causes can often be attributed to changes in mating behaviour (Bertram et al., 2020). Therefore, a relevant research question is whether possible sedative effects of the antidepressant mirtazapine are also seen in aquatic vertebrates. The tiered approach for the effect analysis of neuroactive pharmaceuticals on non-target organisms requires the detailed identification of the underling molecular mechanisms of action. In addition to histological analyses with antibody staining, cell culture could help to quantify effects on another biological level between the physiological and molecular level.

The importance of cell culture studies for human disease analysis like neurodegenerative diseases have already been verified with glial cells from different origin (Jung and Chung, 2018; Lobsiger and Cleveland, 2007). Thus, glial cells from mice are used regularly, especially when analysing effect in relation to humans. The close homologous structures and functions between both nervous system are the main reason for the cross-species analysis (de Abreu et al., 2018). Medical studies have focused on neurodegenerative diseases with glial cells because diseases like alzheimer can easily been visualized at cellular level. In addition to morphological studies at cellular level, the quantification of the gene expression level is an often used endpoint (Van der Ven et al., 2006). In detail, the analysis of changes on gene expression level are important for the elucidation of neurodegenerative diseases on molecular level. However, since most of the protocols are universal and organism independent, they can be used

for the analysis of other substance classes like antidepressants. This new approach is an important step for the development of a future environmental risk assessment of neuroactive substances because most of the standard protocols only focus on morphological changes. Since many effects on behavioural level are already measurable at lower concentrations than morphological changes, the hazardous potential to aquatic non-target organisms is not quantified in all detail. The superordinate neurotransmitter systems that are affected by mirtazapine were already identified in previous studies. Thus, the serotonergic and dopaminergic systems are important junctions between the processing of external stimuli which lead to an internal movement coordination. Furthermore, by analyzing effects at the cellular level, the gap between behavioral change and molecular cause of action could be closed.

Therefore, the study design considers the effect of mirtazapine on *Danio rerio* embryos both at behavioural cellular and gene expression level. Physiologically measurable effects in fish (as models for vertebrates in aquatic systems), such as changes in swimming behaviour, can be carried out at both the protein- and gene-levels. In general, a complete genome analysis is time- and cost-intensive, because even for zebrafish the number of interacting genes for behaviour formation is extremely high (Rubbini et al., 2020). Therefore, a total of four different target genes were selected in this study as elements of central neurotransmitter systems such as the serotonin- and histamine-systems (Airhart et al., 2012; Maximino et al., 2013b). Both, *slc17a6a* and *slc6a4a* were selected as encoding for integral transmembrane transporters with symporter activity (Gaudet et al., 2011). These are central channel proteins that maintain chloride homeostasis in neurons and serve as transporters of the serotonin system in the CNS. These are found in various isoforms in both humans and zebrafish (Calvello et al., 2019). To examine the previously described mirtazapine induced blockade of adrenoceptors, a G-protein-coupled receptor (*adra1aa*) was selected. This also has effects on the cardiovascular system and in cell-cell interactions (Rodrigues et al., 2020). The analysis of the histamine receptor hrh4 focused on the effect of mirtazapine on the histamine system (Gaudet et al., 2011).

Based on the activity decreasing effect of mirtazapine in humans, a similar response in the swimming behaviour of zebrafish embryos in terms of swimming distance was expected. For the elucidation of the molecular causes from the network of different regulatory genes, superordinate neurotransmitter systems and their interfaces were studied. An up-regulation of genes that regulate the neurotransmitter transport in the synaptic cleft was expected. This study therefore examined the hypothesis of whether physiological and molecular level effects in humans after exposure to mirtazapine also show an increased activity in zebrafish embryos. The aim of this investigations is the creation of a link between cellular effects and the molecular modes of action. It is assumed that higher-level neurotransmitter systems are influenced in their gene expression rate by the antidepressant.

In addition, it should be demonstrated in parts whether mirtazapine alter the central interface of higher-level neuronal transmitter systems in zebrafish embryos.

## 2.2 Material and Methods

### 2.2.1 Test pharmaceutical

Mirtazapine ≥98% (HPLC) (1,2,3,4,10,14b-Hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c][2]benzazepine), C17H19N3, CAS Number: 85650-52-8) was supplied by Merck (Merck, Germany). A stock solution with 5.0 mg/mL was prepared in 100 % dimethyl sulfoxide (Merck, Germany) as this solvent has already been previously used in different bioassay studies (Chen et al., 2017).

### 2.2.2 Zebrafish maintenance and embryo exposure

Different breeding groups of wild type zebrafish (West Aquarium GmbH, Bad Lauterbach) were held in glass aquaria according to predefined culture conditions (Braunbeck et al., 2005; Hollert et al., 2003). The exposure assay was initiated at one-hour post-fertilization (hpf) with 20 eggs at the 16-cell stage (Chen et al., 2017) in glass vessels (VWR, Germany) for a total test time of 116 h. In total, a negative control and 12 different exposure groups were tested at three replicates and with 20 embryos each. For testing, only fertilized eggs were selected and transferred in one-step to the prepared exposure solutions. The test stopped 120 h after hatching of the embryos which meant that it could be performed without having to acquire permission for an animal experiment (Strähle et al., 2012). The nominal exposure concentrations of 0.4 µg/L, 0.8 µg/L, 1.7 µg/L, 3.3 µg/L, 6.6 µg/L and 13.3 µg/L were selected according to a typical background environmental conditions with low concentrations, (e.g. as found after wastewater treatment). In contrast, the selection of the higher nominal exposure concentrations of 83.3 µg/L, 166.7 µg/L, 333.4 µg/L, 666.7 µg/L, 1334.5 µg/L, and 2666.9 µg/L were based on a worst-case scenario (e.g., after a heavy rain event and the discharge of untreated wastewater directly into the river because of an overloading of the treatment plant). The compound was spiked in DMSO to give a final concentration of 0.01 % DMSO. One treatment with only 0.01 % DMSO but without any chemical was used as the negative control. A positive control could not be prepared due to the absence of a standardised protocol for physiological changes and the lack of comparable substances with could be used. Embryos were exposed to the exposure concentrations listed at 25 ± 0.5 °C. Every 24 h, lethal and sublethal endpoints (odemas, changes in the heartbeat rate and morphological malformations) were controlled according to the OECD guideline (OECD, 1994). For the analysis of neurophysiological reactions, embryos were used to perform a 30 min locomotor light-

dark routine behaviour assay with 5 min light-dark alternations (Chen et al., 2017; Nüßer et al., 2016). After sampling, embryos were euthanized with 0.4 g/L benzocaine (Merck, Germany) ethanol solution and frozen using liquid nitrogen. Finally, they were stored at minus 80°C for RNA isolation and quantitative real-time polymerase chain reaction.

### 2.2.3 Locomotor light-dark routine behaviour assay

The locomotor assay was carried out with 116 hpf old embryos in 96 well-plates (StarLab, Germany) using the DanioVision™ video-track system (Noldus, Netherlands) following an established protocol (Chen et al., 2017). For testing, a total of 20 embryos per exposure group, as well as of the control groups, were separated with 300 µL exposure solution in a 96-well plate (max. volume 350 µL). The embryos were transferred with increasing concentration levels until the plates were full. Previously, different control approaches were performed with unstressed embryos to test for edge effects. The results were then compared and showed no differences between the different plate positions. All tests were started in the morning at 08:00 a.m. and were finished before 10:00 a.m. in order to maintain the natural circadian cycle. The video recording made it possible to determine in a single step both the total swimming distance as well as the distance to the well centre. The embryos were first acclimatized in the dark for 10 min to avoid falsification of the experimental results. This was followed by a detection period of 5 min for a total of 3 light-to-dark transitions cycles to give a total measuring time of 30 min.

### 2.2.4 Glial cell preparation

The mouse experiments were conducted in strong cooperation with Prof. Dr. Thomas Puffe, Dr. Nicole Schröder and Ms Abenaya Atputharajah from the Institute for anatomy and cell biology. Asterocytes from mouse cortex and mesencephalon were obtained from the Institute for Anatomy and Cell Biology of the University Hospital of RWTH Aachen University. A total of four cortex hemispheres per cell culture replicate and 5 mesencephalon hemispheres per cell culture replicate were used. In total, the experiment was repeated in three replicates. Therefore, cells were dissolved in falcon tubes (VWR, Germany) with Dulbecco's Modified Eagle Medium (ThermoFisher Scientific, USA) as the basic medium mixed with Hepes buffer (Merck, Germany), trypsin (Merck, Germany) and EDTA (Merck, Germany) and then transferred to cell culture flasks (VWR, Germany).

### 2.2.5 Cell culture

The cell culture medium was renewed four times a week and the cells were incubated at 44 °C. Furthermore, the test concentration range of the exposure substance mirtazapine was adapted to the highest concentration of the behaviour assay with 660 and 2600 µg/L. In addition, one cell culture of cortex and mesencephalon cells were incubated without any exposure as control groups. Finally, to avoid contamination effects, 1% Pen/Strep (Merck, Germany) was added.

After 12 days, the cell cultures of one tissue group were combined in a cell tube and centrifuged to distinguish between cell pellet and medium. Since only cells in suspension were collected in this procedure, the cell culture flask was covered with DMEM medium (Merck, Germany) afterwards and a second incubation time started. After a total of four removals, the cell culture flasks were disposed and the falcon tubes with the cell pellets were frozen at minus 80 °C.

### 2.2.6 RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was isolated with the PureLinkTM RNA Mini Kit (Invitrogen$^{TM}$, USA) as described previously (Bräunig et al., 2015). Subsequently, the RNA was transcribed into more stable cDNA using a M-MLV Reverse Transcriptase (Invitrogen$^{TM}$, USA). The target primers (*slc17a6a*, *slc6a4a*, *adra1aa*, *hrh4* for zebrafish and *mRPL13a*, *miFNg* and *mus18S* for the glia cell analysis) were ordered from Eurofins genomics (Eurofins, Germany). For the quantitative real-time PCR (qRT-PCR), PowerUP SYBR Green Master Mix (ThermoFisher Scientific, USA) was used. Finally, calculations of the relative gene expression rates followed the protocol suggested by Livak and Schmittgen in 2001 (Livak and Schmittgen, 2001).

### 2.2.7 AChE activity measurement

The measurement of the acetylcholinesterase (AChE) activity was performed following the protocol of Ellman, 1961 (Ellman et al., 1961). For analysis, one millilitre of sample was frozen at the beginning of each RNA isolation in PBS (Merck, Germany) with 20 individuals in 3 replicates for measurement of AChE activity. Furthermore, for minimizing protein degradation the concentration was measured before starting the test and no further purification steps were performed (NanoDrop, ThermoFisher Scientific, USA). Finally, the total amount of protein and AChE activity was measured photometrically using the Cytation 5 Multi-Mode Reader (BioTek, USA). Total protein concentration was measured in the supernatant using the Bicinchonic acid protein assay kit (Merck, Germany). The activity was also determined photometrically and calculated as enzyme units (U) per mg of protein.

### 2.2.8 Chemical analysis

The chemical analysis was conducted by Kilian Smith and David Kämpfer at the Institute for Environmental Research, RWTH Aachen University. At the end of the exposure period, one millilitre of each sample vessel was collected directly in an amber HPLC glass vial and measured. For the analysis, an HPLC system (1200 Series, Agilent technologies, USA) coupled to a LTQ Orbitrap XL mass spectrometer equipped with a heated electron spray ionization source (HESI; ThermoFisher Scientific, USA) was used. Mirtazapine was chromatographically separated on a Phenomenex Synergi Hydro-RP column (250 x 2 mm; particle size: 4 µm, pore size: 80 Å) using pure water (A) and methanol (B) as mobile phases. Both were acidified with 0.1 % formic acid. A gradient program was used with a flow rate of 0.6 mL/min and a total run time of 17 min, and the retention time of mirtazapine was 9.40 min. The gradient program was as follows: 0 – 1 min, 0 % B; 1 – 9 min, ramped to 65 % B; 9 – 10 min, ramped to 100 % B; 10 – 11 min, 100 % B; 11 – 12 min, reduced to 0 % B; 12 – 16 min, 0 % B). The column was heated to 40 °C and 10 µL volume was injected per sample. To identify and quantify mirtazapine, the transition of the protonated precursor ion with m/z 266 to two fragments (quantifier: m/z 195; qualifier: m/z 209) at a normalized collision energy of 30 was measured in single reaction monitoring mode. For the quantification, an external calibration curve covering a range of 1 to 2000 µg/L was measured, and at least six standards bracketing the actual sample concentrations were used for the quantification.

### 2.2.9 Statistical analysis

Behavioural-, protein- and gene expression data were checked for normality and homoscedasticity, and later analysed with a one-way analysis of variance (ANOVA). The Kolmogorov-Smirnov test was used to test for normality, and the Levene's test was used for testing homoscedasticity. The data were examined with the Turkey´s test using the SPSS software package (version 26.0, IBM, USA). A statistical significance was assigned at $\alpha = 0.05$ and graphs were plotted with GraphPad Prism (version 8, GraphPad Software, USA). In this case, different letters mean that they are statistically different from each other ($p < 0.05$). In addition, the dimensionality of the behavioural data was simplified using principal component analysis (PCA) and then analysed using multiple linear regression (MLR). The F-value was 2,30 and the p-value was 0.05.

# 2.3 Results

## 2.3.1 Behavioural effects

Exposure to mirtazapine altered the activity of 116 hpf old zebrafish embryos in both the light and the dark phases (Figure 2.1) but showed no effects in the fish embryo toxicity (FET) test for any of the treatments (Figure Annex 2.2). After an initial increase in swimming activity at concentrations below 666.7 µg/L, there was a significant decrease in the moved distance at concentrations above 1334.4 µg/L by more than 45 – 50 % compared to the control group ($p < 0.05$; Figure 2.1). In addition, the thigmotaxis reaction in the treatments from 2666.9 µg/L onward showed longer residence times at the edges of the wells than in the central area (Fig. S2). Zebrafish embryos showed reduced movements and swimming distances at an exposure concentration of 1334.4 µg/L mirtazapine and above. Behavioural effects could already be detected in the range of 13.3 µg/L, but stable changes were only detected at higher concentrations of more than 6.6 µg/L ($p < 0.05$, Figure 2.1). The glass vessels were rinsed before every exposure to avoid contamination effects.

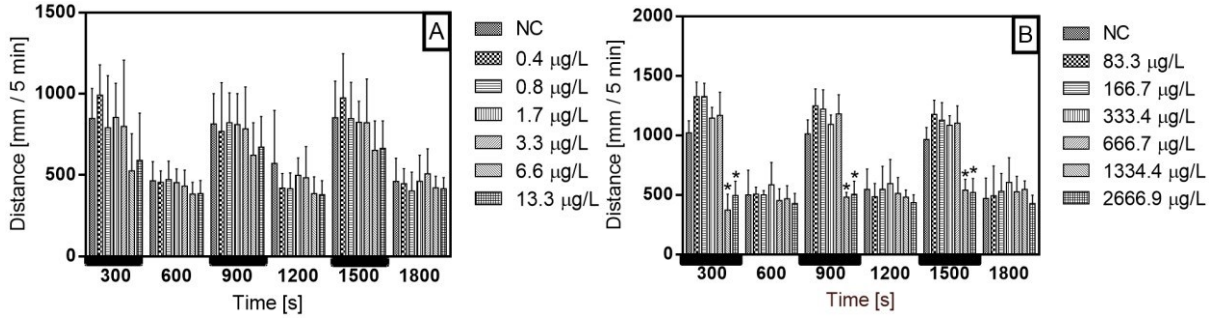



**Figure 2.1: Locomotor activity of zebrafish embryos with three 5 min cycles** of light-dark stimulation after exposure to a control sample (NC) and 12 different exposure batches mirtazapine. Graph type A and B show two different concentration ranges that were tested, and each bar represents 3 replicates with 20 embryos and expresses the mean ± standard deviation. The black bars indicate the different dark phases, and an asterisk represents a significant difference ($p<0.05$) to the control group.

## 2.3.2 Acetylcholinesterase (AChE) activity

A significantly increased AChE activity was found in the exposure group with 83.3 µg/L ($p<0.05$, Figure 2.2). The specific enzyme activity decreased with increasing exposure concentrations and almost reached the level of the control group at a concentration of 2666.9 µg/l. The regression analysis gave a R2 value of 0.61.

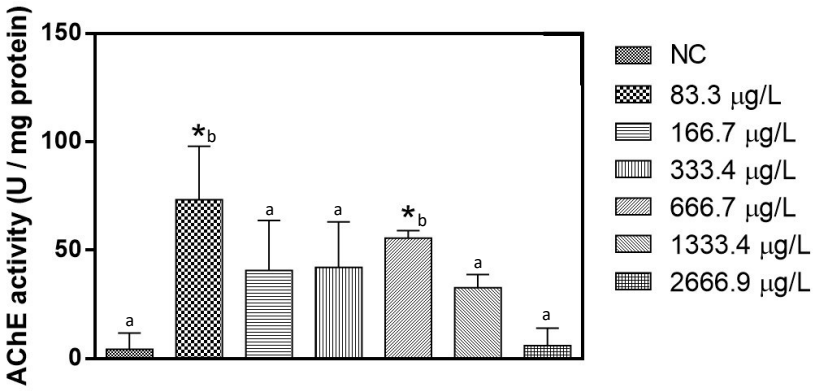



**Figure 2.2: Changes in the specific acetylcholinesterase (AChE) activity** in 116 hpf old zebrafish embryos after the exposure to six different batches of mirtazapine. Each bar represents 3 replicates (each replicate contained 20 embryos) and expresses the mean with standard deviation. An asterisk represents a significant difference ($p<0.05$) to the control group (NC).

### 2.3.3 Increased mRNA expression of genes related to the central nervous system

An increased expression rate of mRNA activity was measured for two closely related genes of the serotonergic system for the highest exposure batches of 666.7 µg/L mirtazapine and above. For the solute carrier family 6-member 4a (*slc6a4a*), an eightfold induced gene activity was detected at the exposure concentration of 1333.4 µg/L (Figure 2.3). The solute carrier family 12-member 5b (*slc17a6a*) showed no significant differences in the gene expression rate ($p < 0.05$) compared to the control group (Figure 2.3). The zebrafish adrenoceptor alpha1Aa (*adra1aa*) showed a significantly higher expression rate ($p < 0.05$) for an exposure concentration of 666.7 µg/L compared to the control group (Figure 2.3). At the exposure concentration of 1334.4 µg/L a significant up-regulation of the histamine receptor H4 (*hrh4*) was detected (Figure 2.3). None of the tested genes showed an alteration of the gene expression rate at exposure concentrations below 666.7 µg/L.

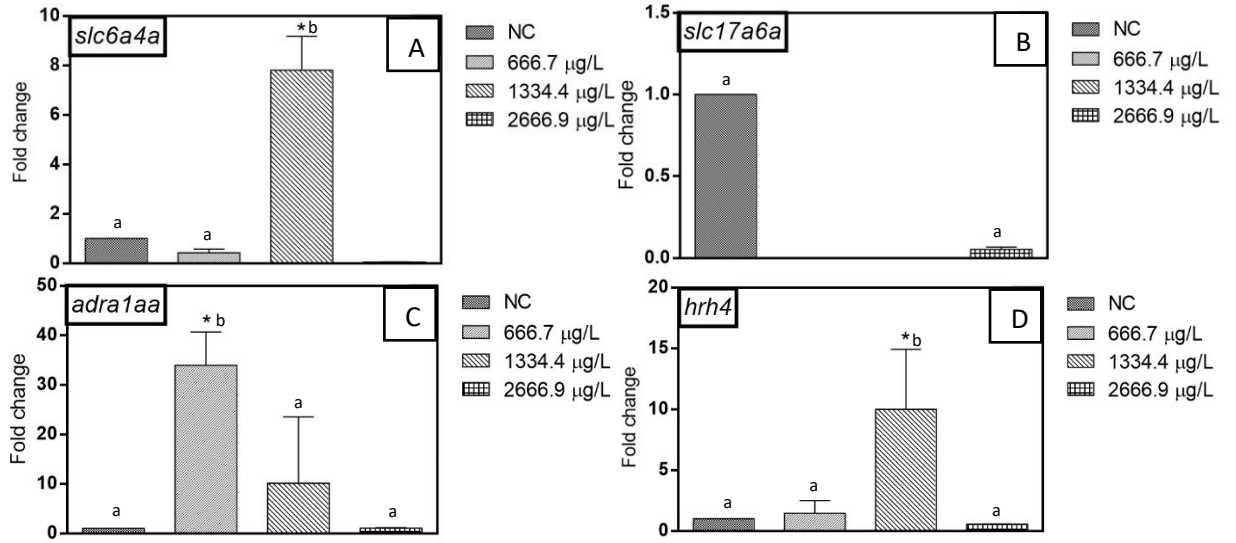



**Figure 2.3: Gene transcription profiles in zebrafish embryos** after 116 hpf exposure. (A) *slc6a3a*; (B) slc17a6a; (C) *adra1aa* and (D) *hrh4.* Each bar represents 3 replicates (each replicate contained 20 embryos) and expresses the average mean ± standard deviation. An asterisk represents a significant difference ($p < 0.05$) to the control group. The graphs show from left to right the control group (NC) and the three exposure concentrations 666.7 µg/L, 1334.4 µg/mL and 2666.8 µg/L.

## 2.3.4 Increased activity of mesencephalon cells

The results of *mus18s* and *miFNg* gene expression behaviour studies showed increased expression levels for stressed mesencephalon cells. In both cases, the increase was higher than the 15-fold expression rate of the control groups (Figure 2.4).

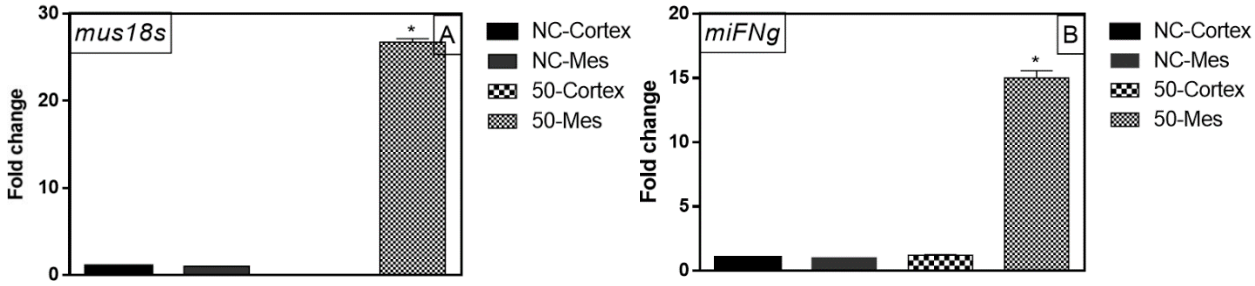



**Figure 2.4: Gene transcription profiles of cortex and mesencephalon cells. (A) *mus18s*; (B) *miFNg*.** Each bar represents 3 replicates (with more than 100.000 cells) and expresses the average mean ± standard deviation. An asterisk represents a significant difference ($p < 0.05$) to the control group. The graphs show from left to right the control groups for the cortex and mesencephalon and the exposure batches with 2666.8 µg/L mirtazapine.

## 2.3.5 Chemical analysis

Chemical analysis of the concentrations after the exposure were done using LCMS/MS to verify the ratio between nominally applied and final concentrations. These measurements were carried out for

the range of exposure concentrations where physiological effects were clearly detectable. Concentrations of mirtazapine measured at the end of the 116 hours exposure period were 81% of the nominal concentration for the highest exposure of 1800 µg/L. The minimum recovery was determined for the 840 µg/L level, but this was still more than 60 %. For the 1120 µg/L to 1680 µg/L nominal concentration range, recoveries were all above 70 %.

## 2.4 Discussion

The behavioural studies were conducted to analyse the effects of mirtazapine in aquatic non-target organisms and to compare the results with observations coming from the clinical use of the antidepressant in humans. The results of our study showed a reduced swimming distance covered in the light-dark transition test.

This finding confirms our research hypothesis that mirtazapine exerts an effect on the swimming behaviour of zebrafish embryos. In addition to the reactions within the dark phase, which are already known from comparative studies with other substances, differences within the light phases could also be measured in some cases. These are comparatively unusual for antidepressant reactions. (Richendrfer and Creton, 2018; Thompson et al., 2017). Similar to the described behavioural alterations of mirtazapine in zebrafish, a study in male Wistar rats showed that mirtazapine led to reduced spontaneous locomotor activity (Salazar-Juarez et al., 2017). The study found permanent sedative effects at low doses equivalent to 15 mg mirtazapine in humans and transient sedative effects at higher doses equivalent to more than 30 mg in humans (Salazar-Juarez et al., 2017). However, the doses applied in this study with rats were significantly higher than the concentrations of mirtazapine that zebrafish embryos were exposed to in our experiment.

The results of the chemical analysis show an average recovery rate of more than 70 % in the concentration ranges relevant for the investigations. This leads to the conclusion that the expected concentration also corresponds to the real concentration and that there were no significant differences, for example due to sorptive or other losses. The analyses show that there is neither sorption effects to the test vessels nor bioaccumulation in the organism. This is supported by medical data suggesting a half-life of 30 h for mirtazapine in humans. In this context, clinical applications confirm that the administration of the antidepressant over several days first causes a reserve to build-up in the body before the drug takes an effect (Stahl, 2013). In zebrafish embryos, a response is already observed after acute administration, especially at concentrations above 2000 µg/L. In summary, it appears that the embryos are continuously exposed to the test chemical throughout the test period.

The study of mirtazapine at the cellular level did not have the same effect strength compared with other neurodegenerative disease studies, making detection of effects much more difficult. Morphological effects have been described for mirtazapine only at comparatively very high concentrations in aquatic non-target organisms, and these could not be detected at the cellular level in mouse glial cells for the investigated concentration ranges.

To our knowledge, no study has explicitly measured the effect of mirtazapine on locomotor activity in humans. However, a logical consequence of increased fatigue and sedation as a side effect of mirtazapine should be reduced locomotor activity in humans. Mirtazapine side effects such as drowsiness or sedation in humans were associated with low daily doses and appeared to diminish in intensity as doses were increased (Anttila and Leinonen, 2001; Fawcett and Barkin, 1998). One explanation is that at higher doses the noradrenergic activation is stronger and counteracts the effect of antihistaminergic activity (Stimmel et al., 1997). However, this complex interaction at therapeutic mirtazapine doses was not evident in the zebrafish of our study at the significantly lower exposure concentrations. In principle, a comparable test design can be identified for different micropollutants, which also include the residue analysis of pharmaceuticals in wastewater samples (de Oliveira et al., 2021).

Studies with antidepressants from other drug classes such as selective serotonin and norepinephrine reuptake inhibitors (SSNRI), or atypical antipsychotics, have also shown physiological changes in zebrafish embryos that are comparable to the reactions observed in humans. A study on zebrafish and the antidepressant venlafaxine, a selective serotonin and noradrenaline reuptake inhibitor, showed activity changes in both frequency of movements and in the swimming distance. This occurred after chronic exposure over several days even at concentrations in the µg/L range (Thompson et al., 2017). This chronic exposure cannot be adequately studied at the laboratory scale due to the limited testing period but provides a basis for the expected effects in the environment. Continuous exposure of aquatic organisms to even lower concentrations of the drug may result in the formation of a reservoir, which may lead to the same effects as seen for acute exposure with high exposure concentrations. For this reason, concentration ranges beyond the currently measured average concentration of mirtazapine in the environment were also considered in the analyses. However, one decisive difference between humans and aquatic organisms is not only the threshold concentration after which any effects occur, but also the significantly higher metabolic capacity of most drugs in humans (Chng et al., 2012). It can therefore be concluded that the observed physiological changes in zebrafish may be causally linked to the exposure of mirtazapine affecting several neurotransmitter systems. An analysis of acetylcholine at the protein level and selected genes at the molecular level is therefore a useful approach to elucidate the underlying modes of action.

Acetylcholine in humans is fundamental for neurotransmission, particularly for cognition. The enzyme acetylcholinesterase (AChE) terminates the action of the neurotransmitter acetylcholine (Stahl, 2013). In zebrafish, acetylcholine plays a decisive role in the transmission of excitation, especially at the motor end plate (Rudolf and Straka, 2019). The results measured in the Ellman assay show that the balance of acetylcholine in the synaptic cleft in the presence of mirtazapine undergoes changes comparable to those in humans. We conclude that although there is no clear dose dependent effect, there is evidence for a general impact of mirtazapine on AChE activity in zebrafish. A consideration of the molecular interfaces at the gene and protein level revealed the high complexity of the interactions between the different systems. Specialization in the comparative elucidation of these effects between different neurotransmitter systems is useful and has already been performed with the help of other substance classes in similar types of studies (Boehmler et al., 2007). For a complete elucidation of the molecular causes of the observed effects on the behaviour of the exposed zebrafish embryos, a comparison between the investigated genes in humans and in zebrafish is important. Both organisms have in common that they have superordinate hormonal control systems that help to regulate their behaviour (Panula et al., 2010). In humans, mirtazapine blocks adrenergic alpha2-receptors, exerts a high affinity to histamine H1 receptors and despite its action on the serotonin system, the affinity to the serotonin receptor 5-HT1A is considered to be low (Anttila and Leinonen, 2001). Therefore, mirtazapine affects histaminergic, serotonergic, and noradrenergic neurotransmitter systems in humans. There are similarities in these targets of mirtazapine between humans and zebrafish, both at the gene- and protein level. For each gene in our study, a significant difference in the gene expression rate could be measured at a specific drug concentration, which did not necessarily correspond to the highest concentration. This illustrates the close relationship between genes and the influence of exposure concentrations on the underlying molecular response in the central nervous system (Guo, 2004). Although current studies on the influence of mirtazapine on the swimming behaviour and the molecular causes in zebrafish embryos are still missing, related studies with other psychotropic drugs give a first insight into molecular effects. For example, Boehmler et al. (2007) detected an activity-reducing effect of the atypical antipsychotic clozapine in zebrafish embryos (Boehmler et al., 2007). Their data suggest that the locomotor effect was mediated by zebrafish D4 dopamine receptor, but clozapine also strongly blocks the histamine H1 receptor. However, although mirtazapine does not block dopamine receptors or dopamine reuptake it still resulted in a decrease activity in zebrafish embryos in our study (Stimmel et al., 1997).

A comparison of the gene and protein sequences of humans and zebrafish showed clear differences in the gene sequences, but at the same time, because of the redundancy of the genetic code, a high overlap of the proteins formed (Ardell and Sella, 2001). These findings indicate that mirtazapine causes changes at a molecular level, at least at the site of the formed proteins, particularly when considering

that an increased gene expression rate is the starting point for an organism's reaction to such a neuroactive compound. From previous studies with other pharmaceuticals, it can be assumed that possible combination effects of mirtazapine with other neuropharmaceuticals will increase rather than decrease the effects of behaviour alterations (Galus et al., 2013a). Field experiments are much more challenging in terms of planning, implementation and analysis, but they also offer new opportunities in interpretation of complex data, which could help to better assess the environmental relevance of the substance (Brodin et al., 2014; Kidd et al., 2007).

These difficulties could already be seen in the analysis of the gene expression levels for the neuronal mouse cells because the large number of possible nodes, did not allow the selection of all relevant genes for testing. In comparison to other aquatic species and biological level, effects of mirtazapine have primary been described at behavioural level in rodents and zebrafish. The high complexity level of different movement patterns that could be influenced by neuroactive pharmaceuticals did not help to find some hints for the underling molecular mechanisms of action. A clear identification has not yet been sufficiently described in literature and the complex interactions at the molecular level are currently still a central part of basic research in mice. Nevertheless, the results show that effects could occur at cellular level of glial and mesencephalon cells and with different extrapolation steps this could be the reason for behavioural alterations.

A detailed linkage of cellular and molecular effects in glial cells could not be conclusively elucidated by the experiments but could be identified in the overall context. The analysis of central nodes between these two biological levels seems purposeful against the generated results. With a specific focus on zebrafish embryos, a stronger focus on cells of this non-target organism should therefore be considered for future experiments, or additional experimental procedures such as histological sections should be included. The results at the cellular level have shown that quantifiable effects occur, but these need to be studied with a higher concentration range to make further conclusions.

Whether this reaction is triggered directly by the drug itself, or whether the effects under consideration are rather a reaction of the organism to the antidepressant, cannot be answered conclusively, at least for zebrafish. The homologous physiological reaction of mirtazapine postulated at the beginning of the research project, i.e., sedation and consequently reduced locomotor activity in humans, was supported by using zebrafish embryos as an aquatic organism. The elucidation at the molecular level illustrated the complex links between the various neurotransmitter systems and show that an isolated consideration of the causes of these effects is not appropriate. For future analyses, a consideration of more complex environmental samples with psychotropic drugs of different drug classes would be of particular interest. Behavioural effects of organisms in these environments should also be quantified, and the molecular effects should be analysed with the help of gene expression

analysis. Current developments show that even for well-established test methods with model organisms such as zebrafish, different possibilities for further optimisation steps exist. They could support the effect analyses at the ecosystem level (Guimarães et al., 2021). A complete risk assessment of mirtazapine in the environment requires further studies. On the one hand, these should quantify the actual environmental concentrations at various time points. On the other hand, these should also consider the physiological changes in behaviour within the context of an entire fish population. In addition, such studies on mirtazapine represent a first step towards effect analysis at higher biological levels (e.g., at population level). For other substance classes such as hormones, an equivalent approach, with ecotoxicological single-substance testing at individual level and physiological effect analysis at the population level, has already led to significant findings at the interface with ecology (Broséus et al., 2009). The relevance of ecotoxicological behavioural studies for risk assessment becomes more and more important for different substance classes (Ford et al., 2021). An analogous approach for antidepressants, including additional classes to the NaSSAs studied here, thus makes sense at the population level.

## 2.5 Conclusions

Our studies with mirtazapine at different exposure concentrations have shown that in contrast to morphological changes, physiological behavioural changes are an important but not yet sufficiently considered endpoint in risk assessment. Especially neuroactive pharmaceuticals whose primary targets are changes in the behaviour are only one group of compounds that illustrate the need for further development of protocols for behaviour measurements. These changes have the potential to endanger the stability of the population and parts of the ecosystem and should clearly be included in future analyses of micropollutants. In this context, our study provides evidence that the dual mechanism of action of mirtazapine also occurs in aquatic non-target organisms and results in behavioural changes. Simultaneously, the elucidation of the molecular causes of action showed that there are different nodes between higher-level neurotransmitter systems and behaviour in the tightly interconnected neural network. This information provides relevant information for the future assessment of the environmental effects of neuroactive psychotropic drugs and demonstrates the importance of these studies, particularly in an ecological context. All this will help to better assess the potential risk of these psychotropic drugs in the future.

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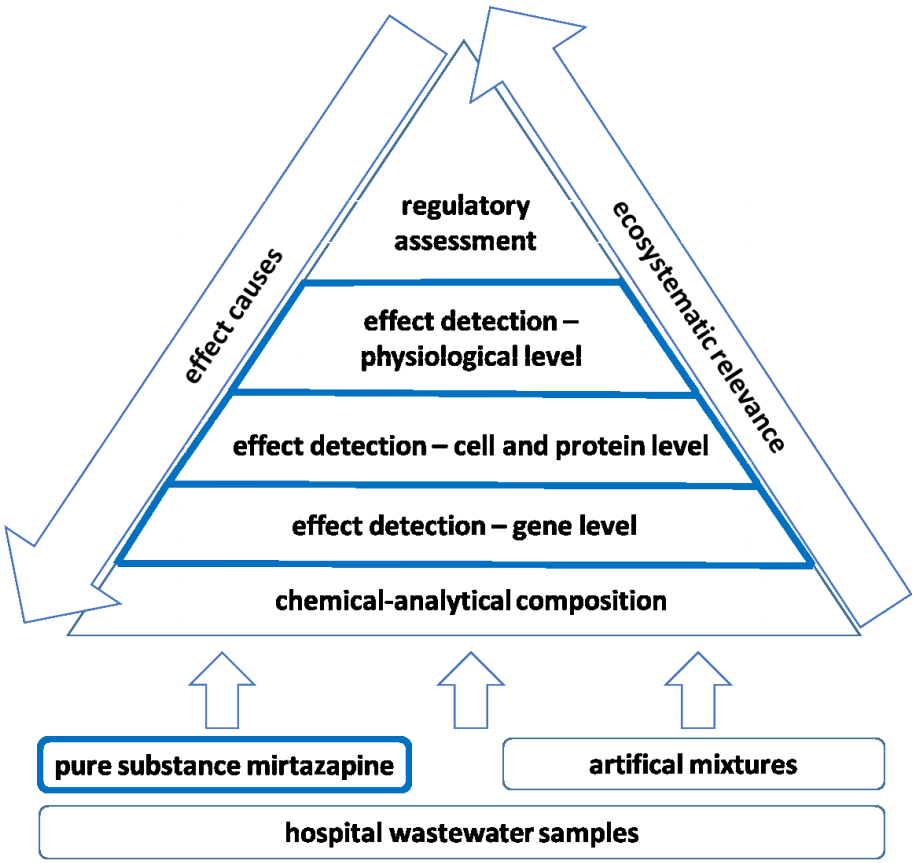
# Chapter 3

# Effects of mirtazapine on the swimming behaviour, gene expression rate and reproductive ability of *Daphnia magna*

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## Abstract

The study of the neuroactive potential of the noradrenergic and serotonergic effective antidepressant mirtazapine has gained considerable attention in recent years due to the increasing use of the drug in the treatment of depression and sleep disorders. The increasing use of the drug has also led to a significant release into receiving aquatic environments. The monitoring of neuroactive pharmaceuticals in aquatic systems has been expanded in recent years, but an analysis of sublethal effects in non-target aquatic organisms has not yet been carried out systematically, especially for invertebrates. The aim of the present study was to evaluate the reproduction, swimming behaviour and molecular responses of *Daphnia magna* exposed to the antidepressant mirtazapine at concentrations of 0.1 to 200 µg/L in 48-h and 21-d tests. An analysis of the swimming behaviour of individuals of the aquatic model organism, after different exposure periods throughout a 48-h test showed an increase in the swimming distance with exposure to 100 µg/L and 200 µg/L mirtazapine. The molecular effects on *Daphnia magna* at the end of the 48-h test were analysed with a special focus on the superordinate serotonergic and dopaminergic systems using a quantitative real-time PCR system. An upregulation of the serotonin transporter and receptor genes were measured after an exposure to 200 µg/L mirtazapine. Furthermore, effects of mirtazapine on the reproduction and length of *Daphnia magna* were investigated in a 21-d reproduction test but no significant results were observed at the tested concentrations. The results from the present study demonstrate that sublethal effects on behavioural changes in *Daphnia* occurred after exposure to mirtazapine. Since swimming behaviour of *Daphnia* is closely linked to essential biological and ecological functions such as feeding, migration, and predator-prey interactions, such sub-lethal effects from exposure to neuroactive pharmaceuticals could have cascading effects on the population and community structures of aquatic ecosystems. Thus, it is important that sub-lethal effects on non-target aquatic invertebrates should also be considered in the environmental and regulatory assessment of potentially neuroactive substances. Large-scale studies of central neuronal systems are still needed to elucidate the underlying molecular causes of action of mirtazapine and other potentially neuroactive substances.


## 3.1 Introduction

*Daphnia magna* occupy a key position in aquatic ecosystems as first-order consumers, not only because they are the food source for many groups of organisms, but also because they play an important role in the accumulation of pollutants in the food chain (Ding et al., 2016; Hou et al., 2013; Tan et al., 2017). Due to their ecological importance as a widespread keystone species, ease of

culturing, and availability of genetic databases, *Daphnia* species have become a standard model organism in ecotoxicology, evolutionary biology, and ecology. Many ecotoxicological studies have focused on the classical acute and reproductive endpoints, such as the number of immobilised individuals or the number of offspring produced with exposure to increasing concentrations of contaminants compared to a control treatment. However, there are a lack of studies on the sub-lethal effects of contaminants for endpoints such as changes in behaviour. Sub-lethal effects can be particularly important when investigating the effects of micropollutants that can cause several effects at lower concentrations typically found in the environment (Kanaujiya et al., 2019; Rogowska et al., 2020; Schwarzenbach et al., 2006). In particular, the number of psychotropic drugs in aquatic systems has continuously increased, both in the number of different medications and in the measured concentrations found in the environment (Golovko et al., 2020; Phonsiri et al., 2019; Yuan et al., 2013).

The use of the antidepressant mirtazapine has increased considerably in the last decade due to its dual mode of action and its small number of side effects compared to older psychotropic drugs (M.E.P.S., 2017). Mirtazapine is a tetracyclic piperazine-azepine that belongs to the group of noradrenergic and specific serotonergic antidepressants (NaSSA) and is used to treat temporary depressive episodes and sleep disorders (Benkert et al., 2002; Holm and Markham, 1999). In humans, it acts as a sleep aid immediately after ingestion and has an activity-enhancing effect the day after consumption. Pharmacologically, many homologous molecular receptors are found in humans as well as in aquatic non-target organisms such as *Daphnia* and zebrafish, where pharmaceuticals can have an effect (de Souza Anselmo et al., 2018; Spitsbergen and Kent, 2003). Most of the receptors of the central nervous system are conserved and can be found in homologous structures in different organisms. This can be used for the retrospective analysis of effects in various non-target organisms, but for the development of new human pharmaceuticals. The investigation of possible negative effects of mirtazapine on non-target organisms are important as zebrafish serve as good biomedical model organisms for human medical research, and *Daphnia* act as a keystone species and important food source in many aquatic ecosystems (Flaherty and Dodson, 2005). Furthermore, there are also possible bioaccumulation effects along the food chain that are unknown for many neuroactive substances, which could again have a negative impact on humans as high order predators (Du et al., 2016). The concentrations of mirtazapine found in the environment range from ng/L to µg/L, depending on the respective drainage area of the study site and on the removal technologies of the nearby wastewater treatment plants (Golovko et al., 2020; Gurke et al., 2015). Due to the pharmacological nature of the antidepressant, there is a concern regarding the neurological and physiological effects on non-target organisms that may be associated with the release of mirtazapine into the environment.

Mirtazapine is a potent antagonist of several receptors including adrenergic, serotonin and histamine, which can affect the central nervous system for example by enhancing adrenergic and serotonergic neurotransmission in the brain involved in mood regulation. Since most invertebrates use serotonin as neurotransmitters, it is likely that a NaSSA such as mirtazapine could result in specific mode of action effects on a variety of different aquatic invertebrates. Different antidepressants that show effects on the serotonergic system have been reported to result in acute and chronic effects in aquatic organisms at environmentally relevant concentrations using traditional endpoints such as survival and reproduction (Black and Armbrust, 2000; De Castro-Català et al., 2017; Johnson et al., 2007). However, physiological endpoints are often more sensitive parameters that can allow a better evaluation of sub-lethal effects. The physiological effects of neuroactive pharmaceuticals that influence major neuronal control systems like the serotonergic system have already been quantified in zebrafish and, in some initial studies, the associated molecular causes have also been determined for selected genes (Boehmler et al., 2007). Since many of these potentially neuroactive substances have a particularly negative effect on the behaviour of individuals, various methods have been developed for recording behavioural changes. However, little information are available about the behavioural and molecular level effects of neuroactive substances on invertebrates. The few behavioural studies available with the model ecotoxicology test species, *Daphnia magna,* suggest that different physiological (e.g., feeding activity, heart rate, respiratory activity) and behavioural (e.g., locomotion, predator avoidance, swimming) endpoints can be analysed through some common and standardised bioassays such as the OECD *Daphnia* immobilisation and reproduction tests (Bownik et al., 2020; Flaherty and Dodson, 2005; Kleiven et al., 1992; Wollenberger et al., 2000). Swimming behaviour is an important endpoint for *Daphnia* species that can be assessed using several parameters (e.g., swimming time, speed, distance travelled, and vertical/horizontal distribution or migration) with exposure to substances that act for example on the nervous system (Bownik, 2017). To assess the movement of *Daphnia*, which takes place in both vertical and horizontal directions, different dimensional approaches are available for estimating possible behavioural changes, including factors such as swimming speed (Abe et al., 2019; Ren and Wang, 2010). When examining the effects of chemicals on the behaviour of an organism it is important to understand the physiological mechanisms through molecular and biochemical analysis.

In the present study, the sub-lethal effects of mirtazapine on the swimming behaviour, reproduction, and molecular level responses of *Daphnia magna* were examined. Based on studies in humans and zebrafish, an activity-enhancing effect of mirtazapine is suspected in *Daphnia magna* that may influence behaviour and reproductive endpoints. Swimming behaviour was assessed at three time points throughout a 48-h acute *Daphnia* test. The elucidation of the underlying molecular mechanisms related to acute exposure to mirtazapine in *Daphnia magna* at the end of the 48-h test was conducted with a special focus on selected superordinate control systems and an upregulation of genes of the

serotonin and dopamine system was assumed. Furthermore, reproductive output was monitored in a 21-d *Daphnia* reproduction test to assess possible long-term effects of mirtazapine on *Daphnia magna*. The aim of the study was to generate knowledge on the sublethal effects of psychotropic drugs, such as mirtazapine, on non-target organisms such as cladocerans, which is lacking about its influence on the reproduction, behavioural and molecular level in invertebrates that may be important endpoints in future risk assessment.

## 3.2 Material and Methods

### 3.2.1 Test pharmaceutical

Mirtazapine ≥98% (HPLC) (1,2,3,4,10,14b-Hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c][2]benzazepine), C17H19N3, CAS Number: 85650-52-8) was supplied by Merck (Deisenhofen, Germany). A stock solution of 0.25 mg/mL mirtazapine was prepared in ultrapure water (Millipore).

### 3.2.2 Daphnids maintenance and exposure

*Daphnia magna* were maintained and tested in-house at the Institute for Environmental Research, RWTH Aachen University, Germany, according to the OECD guidelines 202 and 211 (OECD, 1994; OECD, 2012). In brief, *Daphnia magna* were maintained in permanent breeding groups in different age structures in M4 medium at 20 ± 1 °C and a photoperiod of 16:8 h light:dark. The cultured individuals of *Daphnia magna* were fed with algae, *Scenedesmus subspicatus,* three times a week and the transfer of the *Daphnia* into new medium was done weekly.

Behavioural experiments were conducted with *Daphnia magna* neonates (≤ 24 h old) under static conditions for 48-h in the dark without feeding. Ten neonates from the second brood progeny were exposed in three replicate glass vessels each with 100 mL exposure medium per treatment. Four different exposure treatments with the concentrations of 0.1 µg/L, 10 µg/L, 100 µg/L, and 200 µg/L mirtazapine were examined in addition to a control treatment with just M4 medium. Behavioural observations included the total swim distance (mm) of the individuals, which were recorded at three time points throughout the 48-h test exposure using the DanioVision™ video-track system (Noldus, Netherlands). Methods were adapted from the previously described protocols for behaviour measurement in fish (Chen et al., 2017). In brief, individual animals were placed in wells of 96-well plates (Starlab, Germany) and allowed to settle in the DanioVision™ system for 10 min in the dark prior to recording. Individual animals from each mirtazapine treatment and control (n=30 each) were

recorded three times throughout the test (t = 0, 24, and 48 h) for a period of 30 min with a 15 min dark cycle followed by a 15 min light cycle. At the end of the exposure after the last behaviour measurement (48 h), animals were immediately euthanized in liquid nitrogen and subsequently stored at - 80 °C for subsequent RNA isolation and quantitative real-time polymerase chain reaction analysis.

The 21-d reproduction test was carried out according to OECD Guideline 211 with *Daphnia magna* (OECD, 2012). Three exposure concentrations (10 µg/L, 100 µg/L, and 200 µg/L mirtazapine) and a control treatment (Elend M4 medium) were examined in three replicates with 10 animals in 100 mL each (≤ 24 h old from > the second brood progeny). Animals were fed daily throughout the test and performed with semi-static renewal of test medium every 7 days under culture conditions. The stability of mirtazapine in water was measured after the exposure to zebrafish embryos and showed a recovery rate of more than 70 % after 5 days exposure. The total number of offspring produced per 10 individuals in each replicate was tracked over the duration of the test for each treatment (n=3). Additionally, the determination of body length was conducted at the end of the 21-d test on a random subsample of 5 individual animals per treatment (n=5). For length determinations, individual animals from each treatment were separated in a Petri dish and immobilised through removal of excess water. Body length was measured from the upper edge of the compound eye to the base of the abdominal claw using a stereo microscope (Nikon, Japan).

### 3.2.3 Molecular analysis

The elucidation of the gene expression rates for *Daphnia magna* exposed during the 48-h behaviour test was carried out for selected genes of superordinate control systems of the serotonin and dopamine system. The gene selection and the design of the primers were carried out across species, including genes from *Drosophila melanogaster*, which have clear homologies with *Daphnia magna* in the structure of the formed proteins. First, the 30 daphnids of the same exposure treatment were pooled and digested using the homogenizer VDI 12 (VWR, Germany). The control group and three highest exposure concentrations that show an effect in the behaviour assay (10, 100, and 200 µg/L) were analysed for gene expression. The subsequent RNA isolation for each treatment was performed using the PureLink™ RNA Mini Kit (Invitrogen™, USA) as previously described in recent studies (Chen et al., 2017). Afterward, the RNA was transcribed into the more stable cDNA using M-MLV reverse transcriptase (Invitrogen™, USA). The final quantification of the effects was performed using a quantitative real-time qPCR system and PowerUP SYBR Green Master Mix (ThermoFisher Scientific, USA). The selected primers correspond to genes of higher-level neuronal control systems (serotonin

transporter, transcript variant B (SerT, *DDX54*); tryptophan hydroxylase (Trh, *CG6836*); *slc12a5b;* and *slc17a6a*) and were synthesized by Eurofins Genomics (Eurofins Genomics, Germany).

### 3.2.4 Statistical analysis

For statistical analysis, the SPSS software package (version 26.0, IBM, USA) was used with a significance level of $\alpha = 0.05$. The behavioural data were checked for normality and homoscedasticity and analysed using a one-way analysis of variance (ANOVA) followed by a Dunnett´s test to determine differences among treatments. A t-test was used to check for significant changes in reproductive output and length data for the different treatments of mirtazapine compared to the control. The gene expression data were analysed using the protocol of Schmittgen and Livak (Schmittgen and Livak, 2008).

## 3.3 Results

All *Daphnia* tests fulfilled OECD validity criteria regarding water chemistry parameters and biological criteria for controls. All test concentrations for both the behaviour and reproduction test were sub-lethal as no increased mortality rates were observed for any of the treatments and controls in the tests.

### 3.3.1 Behavioural endpoints

Figure 3.1 shows the measurement immediately at the start of exposure (t = 0h) for the different mirtazapine treatments and control group. The first 15 min period with darkness showed an average swimming distance ranged from 193.5 to 199.1 mm, followed by a lower average swimming distance of 96.8 ± 1.6 mm observed in the 15 min light period for *Daphnia magna* from the control treatment. Swimming distance of *Daphnia magna* was not significantly increased compared to the control within the dark phase for the 100 and 200 µg/L mirtazapine treatments at time 0 h (Figure 3.1 A). After 24 h of exposure, an average swimming distance in the dark phase of 148.3 ± 1.5 mm and 78.2 ± 2.9 mm in the light phase was observed for *Daphnia magna* in the control treatment (Figure 3.1 B). Locomotor activity of *Daphnia magna* followed a dose-dependent response with exposure to mirtazapine after 24 h for both the light and dark phase. The individuals from the highest exposure treatment with 200 µg/L mirtazapine had greater average locomotor activity compared to the control group in both photoperiod phases (200 ± 5.7 mm for dark and 108.6 ± 7.0 mm for light). At the end of the 48 h exposure, the average locomotor activity of *Daphnia magna* from the control group was 151.3 ± 5.6

mm for the dark and 98.3 ± 4.2 mm for the light photoperiods (Figure 3.1 C). A dose-dependent response was observed compared to the control group until the 10 µg/L concentrations in the dark phase. Baseline swimming distance was generally the same in the controls throughout the duration of the 48-h test for *Daphnia magna* in both the dark and light phase.

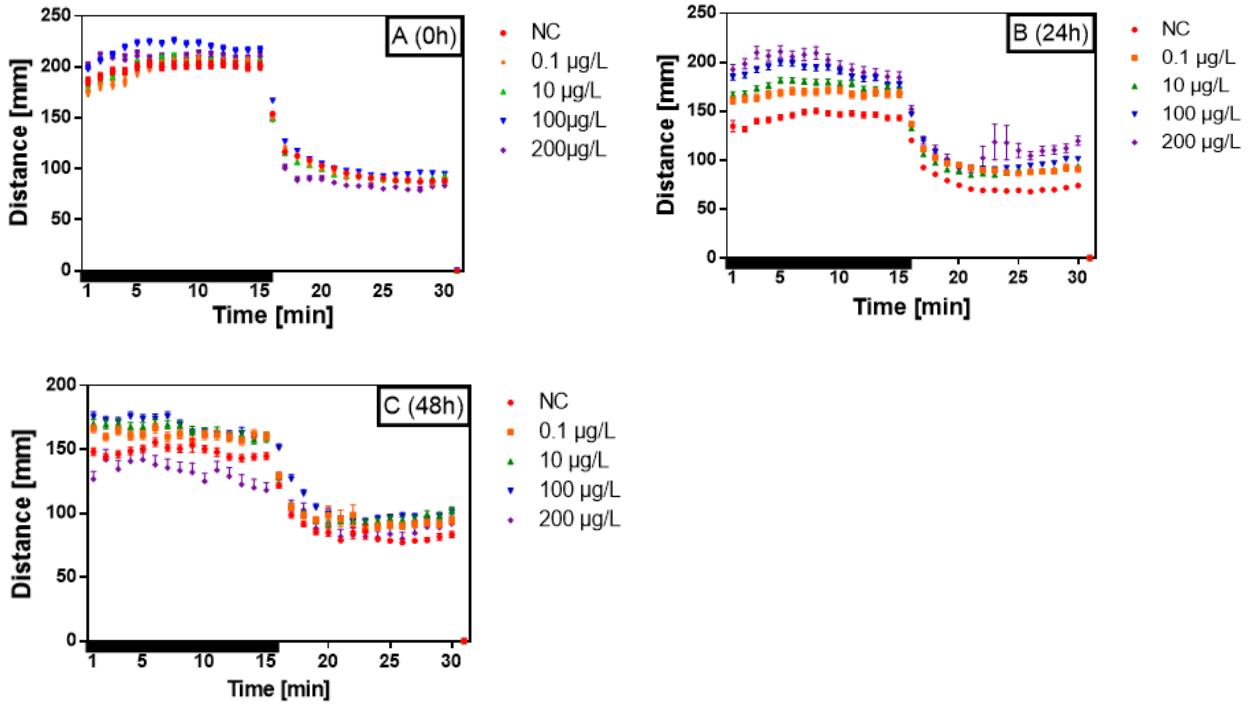


**Figure 3.1: Locomotor activity (swimming distance, mm) of *Daphnia magna*** measured during a 15 min dark phase followed by a 15 min light phase (A) before the exposure at time 0 h, (B) after 24 h exposure, and (C) after 48 h exposure for control (NC), 0.1, 10, 100, and 200 µg/L mirtazapine treatments. Each data symbol represents the average swimming distance of 10 individuals from 3 replicates (n=3) that were recorded for a total of 30 min at 3 different time periods throughout the 48-h test. The results are expressed as mean ± standard deviation and the black bars on the x-axis indicate the initial dark phases of the observations.

## 3.3.2 Molecular endpoints

The results of the gene expression analysis are shown in Figure 3.2 for the three highest mirtazapine treatments of 10 µg/L, 100 µg/L, and 200 µg/L from *Daphnia magna* exposed in the 48-h behaviour test. The results of the gene expression analysis did not demonstrate a clear dose-response relationship with exposure to mirtazapine for any of the examined genes. An induction of gene expression was measured for the serotonin transporter, transcript variant B (SerT, *DDX54*) in Figure 3.2 at the highest exposure concentration of 200 µg/L with a fold change of more than 3.5 ± 1.3. An increased activity with a fold change of more than 4.0 ± 0.5 was observed for the lowest exposure treatment (10 µg/L mirtazapine) for tryptophan hydroxylase (Trh, *CG6836*) in comparison to the other two higher concentrations of mirtazapine that had no statistical difference to the control group (Figure 3.2). The greatest fold change for the gene expression of *slc12a5b* and *slc17a6a*, were observed for

the lowest concentration of 10 µg/L mirtazapine and the second highest concentration of 100 µg/L with fold changes of more than 2.0 ± 1.8 and 6.0 ± 6.2, respectively (Figure 3.2).

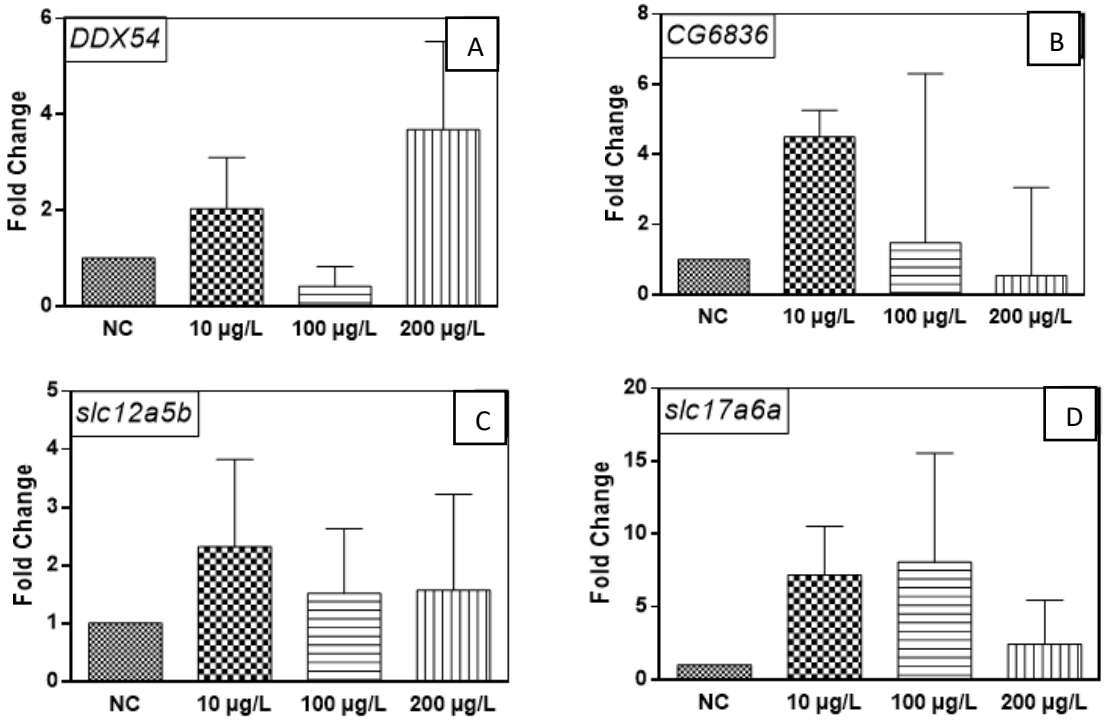


**Figure 3.2: Gene expression profiles of the *D. magna* at the end of the 48 h behaviour test with exposure to three different mirtazapine treatments (10 µg/L, 100 µg/L, and 200 µg/L) and the control treatment (NC) for genes (A) *DDX54*, (B) *CG6836*, (C) *slc12a5b*, and (D) *slc17a6a*.** Each bar represents 3 replicates (with 30 individuals per replicate, n=90) and a cDNA concentration of 3 µg. The bars represent an average fold change in gene expression (mean ± standard deviation) of the 10 individuals pooled per replicate (n=3) for each treatment.

### 3.3.3 Reproduction and length endpoints

The 21-d reproduction test did not indicate any significant difference in the reproductive output of the *Daphnia magna* exposed to the three different concentrations of mirtazapine compared to the control ($p > 0.05$; Figure 3.3). The average total number of offspring produced per ten organisms over the duration of the test was the lowest in the control group, averaging 38 ± 11 individuals, and the highest in the 100 µg/L mirtazapine treatment, averaging 52 ± 14 individuals.

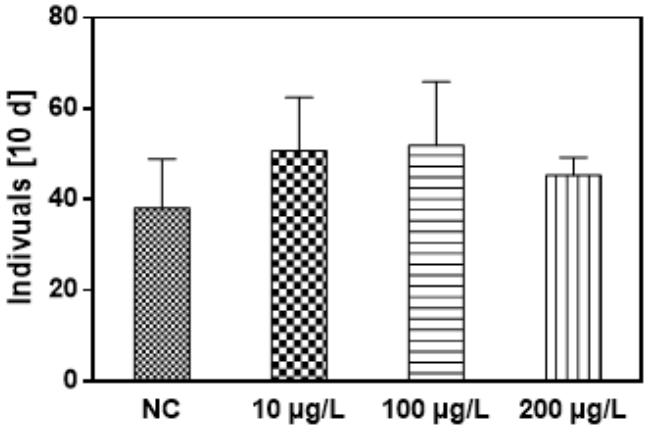


**Figure 3.3: The average total number of offspring produced (per 10 individuals in each replicate) at the end of the 21-d *Daphnia magna* reproduction test with exposure to a control (NC) and three mirtazapine treatments (10 µg/L, 100 µg/L, and 200 µg/L).** The bars represent the mean ± standard deviation of the average number of offspring produced per 10 animals exposed for each of the 3 replicates (n=3).

The mean body length of *Daphnia magna* in the control and mirtazapine treatments are shown in Figure 3.4. No significant differences in the body length of *Daphnia magna* between the individuals of the different treatments were observed, with an overall average body length of 5.9 ± 0.2 mm for individuals across all treatments and control.

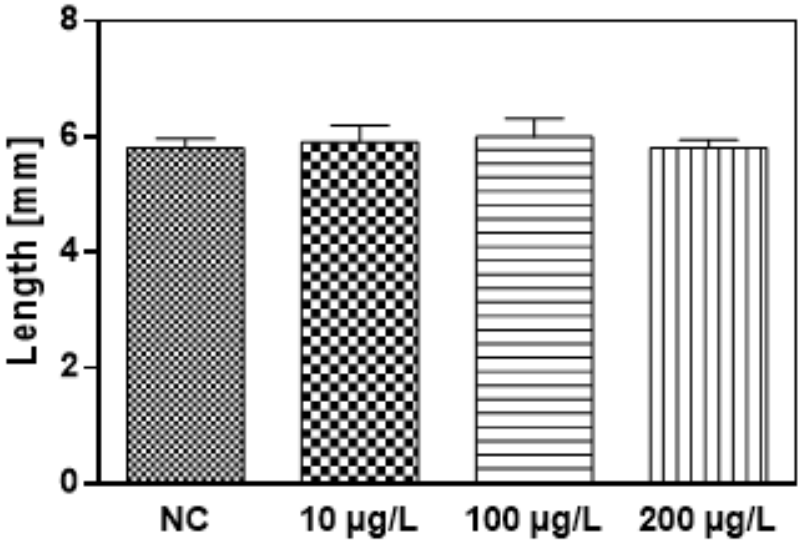


**Figure 3.4: Average body length measurement (mean ± standard deviation) for five individual *Daphnia magna*** after an exposure of 21 days for each of the control and three mirtazapine treatments (10 µg/L, 100 µg/L, and 200 µg/L).

## 3.4 Discussion

This was one of the first studies to investigate the effects of mirtazapine directly on aquatic invertebrates such as *Daphnia* species. Except for a few acute studies with zebrafish embryos, very little information is known about the neuroactive potential of this psychotropic drugs on the physiological processes in non-target organisms like crustacea in receiving aquatic environments. The majority of previous studies on the effects of mirtazapine were conducted in humans with a special focus on the refinement of the molecular modes of action (Chorilli et al., 2011; Davis and Wilde, 1996; Dural et al., 2020).

### 3.4.1 Behavioural changes

The hypothesis of an increased physiological swimming activity reaction of *Daphnia* as central representatives of the aquatic non-target organisms could already be demonstrated for a 2-dimensional movement recording. This means that there is not only a direct influence of the pharmakon on the behaviour of aquatic consumers of higher order, but also on organisms of lower biological levels, which can have a potential influence on the overall stability of the ecosystem.

The results from the present study show that the swimming behaviour of *Daphnia magna* is influenced by mirtazapine in a dose-dependent manner, resulting in an activity-enhancing effect with concentrations great than 10 µg/L of the drug. Although this concentration is too high compared to the actual measured eecologically relevant concentrations (high ng/L), it could be an important aspect in future research. Large-scale monitoring studies are constantly identifying more and more point sources where these concentrations can be reached and, due to the constantly increasing prescription quantities, it can be assumed that the concentrations in the environment will increase (Grabicová et al., 2020; Jaria et al., 2020; Miarov et al., 2020). It was observed that mirtazapine increased the locomotor activity (e.g., swimming distance) of *Daphnia magna* by approx. 45.1 to 7.2 mm during the dark photoperiod when animals were exposed to the highest tested concentration of 200 µg/L compared to the control. The effect strength was higher between the different exposure groups in the dark phases than in the light phases, both after 24 h and after 48 h exposure time. This is part of the natural movement responses of *Daphnia* in the aquatic ecosystem, as hunting by visual predators is much more difficult in the dark compared to hunting in the light (Schönborn, 2003). After 24 h exposure time, the effect strength was greatest, which suggests a degradation of the substance. Nevertheless, comparative studies with zebrafish embryos show that even after 5 days more than 70 % of the originally applied concentration is still present (Gundlach et al., 2021). Comparable effects on swimming behaviour were observed for other aquatic invertebrates, including *Gammarus pulex* and

*Daphnia magna*, with exposure to similar neuroactive substances of the same chemical class (Bownik, 2020; Rivetti et al., 2016; Tkaczyk et al., 2020a; Tkaczyk et al., 2020b). For example, our results align with literature reporting the modulation of swimming velocity of the amphipod, *Gammarus pulex,* with exposure to a selective serotonin reuptake inhibitor antidepressant, fluoxetine (De Castro-Català et al., 2017). For effects on the swimming behaviour in *Daphnia magna*, previous work has suggested that an increased accumulation of a pharmaceutical drug in the nervous system of crustacea can lead to a functional disorder in the coordination between external stimuli and the subsequent behavioural response (Yuan et al., 2020). From the results of the present study and those of the literature, it is likely that there is a shift in the neurotransmitter balance of *Daphnia magna* with exposure to mirtazapine. Such findings are important as they demonstrate that several similar pharmaceutical chemicals may also affect organisms in taxonomically lower classified groups such as first-order consumers.

Behavioural endpoints are often ecologically important, but the limited data available in literature on sub-lethal effects, such as swimming behaviour, is partly due to the lack of standardised methods for testing. A clear method for recording the swimming behaviour of *Daphnia magna* is currently no more established than for behavioural swimming studies of the taxonomically higher *Danio rerio*, for which studies have been carried out much longer (Fleming and Alderton, 2013; Langheinrich, 2003; Zhou et al., 2019). Recent work has provided some steps forward in individual approaches for the standardization of methodological observations for evaluating behavioural changes of *Daphnia magna* (Yuan et al. 2020). In previous studies, inconsistent study design was mentioned as the main barrier for data comparability. In the current study, a special form of data detection was chosen with the 2D recording, which is oriented to the previous procedures for behavioural monitoring in zebrafish embryos. In the current study, a special form of data detection was chosen with the 2D-recording, which is adapted from the previous procedures for behavioural recording of zebrafish embryos (Legradi et al., 2015). An extension to a 3D-recording makes sense for the natural swimming movement of *Daphnia* but is also comparatively costly because of the small size of the individuals. The endpoints (distance travelled) are reasonable considering the known human effect of increasing activity and provide results that allow comparison between the exposure groups and the control group. Investigating *Daphnia* swimming behaviour as a biomarker in ecotoxicological studies is important as it plays an important role in determining predation risk. In addition to different natural factors such as shading or changes of the pH, various substances could lead to changes in the behaviour of the organisms (Bownik, 2017). As one example, different regulations for the protection of the environment have already been defined for pesticides with different ecological data. The REACH regulation has led to more effect data especially for classical sub-lethal effects. In particular, diel vertical migration of *Daphnia* (i.e., movement towards deeper darker areas during the day) is an important antipredator

trait to avoid detection by visually hunting predators such as fish (Boersma et al., 1998), whereas deal horizontal migration (i.e., movement towards vegetated littoral during the day) can be more important in shallow lakes as an antipredator trait (Michels et al., 2007)). Behavioural changes, starting from changes at the individual level, can lead to changes of the population structure. Depending on the relevance of *Daphnia* in the ecosystem as consumers of the first order, changes in the behaviour of *Daphnia* could significantly alter the structure of an entire ecosystem through various top-down or bottom-up effects on the food web (Bownik, 2017). The relevance of behavioural changes in *Daphnia* on the population, as well as on the overall structure of the ecosystem, has previously been confirmed in ecological field studies (Dodson and Hanazato, 1995; Duffy et al., 2004; Miner et al., 2012). Thus, the activity-enhancing effects of mirtazapine observed in the current study on the locomotor activity of *Daphnia magna* poses a potential risk to aquatic systems that have not yet been fully assessed in regulatory terms. However, for a conclusive interpretation and mechanistic understanding of the behavioural results the elucidation of the molecular modes of action with exposure to mirtazapine to *Daphnia magna* are of great importance.

### 3.4.2 Molecular changes

The results of the analysis of the molecular changes in *Daphnia magna* after exposure to mirtazapine confirmed the initial assumption of a change in the gene expression rate of higher-level neurotransmitter systems.

These changes at the molecular level are reflected in behaviour and, as for most groups of organisms, have not yet been quantified in detail for *Daphnia magna*. Elucidation of the effects of mirtazapine on the gene regulatory level was carried out on a special set of genes, which are important molecular linkages to physiological reactions. While no clear dose-dependent trends in the induced gene expression of the serotonergic and dopaminergic system were observed with exposure to mirtazapine, there was still an induction observed at the tested concentrations. Corresponding cross-species studies show that the genes of superordinate control systems examined in the present study play a decisive role in neurotoxicological analysis (Fong, 2001; McDonald et al., 2017; Silva et al., 2012). The increased induction values for the different mirtazapine concentrations observed in the present study could also be found in humans and zebrafish (Stewart et al., 2013), and an overload of the neurotransmitter system (roughly comparable to the serotonin syndrome) can occur in *Daphnia magna*. Even if the observed concentration ranges differ due to different metabolisation rates, activity-increasing effects occur in most of the exposure groups of the study.

### 3.4.3 Reproduction and length measurement changes

The results of the reproduction studies show the expected effects on the number of offspring, but the ecological effects on the overall system cannot be fully assessed completely.

In addition to the specific consideration of the neurotoxicological effects of mirtazapine, the results of the *Daphnia magna* reproduction test show an increased stress level compared to the control group. Previous studies have observed similar increases in the number of offspring produced with exposure to other neuroactive chemicals, with stress levels being particularly evident through the observed abnormal occurrence and increased number of males produced (Heckmann et al., 2008b; LeBlanc and Medlock, 2015; Olmstead and LeBlanc, 2003). The long-term effects of these observations in the natural ecosystem need to be further investigated, especially with regard to the point exposures (Li, 2014; Ternes, 2001; Ternes and Joss, 2007; Zuccato et al., 2006). Due to the steadily increasing use and exposure concentrations of antidepressants and other pharmaceuticals in the environment, mirtazapine has been monitored in wastewater treatment plants for several years with concentrations detected in the range of 0.023 – 0.17 µg/L (Li et al., 2019b; Melchor-Martínez et al., 2020; Wu and Li, 2015).

Overall, the results from the present study demonstrate that behavioural effects are a more sensitive endpoint than morphological or traditional acute and chronic endpoints (e.g., growth, survival, and reproduction) for *Daphnia magna*. Effects on the swimming behaviour were observed at the lowest tested mirtazapine concentration (0.1 µg/L), which is ecologically relevant and was found within wastewater monitoring studies ranging from ng/L and µg/L depending on the respective investigation site (Golovko et al., 2014; Golovko et al., 2020).

## 3.5 Conclusions

As expected, based on the intended pharmacological use in human, an activity-enhancing effect was also observed in *Daphnia magna* with exposure to the NaSSA mirtazapine. Specifically, exposure to mirtazapine led to alterations in the behavioural and molecular endpoints in *Daphnia*. The threshold concentrations for the dual mode of action, as known in clinical use, could not be determined in the study, but the increased physiological activities show that there is an alteration of the neurotransmitter balance. The elucidation of the modes of action also suggests the involvement of higher-level control systems, but a more detailed elucidation of the link between physiological changes and molecular causes of action of mirtazapine in *Daphnia magna* is still required.

It was demonstrated in the present study that the 48-h acute *Daphnia* test was suitable to assess a sensitive endpoint such as swimming behaviour with exposure to a neuroactive substance like mirtazapine. No effects were observed on the reproduction or length of *Daphnia* with exposure to mirtazapine. However, a benefit to the use of swimming behaviour as an endpoint compared to traditional reproduction endpoints is that it can be performed in much less time and that reactions on the behavioural level occur at lower concentrations than morphological or reproductive changes. Further development of behavioural test systems with *Daphnia magna* should be analysed, that in the future it will be possible to examine more complex endpoints in much more detail. Behavioural observations also represent the whole organism, which directly indicate overall fitness of individuals and can easily be used in combination with molecular level analysis of individuals to examine mechanistic effects. Behavioural effects on a keystone species like *Daphnia* can have significant implications on the food web structure of aquatic ecosystems (Muylaert et al., 2006; Straile and Geller, 1998; Wojtal-Frankiewicz, 2012), and thus the protection of this group of organisms is essential. Furthermore, the effects observed in the current study may underestimate the impacts on *Daphnia* that are very likely to be exposed to mixtures of other neuroactive substances. In particular, the combination of neuroactive psychotropic drugs of different drug classes is currently being investigated in various studies with non-target organisms (Nowakowska et al., 2020).

Although no clear patterns in the gene expression were observed in the current study regarding the exposure concentrations, it was still observed that mirtazapine had significant alterations in the gene expression. These behavioural and molecular endpoints represent important observations that have not yet been fully evaluated, but which will become significantly more important in the future, particularly in the context of the discussion on pharmaceuticals. A focus on higher-level central control systems may be useful for future investigations. By integrating results on these class of potentially neuroactive substances on other non-target aquatic organisms, it should be possible to measure effects in the whole ecosystem in more detail. In conclusion, the studies provided important indications that behaviour as an endpoint is important and currently underestimated in the context of risk assessment (Ford et al., 2021). This assessment deficit needs to be closed because is an important step in the protection and future improvements of biodiversity.

## Acknowledgments

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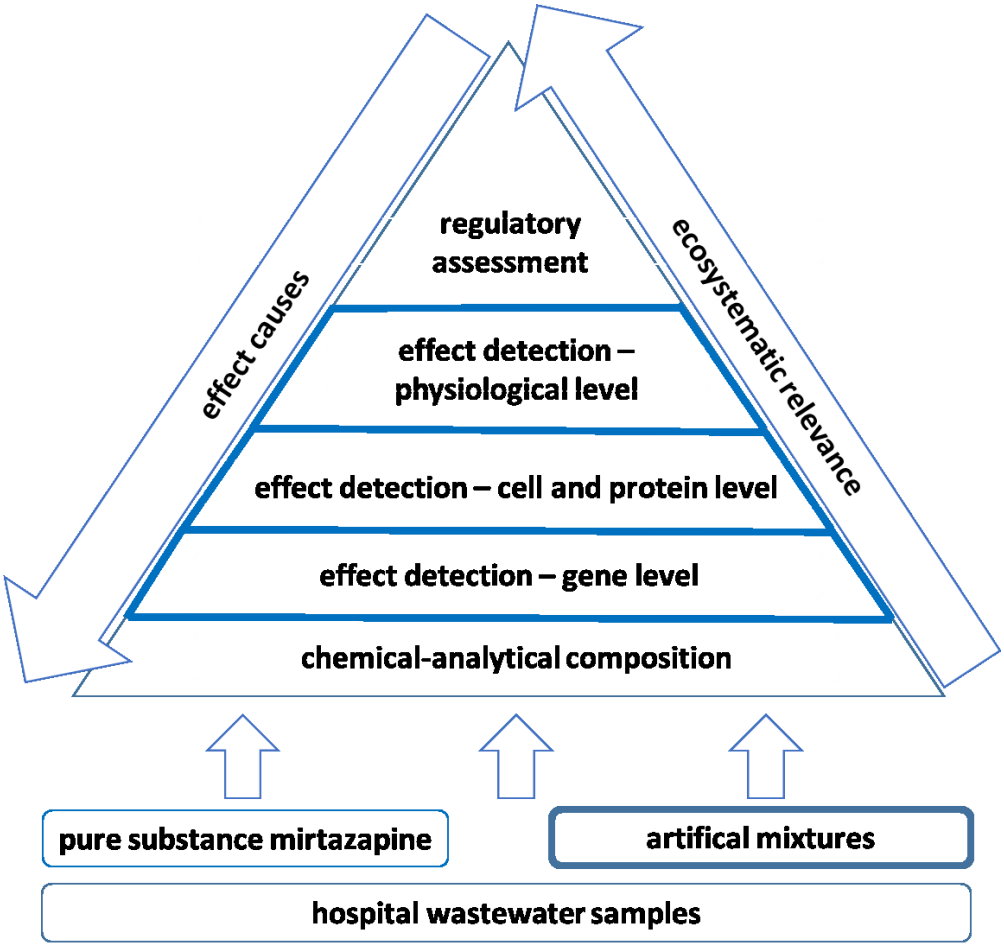
# Chapter 4

## Effects of artificially produced mixtures of psychotropic drugs on the swimming behaviour and gene expression rate of zebrafish embryos




Parts of this chapter will be developed into a manuscript to be published in a peer-reviewed journal.

Gundlach, M., Augustin, M., Paulzen, M., Hollert, H. (2021). Effects of artificially produced mixtures of psychotropic drugs on the swimming behaviour and gene expression rate of zebrafish embryos

## Abstract

Psychotropic drugs with different modes of action occur as complex mixtures in wastewater and have different combined effects on the behaviour of aquatic non-target organisms. A classification of the pure substances into different pharmaceutical groups is necessary for a target-specific analysis. This classification is also necessary for the discussion of behavioural changes caused by neuroactive substances and for the elucidation of the underlying molecular effect mechanisms. The predictions of combination effects are limited even if the reactions of the individual substances are known. An experimental determination of the effect strength, especially for complementary active substances, is useful. Therefore, the combined effects of mirtazapine (a noradrenergic and specifically serotonergic antidepressant), clozapine (an atypical neuroleptic), and duloxetine (a serotonin-noradrenalin reuptake inhibitor) were analysed with zebrafish embryos on behavioural and molecular level. In all cases, a sedative effect on the swimming behaviour was measured with a simultaneous increase in the expression rate of selected genes of the serotonergic and dopaminergic system at concentrations between 0.9 and 19 µg/L. The results indicate the presence of the serotonin syndrome, which occurs when the concentration of neurotransmitters in the CNS is increased. The results provide important information for the analysis of complex environmental mixed samples containing a predominant proportion of neuroactive psychotropic drugs.


## 4.1 Introduction

Neuroactive environmental wastewater samples do not consist of a single pure substance, but rather of a mixture of different pharmaceuticals with various modes of action. Through the selection of the study site, the number of existing active ingredient classes can already be significantly reduced, which simplifies the analysis of the determined effects (Ogungbemi et al., 2021; Pivetta et al., 2020). Another important step for a specific analysis of the neurotoxicological potential is the application of investigated methods that have already been used for single-substance analysis (Ogungbemi et al., 2021).

Often, the analysis is complicated by the structure of the urban wastewater pipes, as there is a collection of wastewater in one drainage system (Umweltbundesamt, 2017; Yang and Chen, 2021). As a result, a combination of different classes of neuroactive substances can get into the environment and pose a potential risk for the aquatic organisms which is higher than the risk of the single substances. In many cities, wastewater from different sources (private households, industry, medical

facilities) is combined and collected for purification in different wastewater treatment plants (Umweltbundesamt, 2017). For reactive substances, such as contrast agents, there are already special cleaning devices at the facilities, but this is not the case for many classes of micropollutants (Di Marcantonio et al., 2020). The efficiency of the treatment depends on the size as well as on the treatment capacity since in case of heavy rainfall events in smaller to medium-sized plants, wastewater discharges without any treatment processes occur more and more frequently (Umweltbundesamt, 2015). This leads to an untreated discharge of wastewater into the aquatic systems and pose a high risk to non-target organisms. The resulting combination effects do not only depend on the concentrations of the single substances but are essentially determined by the sensitivity of aquatic organisms towards the impact on different neurotransmitter systems. A decisive factor for the respective sensitivity is determined by the neuroactive substances and the metabolites. While organisms of a low biological level often take in their food via filtering, bioaccumulation of pollutants often occur due to the predatory lifestyle in organisms of higher biological levels (Dietrich et al., 2010; Fu et al., 2020; Wang et al., 2019).

In the medical application of pharmaceuticals, interactions between the different substance classes are known and different side effects between the substances are documented for each pharmaceutical (Predictable et al., 2006). This usually involves combining a psychotropic drug with pharmaceuticals used to treat other diseases, such as cardiovascular or endocrine disorders.

Even more difficult is the analysis of effects in aquatic non-target organisms due to a lack of neurotoxicological data. The combined effects of psychotropic drugs on physiological and behavioural level of zebrafish embryos have not yet been systematically analysed (Ford et al., 2021). The complex interaction of the various components of the nervous system can be seen from the synthesis of serotonin. *tph1a*, *tph1b,* and *tph2* are basic and important genes for the synthesis of serotonin (Maximino et al., 2013a; Stewart et al., 2010; Stewart et al., 2011). They have a key position in synthesizing the neurotransmitter from tryptophane. At the same time, the serotonergic system is an important linkage between ingested stimuli and physiological reaction (Maximino et al., 2013a). Changes caused by neuroactive substances are therefore particularly negative for the organism.

In most cases, the prediction of possible combination effects between different active substances follow a standardised set of models that assume a simple addition of the individual effects or predict a potentiation of the effects (Altenburger et al., 1996; Vijver et al., 2010). Previous studies with psychotropic drugs are mainly based on the investigation of the combined effect of a classic neuroactive substance with a substance of another class of active substances used in a simultaneous treatment (Siwek et al., 2020).

The side effects of many psychotropic drugs do not necessarily correspond to the summation of the effects, but usually are a mixture of different theories that can only be estimated experimentally (Ogungbemi et al., 2021). The effect strengths also depend on a genetic disposition (Propping and Friedl, 1979). Therefore, predictions for aquatic non-target organisms are difficult and a successful data discussion depend on a tiered testing approach with increasing complexity of the individual substances. At the same time, increasing complexity means increasing test costs (Roos et al., 2012; Schmitt et al., 2010; Siwek et al., 2020). The combination of behavioural tests with the knowledge of the underlying mechanisms of action is one of the most important aspects of future risk assessment (Ford). Thereby, an interpretation of the biological data would be possible for mixed wastewater samples, if enough biological and chemical data are available and if they could be combined. This allows the definition of effect thresholds and the determination of the risk (Kroes et al., 2005). The resulting database with various data sets from bioassay research already corresponds in essential features to the existing databases on the chemical analysis (Gaulton et al., 2012). They can thus also be used for the evaluation of data from different sampling points.

Current studies are using a comparative analysis approach for selected pharmaceuticals in contrast to single substance studies (Rodrigues et al., 2020). When applying this approach to neuroactive substances, it is necessary to include physiological changes, since many substances in this class of active ingredients are synthesized to alter behavioural responses.

The following study should help to close this gap for selected psychotropic drugs at different concentrations. Data from the medical treatment of patients helps with the interpretation of the underlying modes of action at least for a selection of substances. It is assumed that the combined effects distinguish to a simplified sum of the single effects. According to the findings from the combination studies in humans, an overload of the neurotransmitter systems are also assumed in zebrafish.

## 4.2 Material and Methods

### 4.2.1 Test pharmaceuticals

Mirtazapine ≥98% (HPLC) (1,2,3,4,10,14b-Hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c][2]benzazepine, C17H19N3, CAS Number: 85650-52-8), Duloxetine ≥98% (HPLC) ((γS)-N-Methyl-γ-(1-naphthalenyloxy)-2-thiophenepropanamine hydrochloride, (+)-(S)-N-Methyl-γ-(1-naphthyloxy)-2-thiophenepropylamine hydrochloride, (+)-N-Methyl-3-(1-naphthalenyloxy)-3-(2-thienyl)propanamine,

C18H19NOS, CAS Number: 136434-34-9) and Clozapine (8-Chlor-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4]-diazepin, C18H19ClN4, CAS Number: 5786-21-0) were ordered by Merck (Deisenhofen, Germany). The stock solutions were prepared with 8 mg/mL for Mirtazapine, 6 mg/mL for Duloxetine and 4.8 mg/mL for Clozapine in dimethyl sulfoxide (DMSO) as this solvent has already been previously used in different bioassay studies (Chen et al., 2017). Five different mixtures were prepared for the testing of the combination effects. 9.6 µg/mL Duloxetine with 6 µg/mL Mirtazapine (M1), 19.2 µg/ml Duloxetine with 0.9 µg/mL Clozapine (M2), 6 µg/mL Mirtazapine with 1.8 µg/mL Clozapine (M3), 9.6 µg/mL Duloxetine with 1.8 µg/mL Clozapine (M4) and 3 µg/mL Mirtazapine with 1.8 µg/mL Clozapine (M5).

### 4.2.2 Zebrafish maintenance and embryo exposure

According to predefined culture conditions, different breeding groups of wild type zebrafish were held in glass aquaria following the predefined culture conditions (Braunbeck et al., 2005; Hollert et al., 2003). The exposure of 20 eggs was initiated at the 16-cell stage in glass vessels (VWR, Germany) one-hour post fertilization. The total test time for the behavioural and molecular studies was 116 h and the test stopped 120 h after hatching. In accordance with the animal protection act, the individuals are not self-feeding organisms and the test could be carried out without an appropriate permission (Strähle et al., 2012). In total, a control group and 5 different exposure groups were tested at three replicates with 20 embryos each. Morphological changes (coagulation, odema, lack of heartbeat, and morphological malformations) were checked every 24 h according to the OECD guideline (OECD, 2013). The nominal exposure concentrations were selected according to a typical background environmental concentration based on a worst-case scenario (e.g. the discharge of untreated wastewater directly into the river). The mixtures were spiked in DMSO (Merck, Germany) to give a final concentration of 0.01 % DMSO. An exposure only with 0.01 % DMSO was used as a negative control and a positive control could not be prepared because the different behavioural endpoints without any standardised protocols made it impossible to find a suitable substance. First, neurophysiological reactions were measured following a 30 min locomotor light-dark routine behaviour assay and parts of the molecular modes of action were finally analysed using a real-time qPCR system (ThermoFisher Scientific, USA). After the behaviour test the embryos were euthanized with 0.4 g/L benzocaine ethanol solution and frozen at minus 80 °C.

### 4.2.3 Locomotor light-dark routine transition test

The swimming behaviour was measured with the locomotor assay with 116 hpf old embryos in a 96-well plate (StarLab, Germany) using the DanioVision™ video-track system (Noldus, Netherlands). The measurement followed an established protocol that was already used for the effect analysis of other micropollutants (Chen et al., 2017). The embryos were separated with 300 µl test exposure solution in the 96-well plate and incubated for 10 min in the dark at 26 °C to avoid stress effects. All tests were started in the morning at 08:00 a.m. and were finished before 10:00 a.m. to maintain the natural circadian cycle. The swimming distance was recorded by a video camera and was analysed after the end of the experiment. The detection period of 30 min was divided into 5 min section with light-dark cycles.

### 4.2.4 RNA Isolation, cDNA synthesis und quantitative real-time Polymerase chain reaction

The embryos of the behaviour test were euthanized and frozen. Finally, they were homogenized using a rod homogenizer (VWR, Germany) in 5 cycles of 30 seconds each and cooled on ice. RNA was isolated using the PureLink™ RNA Mini Kit (Invitrogen™, USA). For further quantification and storage, M-MLV reverse transcriptase (Invitrogen™, USA) was used for transcription into cDNA. The target primers (*slc17a6a, slc2a2, slc6a3a, eff1a*) were constructed and subsequently produced as a synthesis order by Eurofins Genomics (Ebersberg, Germany). The expression rate was quantified by measurement using PowerUP SYBR Green Master Mix (Thermo Fisher, USA) and the final calculation of the relative gene expression rate followed the protocol of Schmittgen and Livak (Schmittgen and Livak, 2008).

### 4.2.5 Statistical analysis

The data from the behavioural studies were checked for normality and homoscedasticity using a Kolmogorov-Smirnov and a Levene´s test. Finally, they were examined using a one-way analysis of variance (ANOVA) followed by a Turkey's test using the SPSS software package (version 26.0, IBM, USA). A statistical significance was assigned at $\alpha = 0.05$ and the data was illustrated using the GraphPad Prism program (version 8, GraphPad Software, USA). In addition, the dimensionality of the behaviour data was simplified using principal component analysis (PCA). The p-value was 0.05.

# 4.3 Results

## 4.3.1 Behaviour measurement

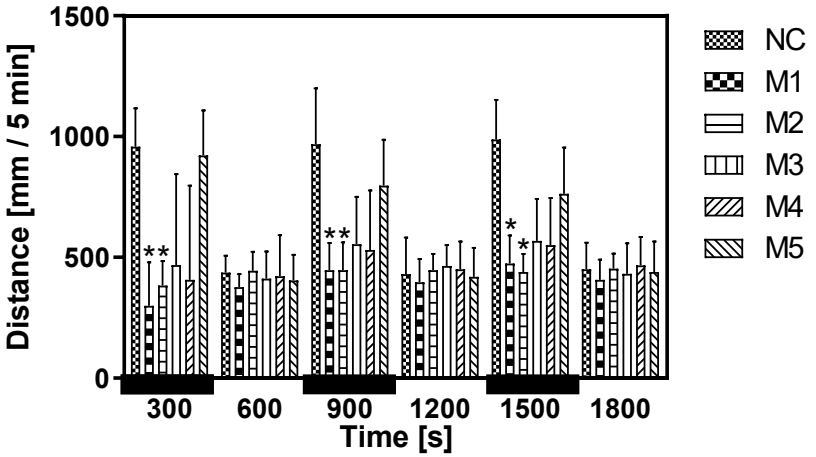



**Figure 4.1: Locomotor activity of zebrafish embryos** in three replicates with three 5 min cycles of light-dark stimulation after exposure to a control sample (NC) and five different exposure batches. 9.6 µg/mL Duloxetine with 6 µg/mL Mirtazapine (M1), 19.2 µg/ml Duloxetine with 0.9 µg/mL Clozapine (M2), 6 µg/mL Mirtazapine with 1.8 µg/mL Clozapine (M3), 9.6 µg/mL Duloxetine with 1.8 µg/mL Clozapine (M4) and 3 µg/mL Mirtazapine with 1.8 µg/mL Clozapine (M5). Each bar represents 3 replicates with 20 embryos and expresses the mean ± standard deviation. The black bars indicate the different dark phases, and an asterisk represents a significant difference ($p < 0.05$) to the control group.

Morphological changes could not be measured for any of the studied combinations.

The maximum value of the swimming distance could be measured in the unexposed embryos of the control group with an average of 990 ± 20 mm in 5 min (Figure 4.1). The artificial mixture M1 showed the strongest sedative effect with a decrease in swimming distance covered by more than 50 % in all three dark phases compared to the control group. Sedative effects in the swimming distance were also measured in the combined reaction mixtures of M2, M3, and M4. Compared to the results of the control group, the distance covered was 40 % below these values in all three dark cycles. The artificial mixture M5 was the only one that did not show any measurable deviations in the swimming distance compared to the control group.

## 4.3.2 Gene expression analysis

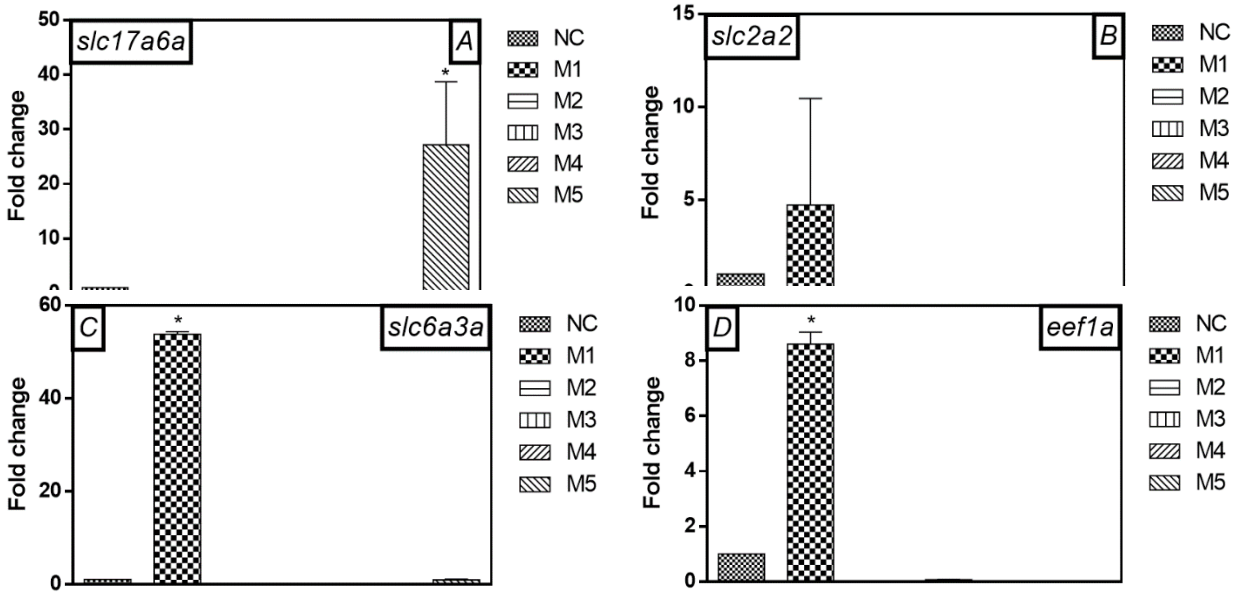



**Figure 4.2**: **Gene expression profiles for 116 hpf old zebrafish embryos after the exposure to different pharmaceutical combination batches.** (A) *slc17a6a*, (B) *slc2a2*, (C) *slc6a3a* and (D) *eef1a*. Each bar represents 3 replicates with 20 zebrafish embryos and expresses the average mean ± standard deviation. An asterisk represents a significant difference ($p < 0.05$) to the control group. The graphs show from left to right the control group (NC) and the five different exposure concentrations M1 to M5.

The values of the gene expression levels are shown for *slc17a6a* in Figure 4.2. A significantly increased expression rate could only be measured in the mixture combination M5.

The other combinations showed no measurable differences in comparison to the control group. For the genes *slc2a2* and *slc6a3a*, a significant increase in the expression level could be measured for the combined effect of mixture M1 (Figure 4.2).

For *slc6a3a* which belongs to the dopaminergic system, the Fold change rate exceeded 50 and was significantly higher than the value for *slc2a2*, which was less than five. It is a gene that is responsible for the development of the central brain structure. For the elongation factor 1-alpha (*eef1a*), an increased gene expression rate with a fold change of more than 8 could be measured for the combination exposure M1 (Figure 4.2).

## 4.4 Discussion

The measurement of behavioural changes in *Danio rerio* embryos, when exposed to an artificial mixture of human psychotropic drugs, has not yet been extensively analysed and only a few data sets are available for acute tests (Cleuvers, 2003; Cleuvers, 2004; Ogungbemi et al., 2021). Possible reasons are the large number of different substance classes used in the treatment of disease patterns, as well as a lack of reliable data on the actual elimination of these substances in sewage treatment plants (Rogowska et al., 2020).

The results of the combination studies show a clear potentiation of the individual physiological effects in some combinations, with the result of an overloading of the entire neurotransmitter system. From a medical point of view, all used drugs are designed to trigger changes in the neurotransmitter balance in the central nervous system and thus leading to changes in behaviour (Holsboer et al., 2008). However, some of the drugs have contrary effects. The results show that some of the pharmaceuticals must have a strong concentration-dependent effect, which increases activity at low concentrations and reduces activity at higher concentrations (Anttila and Leinonen, 2001).

A collection of possible combination reactions of the individual substances when used in humans is compiled in the pharmaceutical index of the yellow list (Jurasovic and Bouvier, 2020). The information in this list are reviewed and provides important information on possible cross-reactions in human. This information can also be used for the interpretation of behavioural data and molecular causes of action due to the close homologies between humans and zebrafish.

In the single-substance studies in zebrafish embryos, a dual-mode of action was identified for mirtazapine and clozapine, which is used in medical disease treatment in humans (Gillman, 2006; Kremer et al., 2018). For mirtazapine, a sedative effect was measured in 116 h old zebrafish embryos in a low concentration range of up to ng/L, and an increase in activity was measured from concentrations of µg/L range. This property is already known for different low-molecular-weight active substances and thus indicates, in addition to the factor of the timespan for exposure, that the actual active concentration in the zebrafish also plays a decisive role in the effect (Bruni et al., 2016). In clinical use, mirtazapine is administered the day before as a sleep agent and has an activity-increasing effect when the plasma concentration is altered the following day (Anttila and Leinonen, 2001). These activity-increasing observations at low µg/L and sedative effects at high concentrations from ng/L could already be measured in some preliminary studies (Gundlach et al., 2021).

The combined effect of mirtazapine and duloxetine or in combination with the atypical neuroleptic clozapine, which occupies different neurotransmitter systems, can therefore only be predicted with

inaccuracies. The actual concentrations in the central nervous system are difficult to estimate, but the results of an exclusively sedative effect of the combination effects suggest that it is an overload of the different neurotransmitter systems (Bachour et al., 2020). Particularly affected by such an overload is the serotonin system, which has also been described in earlier studies in zebrafish as the serotonergic syndrome (Stewart et al., 2013). This is a serious dysfunction of the serotonin system in both zebrafish and humans. Previous studies have shown that the development of this disease pattern is mainly due to the combined administration of an antidepressant and an MAO inhibitor, which leads to an accumulation of the neurotransmitter in the synaptic cleft (Sallinen et al., 2009).

At the gene regulatory level, a selection of genes was chosen that provide information about higher-level neurotransmitter systems with a key position between physiological reaction and molecular modes of action (Maximino et al., 2013a; Wong et al., 2013). The results of previous studies which are summarised in the database of the National Centre for Biotechnology Information (NCBI) listed more than 1000 serotonin genes, more than 500 dopamine genes, and more than 200 genes related to the histamine system in *Danio rerio*. Every gene has an important regulatory function in the CNS and is involved in stimulus processing. The high activity of the mixture sample M1 provides important evidence that the physiological reactions are an overload of the neurotransmitter systems and thus symptoms of the serotonin syndrome. Both drugs have stimulant properties and share some of the same target receptors in the CNS (Ablain and Zon, 2013). Previous studies have demonstrated that different combinations at the molecular level led to the same physiological reactions (Egan et al., 2009; Jesuthasan, 2012; Oggier et al., 2010). This is used in medicine for patients with intolerances to specific substances.

The study results of the artificially produced mixtures show an overload of the serotonin system in some combination reactions. The interpretation of complex mixture data is not entirely possible without a simplification of the test design. The cross-reactions that occur between the substances are complex and cannot be predicted with previous mixture models. In particular, the combination of active substances with contrary mechanisms of action lead to unpredictable effects in aquatic non-target organisms. Since risk assessment is carried out continuously for pure substances but not for mixtures, there is a risk assessment gap that has not yet been sufficiently closed yet. In the current discussion on environmental protection, the demand for a more targeted use of behavioural tests and the elucidation of the underlying molecular mechanisms of action are an important step. There is still a lot of research work to be done, especially for neuroactive psychotropic drugs for the correct discussion of the effect data on behavioural and molecular level.

## 4.5 Conclusions

The conducted studies on the combined effect of mirtazapine, duloxetine, and clozapine provided important insights into the sedative effect of all substances in combination, as well as the serotonin syndrome that occurs. The results were confirmed by the increased induction values of serotonin-associated channel and receptor genes. It could be seen on the gene level that the organism reacts to the increased neurotransmitter concentrations with an increased expression rate.

The results have shown that a comparison of the effect strength with single-substance reactions is only possible in individual cases and that combination effects cannot be estimated completely. For future studies, other neuroactive substances should therefore be investigated in their combined effect, so that the evaluation of the physiological effects of a native environmental sample could be determined and the molecular modes of action could be analysed accurately. The procedure and the results of the investigations carried out within the framework of the study show a possibility for the implementation and analysis of these test procedures, which have not yet been validated with standard test methods. The inclusion of behavioural and gene expression data in risk assessment of neuroactive substances represents an important step for the future handling of these substances. Especially in the context of current discussions on the preservation of biodiversity in the environment, new insights into the actual hazards must be generated through combination studies with different substance classes.

## Acknowledgements

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# Chapter 5

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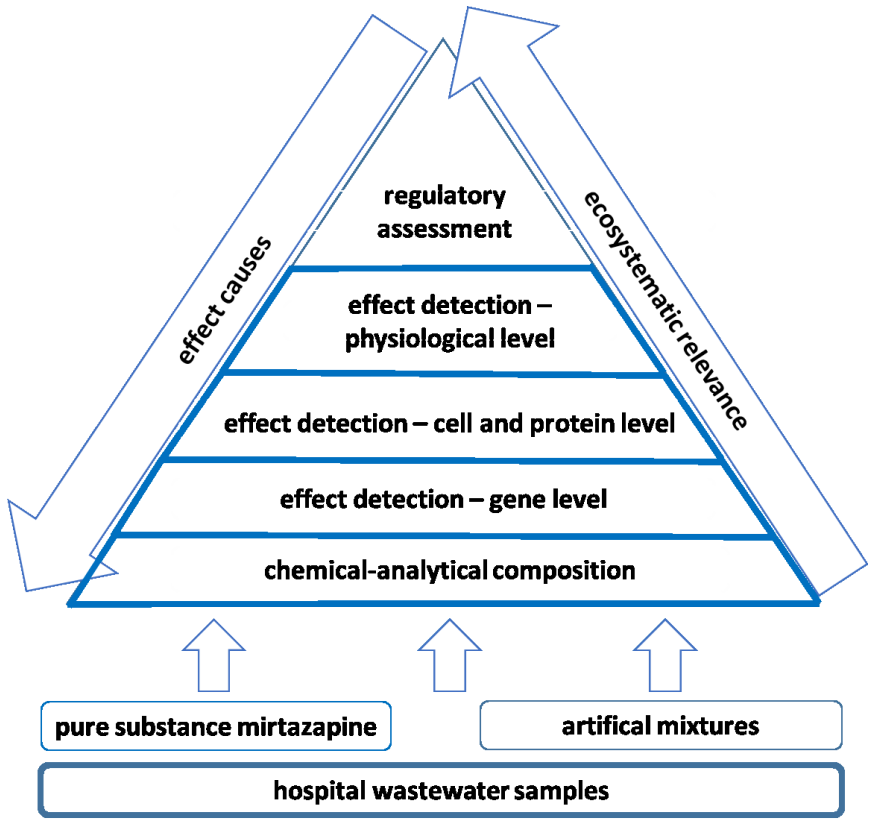
# Effects of neuroactive wastewater samples of a psychiatric hospital on *Danio rerio* embryos - the involuntary treatment of aquatic non-target organisms




This chapter is based on the following manuscript to be submitted for publication in an international journal as: Gundlach, M., Augustin, M., Finckh, S., Krauss, M., Roß-Nickoll, M., Brack, W., Linnemann, V., Paulzen, M., Kiessling, F., Hollert, H. (2021) Exposition of *Danio rerio* embryos to a neuroactive wastewater sample – Influence on behaviour and gene expression rate. To be submitted to publication in Water Research.

## Abstract

The constantly increasing number of patients with psychological disorders in hospitals lead to an increasing recovery rate of neuroactive psychotropic drugs in aquatic ecosystems. The analysis of complex untreated wastewater samples containing substances with different mechanisms of action pose new challenges in determining the risk potential of this mix for the environment. Previous validated bioassays, which are useful in assessing lethal and sub-lethal effects, mostly at the morphological level, have only limited applicability for assessing neurotoxicological effects. Studies with *Danio rerio* show that physiological and molecular endpoints could be analysed and used for the evaluation of the risk potential of substance classes, which primarily show a reaction in the nervous system. Untreated hospital wastewater was used as a point source for the discharge of neuroactive pharmaceuticals and was tested using a test battery consisting of medical, analytical and ecotoxicological data.

Distributed over different sampling times, various compositions of sedative and activity-enhancing neuroactive substances led to sedative physiological behavioural responses with activity changes of more than 20 % compared to control groups. Differences at the molecular level could primarily be attributed to changes of genes of superordinate control systems of the serotonergic and dopaminergic system with Fold changes more than 15 times higher than the control group for *slc2a2*, *slc6a3a* and *eef1a*. In addition to behavioural changes and parts of the underlying molecular modes of action, a chemical residue analysis, a quantification of the activity of acetylcholinesterase activity, and an antibody staining were also measured. In the analytical measurement, most of the substances used could be recovered in concentrations of 1.0 – 5.0 µg/L. At the cell and protein level, increases in activity could be measured for samples that have shown behavioural alterations. Therefore, it could be concluded, that these substances pose a risk to aquatic non-target organisms, especially in their mixture effects, which has not yet been fully considered in the legal environmental risk assessment.


## 5.1 Introduction

The amount of patients with major depressive disorders have overtaken a rate of more than 20 % of all diseases in the EU due to constantly increasing challenges in everyday professional and private life (WHO, 2021). Increasing challenges at the limits of mental capacity led to a steadily increasing number of patients who need a psychological therapy. In most cases this treatment, which at least in main parts takes place in hospital, is combined with the administration of neuroactive psychotropic drugs (M.E.P.S., 2017). These substances finally end up metabolized or as parent substances in the aquatic

environment (Santos et al., 2020). Current studies show the possibility of the quantitative detection of more than 420 chemicals in the environment, and for 65 % the underlying molecular mechanisms of action are known (Busch et al., 2016). Furthermore, 13 % of this substances with known molecular modes of action, are substances with a neuroactive potential (Busch et al., 2016). This detection results underline the importance of a detailed risk assessment for neuroactive substances in the environment but only limited information are available on the effects in non-target organisms. In this context, hospitals are point sources for neuroactive substances because they are central facilities for the treatment of patients with mental disorders (Thomas et al., 2007). The effluent quantities show significant differences between the various facilities, which is the result of different occupancy levels during the year (Kot-Wasik et al., 2016). In particular, an increased number of patients with mental disorders are treated in autumn and winter, which means that also an increased number of neuroactive substances enter the municipal wastewater treatment plants (Mehdi et al., 2021; Singh et al., 2005). Current studies on modernization of existing facilities with additional purification stages show improvements in the removal rates of micropollutants which include the elimination of neuroactive substances. Nevertheless, there are currently no sufficient information about the real elimination rates of these substances in the facilities, and for many substance groups there are less detailed information about the effective concentrations for morphological, physiological and molecular changes in aquatic non-target organisms (Kienle et al., 2019; Loos et al., 2013; Sacher et al., 2008; Schwarzbauer and Heim, 2005). In the context of the current discussions about the impact of climate change on different facilities, especially capacity difficulties of small regional wastewater treatment plants after extreme weather events will become more and more important soon. In order to protect these facilities against an overloading and a following flashover of the treatment plant, the unfiltered wastewater will directly be discharged into the following aquatic systems. A major group of substances that have various negative consequences with sub-lethal criteria for the aquatic environment are neuroactive pharmaceuticals (Rivetti et al., 2016).

In general, neuroactive pharmaceuticals are an inhomogeneous group of substances with different mechanisms of action within the group of organic micropollutants. They can interact with each other, and the effects are contrary to the objectives of the Water Framework Directive (WFD). The WFD (Directive 2000/60/EC) requires a good ecological and chemical status for aquatic systems. The aims of this guideline are not achieved for most of the monitored ecosystems because a complete chemical-analytical surveillance cannot be carried out at all sampling sites. In addition, many biological effects on aquatic non-target organisms have not been described in detail yet. That also means that a complete risk assessment cannot be carried out for most of the neuroactive substances (Carvalho et al., 2019; Kallis and Butler, 2001). Neuroactive pharmaceuticals like antidepressants are tested in standard procedures for their acute and chronic effects using OECD-validated standard tests. In

contrast, behavioural effects that occur at much lower concentrations in the ng/L range are not tested generally (Ford et al., 2021). But even for substance groups with physiological effect data, the elucidation of the underlying molecular modes of action have different challenges such as the lack of comparability due to different test protocols (Götz and Walz, 2017). In general, an intelligent study design can help to simplify the evaluation and develop the interpretation of the bioassay results. The selected study site offers only a limited number of possible substances in the water samples and most of them are neuroactive substances. The subsequent bioassay battery consists of chemical-analytical detection methods and bioassays on physiological and molecular level with the experimental organism *Danio rerio* and allow a better interpretation and consolidation of the available data. This concept has already been successfully investigated in parts for other substance classes like pesticides.

Choosing a hospital section with patients that have mental diseases is one advantage of the study site. It reduced the amount of potentially recovered substances in the wastewater and increase the available information about the administered drugs. That means that information on possible molecular modes of actions from humans are partly known which could be used for the study design of suitable primer and for the final effect discussion.

*Danio rerio* embryos which have already been successfully used in ecotoxicological research for the effect analysis of different substances and is also a suitable model organism for the effect analysis of neuroactive samples (Nowakowska et al., 2020) The high number of transparent eggs and the complete sequenced genome are only two advantages of this commonly used model organism for effect evaluation (Parng et al., 2007; Ton et al., 2006). By quantifying physiological responses such as the swimming distance, it is possible to investigate a more suitable endpoint that is influenced by neuroactive substances than morphological changes. Visible physiological reactions to an environmental stimulus are key events for the analysis of the neurotoxicological potential of the environmental sample and are important for the correct decision which genes and proteins are the main driver for the reaction. Despite obvious differences in the physiology and phenotype of zebrafish and human, zebrafish complement murine animal models to study genetically tractable human diseases (Lieschke and Currie, 2007). A primary reason for prescribing psychotropic drugs in medical treatment is the manipulation of physiological processes that usually accompanied by changes of the patient's behaviour. The analysis helps to elucidate the underlying molecular modes of action by quantitative gene expression studies (Beliaeva et al., 2010; Dai et al., 2014).

The cross-sectional information on the molecular effect causes in humans, are an important information source that can also be used for the result discussion of effects with zebrafish embryos (Choi et al., 2021). The medical classification of neuroactive pharmaceuticals, which is already commonly used in medicine can be used for the application. In contrast to other substance classes, the

investigation of the chemical structure of psychotropic drugs only allows very limited information about the effects at the molecular and, in particular, physiological level (Gago-Ferrero et al., 2020). The complexity of behaviour formation is the result of the interaction between different sub-processes. The synapse is the most important morphological part of the CNS with target receptors for neuroactive substances (Rihel and Schier, 2012). This structure can be identified in all organisms with a chemical information transmission, which means that different organism groups can be affected by an exposure. Furthermore, with a special view on behaviour as the final visible endpoint, it is linked to success in reproduction and defence against predators. That means, that even small changes in the movement sequences can lead to changes of the population structure (da Silva Santos et al., 2018; Dodson and Hanazato, 1995; Ford et al., 2021). The development of a combined test battery for neuroactive substances using zebrafish embryos represents a first step in effect analysis, which must be refined in the future with help of other aquatic organisms. Previous studies with *Daphnia* have shown that organisms of lower trophic levels also show physiological responses to exposure to these pharmaceutical groups and possible holistic effects at the population level cannot be assessed yet (Bownik, 2017). A particular challenge is the analysis of complex environmental samples with complementary active components (Rodrigues et al., 2020).

The results of previous studies showed that the detailed analysis of behavioural data is an essential step in the assessment of the environmental risk potential of neuroactive substances. Standard test systems are only insufficiently suitable for this detection, as they have been optimised for the measurement of acute and chronic effects (Cunha et al., 2019).

Real-time qPCR has proven to be universally applicable and significantly more cost-effective compared to large-scale genome analysis (Piña et al., 2007). This method provides a useful overview of possible changes at the molecular level and has already led to a clear link between behavioural data and molecular causes in various single-substance studies with focus on a specific gene region (Franco et al., 2019; Rodrigues et al., 2020). Nevertheless, this method skips the cellular level. For this purpose, various methods of tissue section analysis have developed in the last decades.

These methods are developed and used in clinical diagnostics for many decades and for different types of tissues which means that the protocols can be used for the tissue analysis of other species (Blake et al., 2003; Burns, 2006). The strong connections between morphological composition and physiological reactions in the central nervous system are difficult to quantify and histological studies can only represent a single moment image. Nevertheless, molecular histological methods can be used to derive important findings based on an interaction between antibodies. It is possible to identify CNS regions with high activities by histological methods by the analysis of different single-moment recordings

(Copper et al., 2018). This is possible because the more tissue is affected by a substance the stronger is the following reaction.

These antibody staining methods are suitable for effect analysis of psychotropic drugs in aquatic non-target organisms and have been successfully used for different drug classes in the detection of effects at the cellular level (Ishchenko et al., 2017). In humans, antibody staining identified various neurotoxicological disease patterns at cellular level which are important also for a cross-species analysis with aquatic non-target organisms.

It is assumed that the physiological reactions in correlation to the activity-increasing and sedative effects known from humans are detectable in zebrafish embryos. In addition, the molecular mechanisms of action should also be comparable with the known information in humans for the higher-level neuronal control systems. A comparison between different native wastewater samples with variable composition of neuroactive substances should show a higher activity of the serotonergic system at molecular and cellular level. Especially for samples with high concentrations of specific neuroactive pharmaceuticals or with a high composition of substances with different modes of action the cellular causes for behavioural alterations should be identified. The primary objective of this study is to develop a suitable test battery of chemical-analytical and ecotoxicological test methods with special focus on neuroactive pharmaceuticals. The developed experimental setup at a specific selected study site will be discussed with special focus on the combination of different information sources. A particular focus will be on the assessment of the available medical information on the application amounts and the modes of action in humans in combination with the chemical-analytical recovery and the bioassay results. In this context, the study is designed to present more detailed physiological and molecular effect data in contrast to currently available standard test systems. This should help to validate new test procedures for the risk assessment of environmental relevant neuroactive mixture sample.

## 5.2 Material and Methods

### 5.2.1 Sampling and preparation

Samples were collected at the modular building of the Department of Psychiatry, Psychotherapy and Psychosomatics of the RWTH Aachen University hospital on 06.01.2020 at hourly sampling times from 10:20 am to 2:30 pm (named T1 to T5). A pooled sample was mixed from all subsamples (T1 to T5) in equal proportions bevor the extraction process. In each case, two litres of native wastewater sample were sampled and stored in aluminium bottles (VWR, Germany) at minus 20 °C. 500 mL sample was

concentrated on an HLB column (VWR, Germany) and eluted in a defined volume of 2 mL methanol (Merck, Germany). Half of this sample was transferred to the Centre for Environmental Research Leipzig-Halle (UFZ) in methanol for chemical analysis. The remaining sample was evaporated and dissolved in one millilitre DMSO (Merck, Germany) for bioassay testing as this solvent has already been previously used in different bioassay studies (Chen et al., 2017).

### 5.2.2 Prescription details

This single building of the psychiatric clinic has three open stations with a regular capacity of 57 patients. The wastewater is disposed by patients, staff, and a limited number of visitors. For the sampling day (January 6, 2020) as well as for the two days before (January 4, 2020 and January 5, 2020), the anonymized drug quantities of all patients of the clinic was retrieved. A chart of the administered medications is provided in table Annex 5.

### 5.2.3 Chemical analysis

The chemical analysis was conducted by Saskia Finckh, Martin Krauss and Werner Brack at the Helmholtz Centre for Environmental Research – UFZ, Department of Effect-Directed Analysis. The samples were analysed by chemical target screening of 493 compounds by liquid chromatography high resolution mass spectrometry (Thermo Ultimate 3000 LC coupled to a Thermo QExactive Plus MS). For the analysis two dilutions were prepared: A first dilution of REF 40 from 10 µL sample extract (REF 250) and 52.5 µL methanol, and a second dilution of REF 0.8 from 10 µL pre-dilution (REF 40) and 490 µL of methanol. For the measurement, 1 mL of ultrapure water was then transferred into a 2 mL autosampler vial (Phenomenex VEREX) and mixed with 25 µL of each dilution (giving two sample aliquots of REF 1 and REF 0.02), 25 µL of an internal standard mixture (containing 40 isotope-labelled compounds, 40 ng/mL in methanol) and 10 µL of a 2M ammonium formate buffer (pH 3.5). In the same way, calibration standards containing the target substances at different concentrations (1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/L) were prepared, using 1 mL of water from a pristine stream and 25 µL of the respective target stock solutions instead of the diluted sample extracts.

After direct injection of 100 µL, compounds were separated using a Kinetex C18 EVO column (50×2.1 mm, 2.6 µm particle size, Phenomenex, pre-column 4x2.1 mm and in-line filter 0.2 µm) and a gradient elution with 0.1% of formic acid and methanol containing 0.1% of formic acid at a flow rate of 300 µL/min. The LC-HRMS raw data was further analysed using the software MZmine (Villar-Briones) (peak detection and annotation) and an in-house R-script (quantification). In addition, chemicals from the

medication list were rechecked using the vendor software TraceFinder 4.1 (Thermo). Both the LC-HRMS analysis and the data processing steps performed are based on the method described in detail in Neale et al. (Neale et al., 2020). Compound concentrations in the sample aliquots of REF 1 which exceeded the upped calibration limit for more than 20% were verified in the diluted sample aliquot of REF 0.02.

### 5.2.4 Zebrafish maintenance and embryo exposure

Wild type zebrafish (West Aquarium GmbH, Bad Lauterbach) were held in different breeding groups. Exposure was performed with fertilized eggs one-hour hpf with 20 eggs per exposure group in a 16-cells stage in glass vessels (VWR, Germany). The maximum exposure time was 116 h and the test stopped at 120 hpf. That means that the test could be performed without a permission for an animal experiment because the organisms were self-feeding (Strähle et al., 2012). The sample extracts were diluted in the glass dishes to the original environmental concentration in artificial water. The medium was enriched with different salts ($CaCl_2$, $MgSO_4$, $NaHCO_3$, KCL), which provides optimal basic test conditions for the zebrafish eggs. In contrast, the use of untreated wastewater was not possible because of the low oxygen concentration of the subsamples. The embryos were exposed for 116 h at 26 ± 0.5 °C.

Morphological changes were observed every 24 h according to the OECD guideline protocol (OECD, 2013). Lethal endpoints (e.g., coagulation and lack of heartbeat) and sublethal endpoints (e.g. odema) were documented. At the end of the exposure period of 116 h, larvae were separated in a 96-well plate (StarLab, Germany) and transferred for behavioural measurement. The samples were spiked in DMSO with a final concentration of 0.01 % DMSO. Therefore, a negative control without sample but with 0.01 % DMSO (Merck, Germany) was prepared and measured in every replicate. After performing the behavioural test, the embryos were euthanized with 0.4 g/L benzocaine ethanol solution (Merck, Germany), frozen in liquid nitrogen and stored at minus 80 °C.

### 5.2.5 Locomotor light-dark routine behaviour assay

Behavioural changes were measured with the locomotor assay in three replicates after a 116 h exposure period with 20 individuals per exposure group. The embryos were transferred with 350 µl exposure medium to a 96-well plate for recording the movement data and the swimming distance of each individual embryo over a total test period of 30 min using the DanioVision™ video-track system

(Noldus, Netherlands). Following the protocol of Chen 2017, the embryos were tested with an alternating light-dark rhythm, but in contrast to the protocol, the total test time was reduced to half an hour and to 5 minutes of the individual light and dark phases (Chen et al., 2017). The embryos were first acclimatized in the dark for 10 min to avoid falsification of the experimental results. The test temperature of the water was constantly regulated to 26 °C ± 1 °C to exclude possible temperature effects.

### 5.2.6 RNA isolation and quantitative real-time polymerase chain reaction

In preparation for testing molecular changes on gene level, RNA from 20 zebrafish embryos in three replicates was isolated with a PureLink™ RNA Mini Kit (Invitrogen™, USA) and then transcribed into more stable cDNA following the protocol of Bräunig and colleagues, 2015 (Bräunig et al., 2015) . The total RNA concentration was measured using a Biodrop Station (Biochrom, Germany) and then transcribed into more stable cDNA with a M-MLV Reverse Transcriptase (Invitrogen™, USA). For the following main expression analyses, a cDNA amount of 200 µg/L and a purification factor of 2 was used. Finally, the gene expression levels for selected genes were determined using the PowerUP SYBR Green Master Mix (ThermoFisher Scientific, USA) and then analysed following the protocol of Livak and Schmittgen (Livak and Schmittgen, 2001). The required primers (*slc6a3a, slc12a5b*, *slc2a2*, *eef1a*, *cyp1a, cyp2a*) were ordered from Eurofins genomics (Eurofins, Germany). The main studies were carried out using a qPCR-system (ThermoFisher Scientific, USA).

### 5.2.7 AChE activity measurement

The activity of the acetylcholinesterase (AChE) was measured for all samples following the protocol of Ellman 1961 (Ellman et al., 1961). First, one millilitre of sample was frozen during the RNA extraction process for minimizing protein degradation. The protein concentration was measured before the experiment started and no further purification steps were performed. The protein activity after addition of acetylcholine iodide (Merck, USA) was photometrically determined at a wavelength of 412 nm using the Cytation5 Multi-mode Reader (BioTek, USA). The activity was calculated as enzyme units (U) per mg of protein.

### 5.2.8 Microtome slices and fixation

In total 15 embryos of each exposure group and the control group were exposed and processed for the following histological steps. Embryos were exposed to environmental wastewater samples (T1 to T5 and Mix) for 116 h in glass dishes at 26 ± 0.5 °C. The concentrations corresponded to the actual environmental concentrations for the sampling times 10:20 to 14:20, analogous to the behavioural studies. The embryos were euthanized and frozen at minus 80 degrees Celsius. They were embedded horizontally in Tissue Tek (VWR, Germany) in the centre of a cube. The slice thickness of the section cuts with a microtome was 50 µm. Finally, the tissues were mounted on slides and stored at minus 20 degrees Celsius. The tissues were fixed with an 80 % methanol solution followed by a 20 % acetone solution. The process was performed in a glass chamber where the slides could be clamped individually and fixed for 10 min. Finally, the slides were cleaned in three repetitions of 10 min each in PBS and incubated with BSA for 30 min at room temperature for the antibody staining.

### 5.2.9 Antibody staining and microscopy

The slides were washed in PBS for 10 min and dried before the two steps application with primary and secondary antibodies at room temperature begin. The incubation with the primary antibody (anti-serotonin (5-HT) transporter (602-622) Rabbit pAb (Merck, Germany)), and with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, CAS: 28718-90-3) was performed for 2 h. After a short purification step in PBS, tissues were stained with the secondary antibody for 45 min. The secondary antibody was antigen: IgG (H+L), Alexa Fluor 488, Donkey (CAS:712-546-153). The analysis of the slides was carried out on the fluorescence microscope axioscope 5 (Zeiss, Germany) at a wavelength of 568 nm (green) and 480 nm (blue) and an exposure time of 2 s. The quantification of the results was done with ImageJ 1.53 (National Institutes of Health, USA).

### 5.2.10 Statistical analysis

Statistics were performed for all test systems using the SPSS software package (version 26.0., IBM, USA) and then visualized using GraphPad Prism (version 8, GraphPad Software, USA).

Behavioural data were checked for normality and homoscedasticity using the Kolmogorov-Smirnov and Levene´s test. Then, they were examined with a one-way analysis of variance (ANOVA). A Turkey's test was then applied to quantify possible effects. A statistical significance was assigned at $\alpha = 0.05$

and the p-value was 0.05. In addition, the dimensionality of the behavioural data was simplified using principal component analysis (PCA).

## 5.3 Results

### 5.3.1 Chemical data

The results of the chemical analysis (Figure 5.1) show with special focus on neuroactive pharmaceuticals, that these substances can be divided into different classes, which differ in their molecular mechanisms of action. The classification is based on the medically used definition of the drug classes. The following descriptions refer to samples with an increased concentration of psychotropic drugs. The subdivision according to the molecular modes of action showed for citalopram a detected concentration of 1.92 mg/L in sample T1, and in the mixture sample a concentration of 0.74 mg/L. Fluvoxamine was detected as a neuroactive pharmaceutical with an influence on serotonin transporters with a concentration of 1.23 mg/L in sample T3, 0.17 mg/L in T5 and 4.05 mg/L in the mixture sample. As an antagonistic substance towards the serotonin transporter, mirtazapine could be detected with a concentration of 1.05 mg/L in T1, 0.61 mg/L in T4, 1.71 mg/L in T5 and 1.06 mg/L in the mixture sample. In addition, melperone also shows antagonistic properties towards serotonin transporters in T1 with 0.54 mg/L, T3 with 0.33 mg/L, T5 with 2.26 mg/L and in the mixture sample with 0.66 mg/L. Closely linked to the serotonergic system, it was possible to show an influence of some neuroactive psychotropic drugs on norepinephrine transporters. As an atypical psychotropic drug acting antagonistically to the norepinephrine transporters, clozapine could be detected in sample T1 with 1.06 mg/L, sample T2 with 9.94 mg/L and in the mixture sample with a concentration of 0.93 mg/L. As reuptake inhibitors and thus activity-increasing substances of the norepinephrine system, first amitriptyline could be detected in sample T4 with 2.19 mg/L and in the mixture sample with 4.05 mg/L. Second, bupropion could be measured in sample T3 with 4.14 mg/L, T4 with 2.98 mg/L, T5 with 6.47 mg/L and in the mixture sample with 4.88 mg/L.

The chemical-analytical data focused primarily on neuroactive psychotropic drugs, but other substances were detected such as beta-blocker. The highest concentrations of non-neuroactive pharmaceuticals of all sampling times were found for blood pressure medications. However, various painkillers and contraceptives were also detected in some samples.

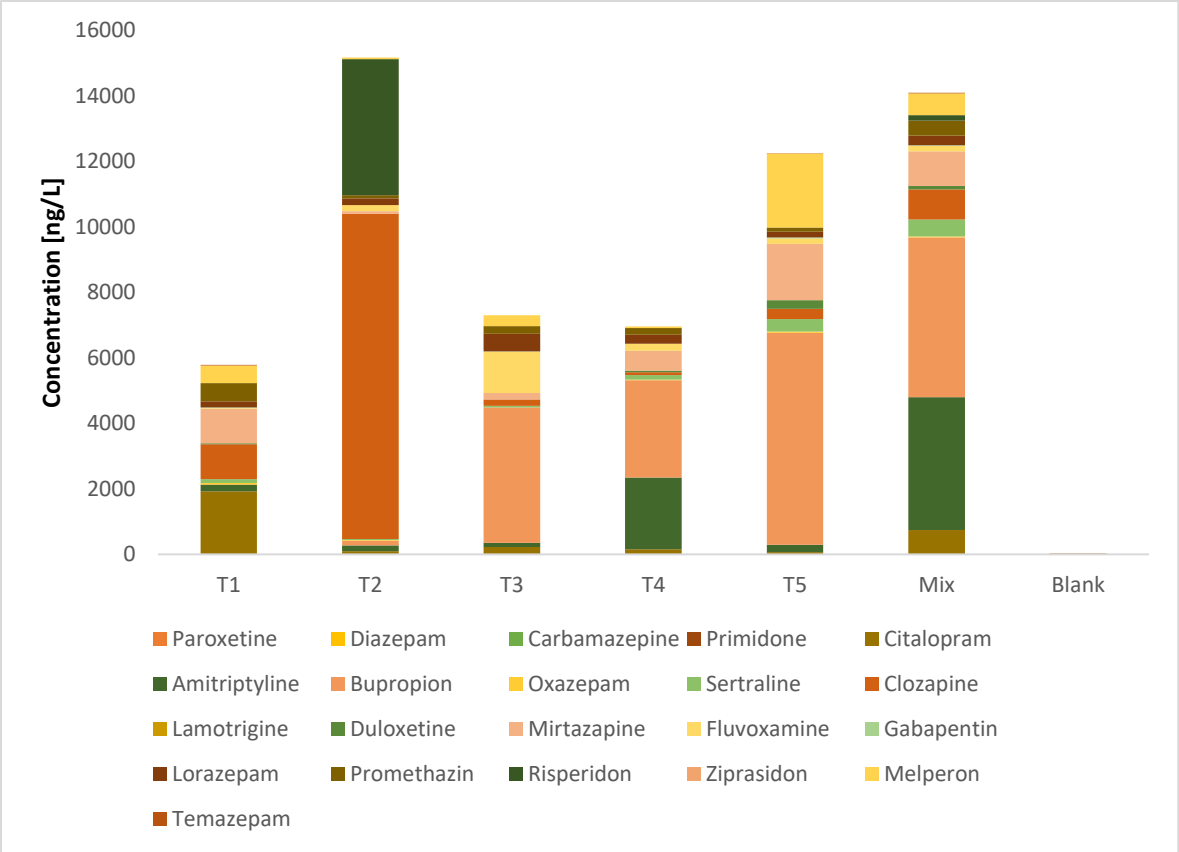



**Figure 5.1: Data of the chemical analysis.** The stacked barplot shows the summed concentrations for all time points for the original environmental concentration without dilution steps. The graph shows the recovery of psychotropic drugs and selective $\beta_1$ - adrenoreceptor blockers. Concentrations are given in ng/L.

## 5.3.2 Behavioural results

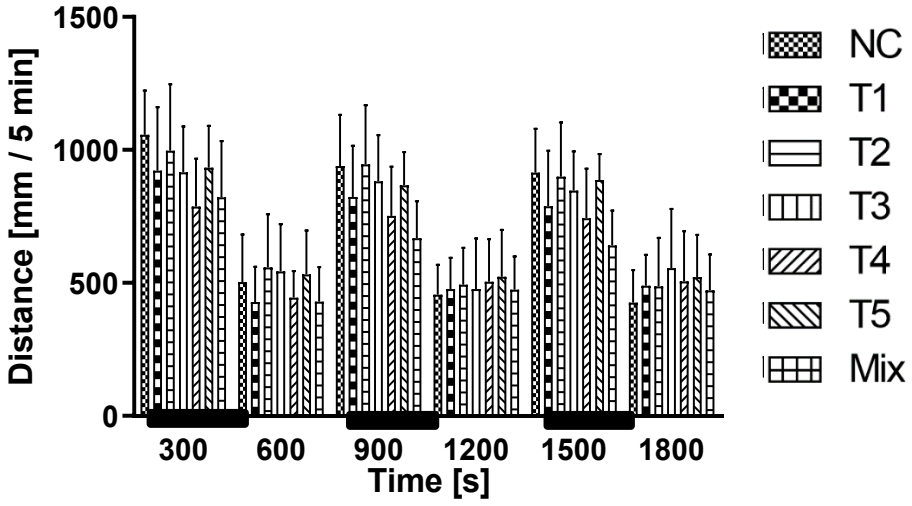



**Figure 5.2: Data of the light-dark transition test with 5 min light-dark cycles** and a total test time of 30 min. It shows the control group (NC) and the data of the environmental concentrations of the five sampling times and the mixture sample for the original environmental concentration without any dilution steps. Each graph represents 20 embryos in 3 replicates, expressed by mean ± standard deviation.

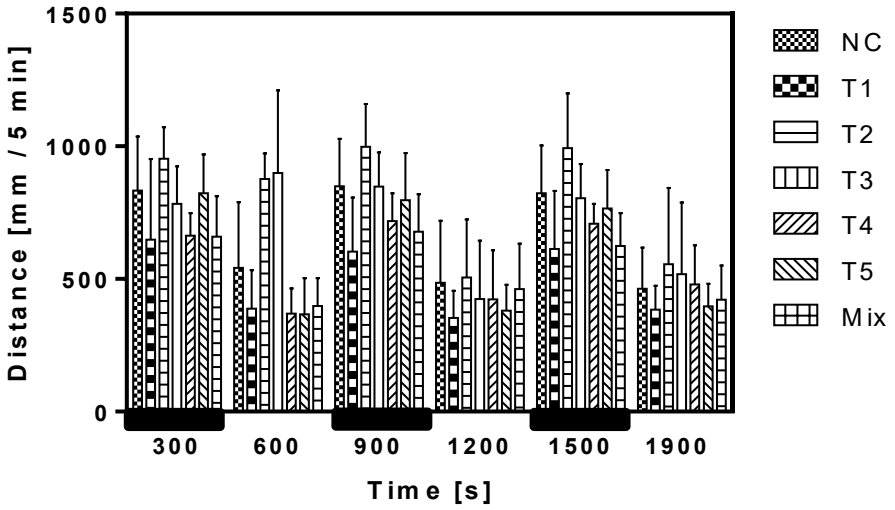



**Figure 5.3:** Data of the light-dark transition test of half-concentrated samples with 5 min light-dark cycles and a test time of 30 min. It shows the control group (NC) and the data of the environmental concentrations of the five sampling times and the mixture sample. Each graph represents 25 embryos in 3 replicates, expressed by mean ± standard deviation.

Figure 5.2 shows the measured swimming distance covered at different sampling times, when exposed to the native environmental sample. The values of the three light and dark phases are summed up to 5-minute sections. The sample time T1 showed an activity-sedating effect in all three dark cycles with a 20 % lower average activity compared to the embryos of the control group. In the measurement of a dilution of 50 % of the native concentration (Figure 5.3), the sedating effect was still detectable. The behaviour results of the time point T2 showed compared to the results of the exposure group T1 a less distinctive sedative effect. In total, the distance travelled was only 10 % lower than the data of the control group. The data of the swimming distance of the exposure group T3 showed a sedative effect in the swimming behaviour of the embryos after exposure to the native environmental sample, but an activity enhancing effect when exposed to a 50 % dilution sample. Except for the behavioural data of the mixture sample, the highest sedative effect could be measured for sampling time T4 for the native sample and the dilution exposure. The effect expression of the sedative effect of the last sampling time T5 was lower than the time points T1 and T4. The decrease was only 10 % below the movement data of the control group and a sedative effect could also be determined for this time point for all three light cycles. In summary, when measuring the activity of the 50 % dilution, an activity decrease could be measured for the time points T1, T4 and for the mixture sample with an average of 10 %. The final analysis of the mixture sample showed a sedative effect in swimming behaviour of more than 40 % compared to the control group.

## 5.3.3 Protein analysis

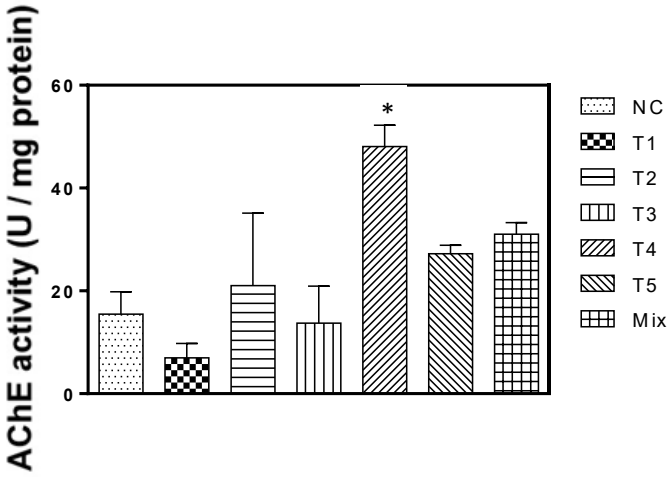



**Figure 5.4**: **Specific activity of acetylcholinesterase** for the exposures of the samples of different sampling times. Each bar represents the protein measurement of 20 embryos (with a total of three replicates) and was presented as mean with standard deviation.

Figure 5.4 show the results of the protein analysis as specific activities (nmol/min*mg) for the different sampling times.

The highest increase with more than 20 % was measured for the time point T4 with more than 40 nmol/min*mg in total. Furthermore, the sample times T2 and T5 with an average activity of more than 20 nmol/min*mg also showed an increased acetylcholinesterase activity. The results of the mixture sample, with an activity of more than 30 nmol/min*mg, were above the control group with less than 20 nmol/min*mg.

## 5.3.4 Gene expression Analysis

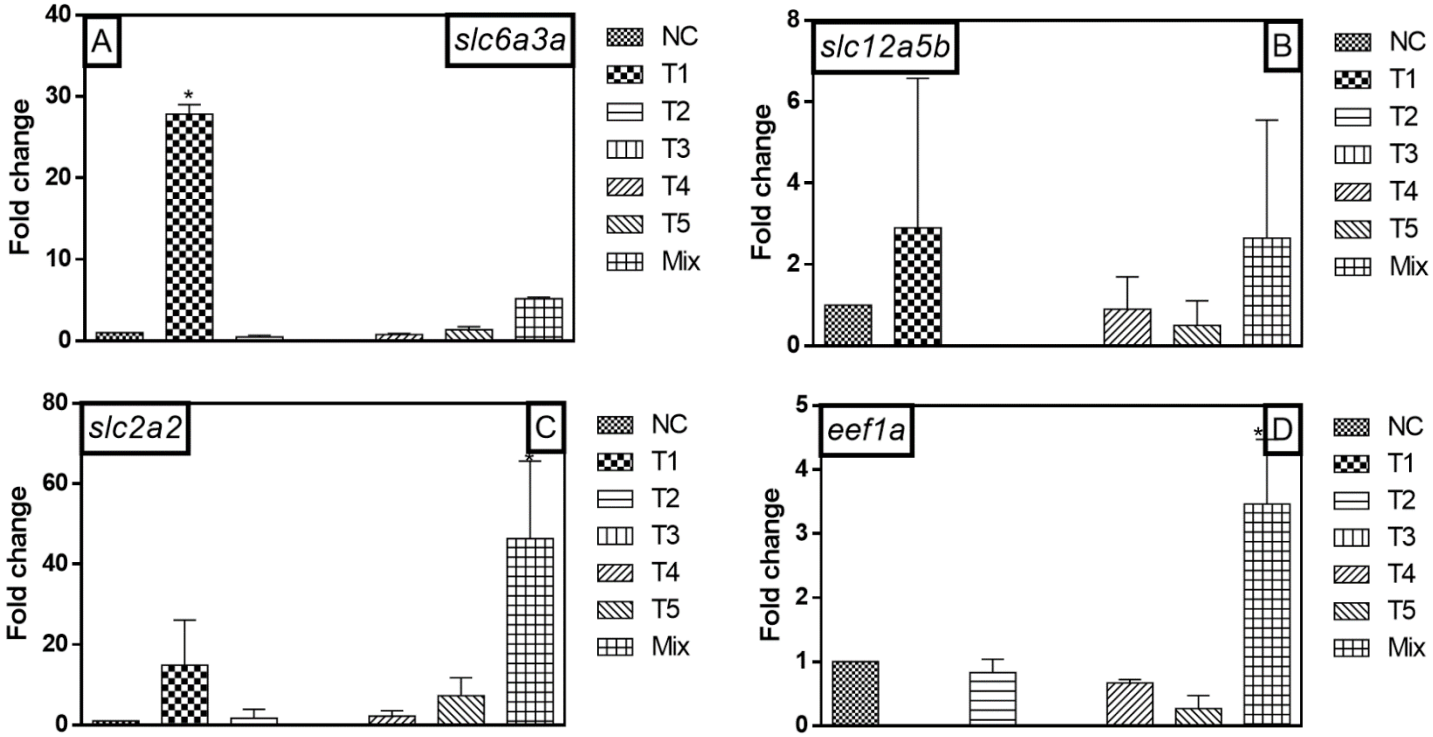



**Figure 5.5: Representation of the gene expression profiles of selected genes of 116 hpf old zebrafish embryos** after exposure to the environmental wastewater samples of different sampling times. (A) *slc6a3a*, (B) *slc12a5b*, (C) *slc2a2*, (D) *eef1a*. Each bar represents the gene expression profile of at least 20 larvae with a total of three replicates as mean ± standard deviation. Significant differences ($p < 0.05$) in the expression profile compared to the control group are marked by an asterisk (*). The graphs show from the left to the right the control group, the five different sampling times and the mixture sample.

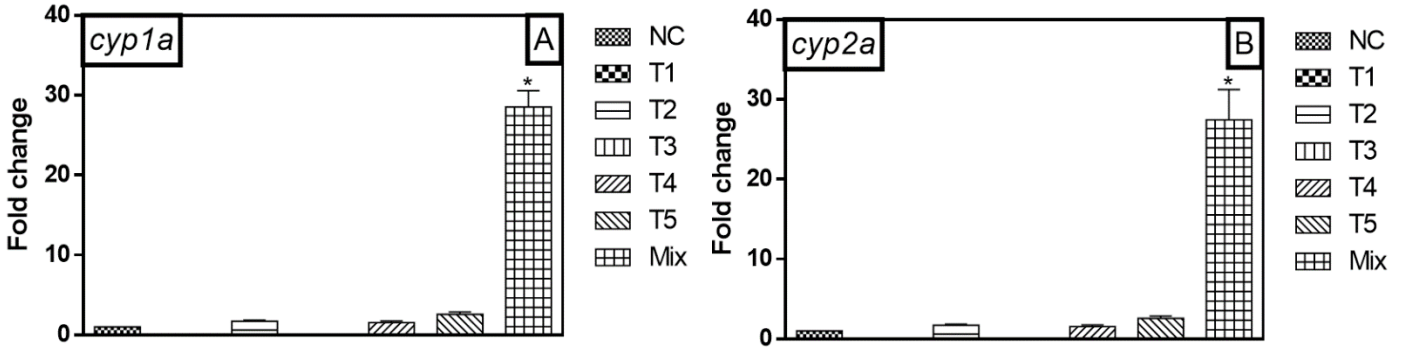



**Figure 5.6: Gene expression profiles of selected general genes of 116 hpf old zebrafish embryos** after exposure to the environmental wastewater samples of different sampling times. (A) *cyp1a,* (B) *cyp2a*. Each bar represents the gene expression profile of at least 20 larvae with a total of three replicates as mean ± standard deviation. Significant differences ($p < 0.05$) in the expression profile compared to the control group are marked by an asterisk (*). The graphs show from the left to the right the control group, the five different sampling times and the mixture sample.

For both *slc6a3a* and *slc12a5b* (Figure 5.5), the highest induction rate could be measured for T1. The induction rate for the channel protein *slc6a3a* showed a value of less than 30 and was much higher than the value of the mixture sample with 5. For *slc12a5b*, these two time points also showed the highest induction rate compared to the negative control, but in both cases a comparatively high standard deviations could be measured.

A decreased induction rate was measured for sampling times T4 and T5. For the genes *slc2a2* and *eef1a*, the highest induction values (with Fold changes higher than 20) could be measured for the mixture sample.

For *slc2a2*, the induction rate of 40 was lower than the induction rate of time point T1 with 20, but higher than the induction rates of the time points T4 and T5, which were below 10. For *eef1a*, the maximum expression rate could also be measured for the mixture sample with an induction of more than 3, while the remaining samples of the different sampling times were at the same level as the control group. Compared to these genes strongly associated with the serotonergic system, increased activity for the genes indicating general stress (*cyp1a* and *cyp2a,* Figure 5.6) could be measured in the mixture samples. All other sampling times were at the level of the control group.

### 5.3.4 Antibody staining

The results of the serotonin images are shown for all histological slides in Figure 5.7. The table show the percentages of the slides with an increased activity of the serotonergic system. They include all potentially neuron-affected areas of central nervous system and the spinal cord.

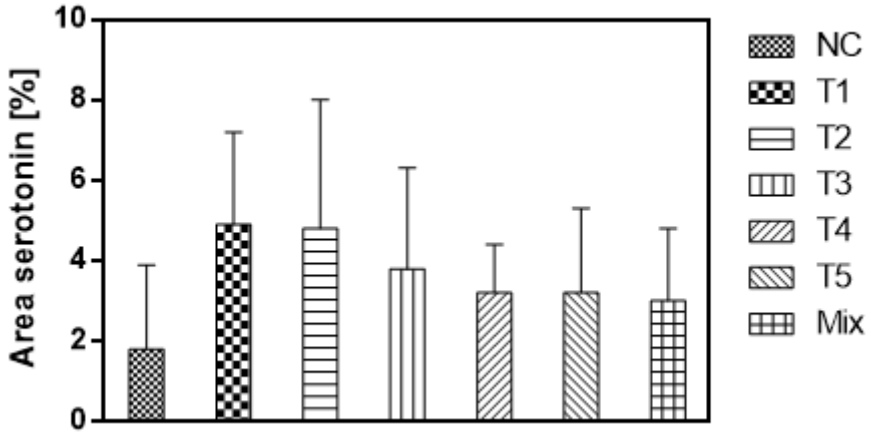



**Figure 5.7: Quantification of the activity of the serotonergic system of the zebrafish embryo for the control group, the five different native hospital wastewater samples and the mixture sample of all five samples.** The graph shows the area with an increased serotonin activity compared to the surrounded tissue.

An increased activity of the serotonergic system could be measured on cell level for all tested native wastewater samples. The highest serotonergic activity was measured for T1 with more than 4.8 ± 1.8 % of the total areas. The affected areas of T2 and T3 were also comparatively high with values higher than 4.7 ± 1.6 %% compared to the surrounded tissues. For the control group an average of 2.0 ± 1.5 % could be measured.

## 5.4 Discussion

The analysis of different neuroactive native samples with various compositions of psychotropic drugs indicated that some of the ingredients have been absorbed, metabolised and excreted in parts. The sampling site with a reduced number of pharmaceutical classes enabled in the analysis of further effect causes on molecular levels central mechanisms of action that allowed a structured classification of the individual substances. A comparison of the chemical-analytical recovery rates with the administered medications allowed the assumption that additional medications have been entered by daily visitors. Furthermore, an incorrect intake and disposal of the drugs or metabolism and excretion are possible reasons for the differences of the results (Umweltbundesamt, 2017). The analysis of the residues in comparison to the behavioural data revealed complex interactions between the different substances. Balancing sedative and activity-enhancing effects in model predictions did not lead to an explanation of the observed effects. The effect data on individual substances are only available for selected and frequently used neuroactive psychotropic drugs and are limited in their comparability due to an inhomogeneous test strategy (Ford et al., 2021). Nevertheless, various physiological studies with individual substances show, that the effects in aquatic non-target organisms corresponds to the molecular mechanisms of action in humans for specific concentration ranges (Fraser et al., 2017; Kidd et al., 2007). In comparison, the native hospital effluent samples are complex composite samples containing various activity-enhancing and sedative constituents, which prevented simple effect predictions. Most of the previous studies have focused on the recovery rate of psychotropic drugs at different sampling sites in the environment or investigated the acute and chronic consequences of selected neuroactive individual substances in aquatic non-target organisms (Roberts et al., 2016; Sim et al., 2010). The combination studies of behavioural changes and molecular causes has been carried out for selected individual substances, but the results can be used for the analysis of more complex mixed samples (Backhaus et al., 2011). The establishment of comprehensive standards for data evaluation is currently a component of various projects with the general aim of enabling the separation of individual natural differences from actual effect characteristics (Legradi et al., 2018).

The physiological reaction of the movement sequences in zebrafish can be compared to the main processes in the human brain. In both cases, the use of neuroactive psychotropic drugs leads to behavioural changes (Boehmler et al., 2007; Franco et al., 2019). Higher-level neurotransmitter systems such as the serotonergic and dopaminergic system provide an interface between the received stimuli and the physiological reactions. The complex processes that lead to a movement reaction have not yet been fully investigated, neither for humans nor for zebrafish, but neuronal nodes can be identified in sections where parts of such reactions are controlled (Drapeau et al., 2002). The detected psychoactive drugs lead to changes at these molecular levels, as shown by the results of selected

transporters and receptors at the gene level, either by preventing the reuptake of the neurotransmitters and leading to permanent excitation or by preventing their release altogether (Yang et al., 2021b). A possible explanation for the consistently visible physiological sedative effects in the native environmental samples would be an overload of the neurotransmitter system. This syndrome, which is known from human diagnostics, is called the serotonin syndrome and has already been demonstrated in zebrafish for selected neuroactive psychotropic pharmaceuticals (Stewart et al., 2013). Especially for mixtures of different psychotropic drugs with diverse molecular mechanisms of action, as in the investigated mixed sample, such a reaction is possible. Nevertheless, not all observed effects could be described by the serotonin syndrome. Especially in the samples of the T1 und T2 sampling times, only a few classes of active substances were present, and the hypothetical predicted effect strength was too high in comparison to the results of existing single-substance investigations. Even the predictions from common models of mixing theory do not provide reliable and comparable results to the observed effects (Galus et al., 2013b). The difficult predictions about how the pharmaceuticals will react in aquatic non-target organisms and the correct confirmation of this theory pose a particular challenge for the risk assessment of these substances. Reliable statements can only be provided with a reliable data prediction of the recovered environmental concentrations and with detailed biological effect data.

The study results are an important linkage between the physiological movement effect data and the underlying molecular modes of action because all neuronal processes are transmitted on cellular level. That also means, that pharmaceuticals could have a negative impact on cellular level at the synapse because it is the node between two different neurons and the chemical signal transmission offered different target points for this substances (Richelson, 1996). A comparison of the mechanistic results at cellular level with the physiological behavioural changes, showed differences that clarified that the connections between the various biological levels are complex and multi-dimensional. Thus, especially the mixture sample showed the strongest sedative effect of all exposure groups on a physiological level, but this effect could not be verified and explained on cellular level (Murrough, 2012). This complexity is only one reason for the prioritization of the analysis on the serotonergic system as a major regulatory system for the behavioural changes. In this context, two major pharmaceutical classes have an effect on the serotonergic system. First, the group of serotonin reuptake inhibitors (SRI) and second the serotonin-norepinephrine reuptake inhibitors (SSNRI) (Maximino and Herculano, 2010; Park et al., 2012).

However, the environmental samples are complex wastewater mixtures that consist of different classes of active substances and that could influence the functioning of different neurotransmitter systems (Pivetta et al., 2020; Reichert et al., 2019). Previous studies focusing on pure substance

analysis with aquatic non-target organisms made clear that the combination effects of different single-substances with a neuroactive potential can lead to an overload the neurotransmitter system (Cheresiz et al., 2020; Rubinstein and toxicology, 2006; Stewart et al., 2013). Nevertheless, it is very difficult to localise a specific behavioural reaction to a specific region of the nervous system. That means, that antibody staining methods are one of the most effective ways to compare morphological changes and molecular causes on cellular level (Kandemir et al., 2014; Retamero et al., 2020). The use of zebrafish tissue for antibody staining was already successfully demonstrated for the effect quantification of other substances with a negative impact on the aquatic non-target organism. Especially endocrine substances were analysed with a special focus on cellular damage and standard guidance protocols already exist for some of the applications (van der Ven et al., 2003). In comparison to most of the studies, the serotonergic system is a decentralised network of different neurons and the connection between molecular modes of action and physiological reaction has not been investigated completely (Lillesaar, 2011). A methodically very promising approach for closing this gap between physiological behavioural reaction and cellular effect causes are fluorescent labelled neurotransmitters or tissues. Although this are only single moment results it is possible to get important information about the cellular responses in aquatic non-target organisms for neuroactive samples with different compositions (Qian et al., 2015). The optimised experimental protocols were suitable for an application in zebrafish embryos. However, the elucidation of the behavioural alterations on cellular level could not be confirmed. This highlights the complexity of the molecular process and show that behaviour is a complex reaction with different molecular causes and interactions between internal and external stimulus (Ogungbemi et al., 2021). In general, the results indicate an overload of the neurotransmitter system at different target sites. Further detailed studies of the detailed modes of action of more target sides would be a useful approach for future projects. However, although some of the effects can be extrapolated from the response in humans, it is important to consider that humans are not usually exposed to such a drug mix over a long time period.

For the systematic solution of this problem, it is necessary to have a developed test battery with an intelligent sampling site. The information from the chemical analysis is complemented by information from the medical administration data and both data sets help by the interpretation of the physiological and molecular bioassay data. Many medical application data can be used as additional data sources, which help to elucidate the underling molecular mechanisms of action (Barbazuk et al., 2000).

The studies helped to compare the physiological effect data between human and zebrafish. A summation of the biological effects of the individual substances does not provide any exact information about the quantitative effects of the used test organisms (Ogungbemi et al., 2021). Even the effect expressions cannot be determined from the chemical-analytical components, but rather

showed a complete overloading of the entire system. The superordinate molecular structures were found both in humans and in zebrafish and the induction of specific genes of the serotonergic and dopaminergic system confirmed the assumption that changes are already occurring at the highest molecular levels. The composite test battery is a suitable tool for sample analysis and provided important insights into the environmental effects on different biological level. The development of a bioassay battery with ecotoxicological, analytical and medical information could be achieved. Nevertheless, further standardization, especially for behavioural effects recording, are necessary for the development of an effective and replicable regulatory experimental procedure in the future.

## 5.5 Conclusions

The final evaluation of the study site shows more advantages in comparison to the existing limitations. It allowed a comprehensive analysis of medical, chemical-analytical and ecotoxicological data for the assessment of the risk potential of wastewater containing neuroactive psychotropic drugs. In general, the study design is another step forward to future solutions for the handling of micropollutants. The possibility of discussing results at different levels from visible physiological responses to the evaluation of molecular mechanisms of action are only possible through the combined consideration of medical, analytical and ecotoxicological data. In particular, the advancing developments in the field of elucidating specific gene responses provide important approaches for a holistic view of the molecular causes, which could only be considered so far in a small set of selected genes of superordinate systems.

The histological studies are only one part of the analysis of the underlying effect causes on cellular and molecular level for aquatic non-target organisms. Especially the challenges of a high complex test protocol which had to be optimised for a very small organ could be solved by the experiments. Nevertheless, they demonstrate that changes could occur at the cellular level that cannot be explained by a single structure-reaction relationship and that further analysis should concentrate on this research gap especially with other fluorescent antibodies. A complete analysis of the cellular responses towards a neurotoxicological exposure are one important step on the final elucidation of the underlying molecular effect causes that are responsible for a behavioural reaction.

Nevertheless, it could be seen from the discussion of results that the current methods only represent a specific part of the physiological effect analysis and the elucidation of the underlying molecular causes. It is very difficult to develop and establish standard protocols for these experiments because in many cases the individual differences overlay the effect responses. Still, this work helped to support

the analysis steps and solve many problems by a useful and intelligent combination of bioassays and chemical residue analysis.

In this way, it is possible to better assess the environmental impact of neuroactive substances to ensure that the changes they cause in aquatic ecosystems can be better assessed and limited by regulation. In summary, the analysis of ecotoxicological endpoints at different biological levels and the analytical measurement of the mixture components has different advantages compared to the use of selected individual assays. However, a prediction of the occurring effects based on the mechanisms of action in humans is only possible to a limited extent. The overloading of the entire neuronal system and the potentiation of individual effects make a precise prediction of the effects impossible in many cases. Nevertheless, the experiments show that significant progress in the analysis can be achieved through a targeted linking of individual studies. Finally, there are different opportunities for further improvements in the investigated methods. The main aim is a step-by-step analysis of the protocols to fulfil the picture of a future environmental risk assessment of neuroactive substances.

## Acknowledgements

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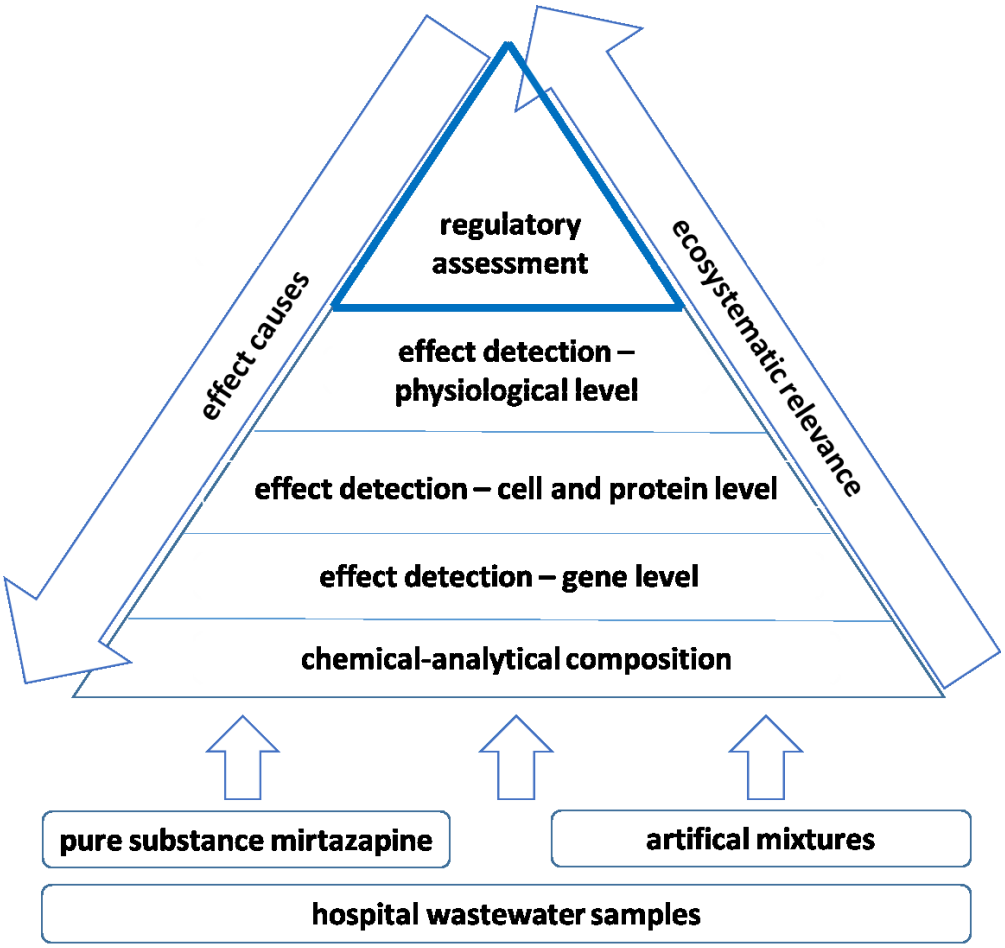
# Chapter 6

## General Discussion



The discharge of micropollutants into aquatic ecosystems has led to increasing rates of neurotoxicological effects (Kumar et al., 2020; Rhee et al., 2013; Weichert et al., 2017). At the same time, a complete surveillance of the substances is not possible because their complex mechanisms of action prevent the use of substance-targeted monitoring in all cases. Previous studies have shown the difficulties of the effect quantification of single neuroactive substances and complex mixtures with different molecular target points (Beckers et al., 2020; Milman and Zhurkovich, 2017; Park et al., 2018). A model prediction of the effects is not possible for most of the substances. In most cases of complex mixtures, a simple addition of the effects of each substance is also not possible but rather, a complex interaction on the molecular level is the reason for the visible physiological effects (Ogungbemi et al., 2021). These effects occur at a much lower concentration level than morphological changes, which can occur at hundredfold higher concentrations for some substances (Maximino et al., 2010; Shams et al., 2018). Such behavioural effects are, however, not yet systematically employed for the regulatory assessment of the environmental hazard potential of these substances (Miklósi and Andrew, 2006; Scholz et al., 2008). This is because of methodological difficulties as well as the lack of important information about the linkages between various biological levels. Current studies do not work with validated standard procedures for the recording of behavioural effects because different viewpoints exist about the quality of the most suitable endpoint (Scholz et al., 2008). In addition, all aquatic organisms show individual characteristics of the physiological reactions to micropollutants in comparison to more or less the same morphological properties. This is reflected by the much lower variations of the threshold concentrations for morphological changes. Still, various tools could be used for the identification of physiological and molecular effects caused by neurotoxic agents. On the physiological level, different endpoints such as, for example, the total swimming distance or the number of changes in the swimming direction, can be used as effect markers.

For the quantification of the physiological endpoints, several commercially available devices are offered by various companies. In detail, a capture in 2-dimensional, as well as in 3-dimensional formats is possible for various organisms and in order to elucidate the underlying molecular mechanisms of action, different devices offer quantitative real-time PCR applications for the measurement of cT levels.

The experiments of this thesis were planned and conducted with these basic devices and established basic methods. At the same time it was necessary to optimise the standard test protocols for neuroactive substances. The aim of the optimised study design was the development of an effective bioassay battery with the possibility to distinguish between organism-dependent differences and effect responses. The optimisation followed a step-by-step approach, starting with the investigations carried out on various individual substances, continuing with the analysis of prepared mixtures with few individual substances, and ending with the evaluation of the effects of a complex sample of

hospital wastewater. At each stage existing methodological suggestions from the literature and previous studies were incorporated for an optimal test design. This method optimisation will be discussed and compared with other approaches in the following section 6.1.

In addition to the analysis of appropriate methods, the use of suitable aquatic non-target organisms for testing was of critical importance. Therefore, a selection of testing organisms was made, whose effects could be extrapolated to other aquatic species who are potentially influenced by the drugs studied. Various aquatic model organisms that have already been established in other studies are suitable for this purpose, and were critically selected at the experimental design stage. Finally, *Daphnia magna* as a primary consumer of the first order, and *Danio rerio* as an aquatic consumer of a higher order, were selected for the studies. This selection will be discussed in section 6.4, also in comparison with literature data.

Finally, it is necessary to assess the benefit of the developed biotest battery for the assessment of the environmental hazard potential of neuroactive pharmaceuticals. A maximum gain in knowledge can result from further developments of the battery for the assessment of neuroactive substances and its optimisation with other test systems. A long-term success would be the implementation of these methods in the standardised environmental risk assessment of psychotropic drugs. An outlook of possible applications and desirable improvements is given in sections 6.5 and 6.6.

## 6.1 Overall assessment of the test methods

Current procedures that have already been proven to be target-orientated for the investigation of other substance classes have demonstrated the effectiveness of the range, and properties of swimming behaviour as a physiological endpoint. These quantitative recording of swimming distance has proven to be a comparatively stable criterion under certain conditions, especially in various investigations of microplastic research (Chen et al., 2017). The conditions include a sufficient number of experimental organisms in independent replicates, and alternating light-dark rhythms in the test procedure. The optimised study results show aspects which have various advantages in comparison to previous studies of neuroactive substances.

Previous behavioural studies have indicated total swimming distance to be one of the most robust endpoints for the assessment of physiological changes caused by neuropharmaceuticals (Boehmler et al., 2007; Oliveira et al., 2021). Nevertheless, an optimisation of the test method was particularly necessary for total measuring times and data evaluation protocols. Nearly all behavioural studies that have already been developed are based on non-standardised test procedures and often consider

individual-specific characteristics for the test design (Ågerstrand et al., 2020). Therefore, the data are often used only very sporadically for the assessment of these substances for regulatory purposes, as is discussed in sections 6.4 and 6.5. One positive consequence of a shorter test time was an increased number of individuals per replicate, which strengthened the robustness of the final evaluation steps. Furthermore, the quantification tests on a gene regulatory level also benefited from individuals which were as unstressed as possible. This offered the possibility to distinguish between neurotoxicological effects and general stress symptoms (Li et al., 2019a).

In addition, data evaluation was improved by the results of single-substance studies in human and for some special cases for zebrafish embryos (Richelson, 2001; Tatsumi et al., 1997). However, the design required optimisation and adaption for the planned biotest battery and most of the existing protocols showed various experimental limitations. Thus, the method evaluations for the pure substance mirtazapine on physiological and gene regulatory level were the first steps towards a hypothesis-driven analysis for more complex samples. In addition, the results of the pure substance analysis offered novel approaches to data evaluation for more complex data sets with various mechanisms of action. In particular, the tiered test approach, with as much information as possible about the substances and effects at each test level, enabled an interpretation of complex results.

Different substance classes with partly contrary mechanisms of action showed the need for another evaluation step in the biotest battery with less complex samples. Therefore, laboratory mixtures of known composition and with only two different substances were employed, first. The findings from these studies only confirmed in part the predictions from common models for mixtures. Especially the increased sedative effects of substance mixtures with normally decreased effects in humans, was not able to be predicted before. These physiological outcomes showed the importance of a detailed elucidation of the underlying molecular mechanisms of action for all tested samples. This evaluation step showed that it is not possible to analyse even a sample mixture with only two neuroactive substances without using molecular data.

In addition, a cross-species comparison between zebrafish and *Daphnia* identified different defense mechanisms that could influence the impacts of neuroactive pharmaceuticals. To conclude, therefore, it is not possible to predict all the combination effects that can occur between two substances. This means that some of the predictions and models that are used for a final regulatory assessment of such combination effects could underestimate the risk for the aquatic environment.

For a future re-orientated assessment of these substances on a regulatory level, it is necessary to minimise the uncertainties of commonly used model predictions. This includes a systematic elucidation of the molecular effects on aquatic non-target organisms which can be applied across species. Thus,

during the identification of target genes, some higher-level neurological systems were identified that serve as central interfaces between external stimuli and internal physiological reactions (Maximino et al., 2013a; Maximino et al., 2016). These included sections of the serotonergic and dopaminergic systems as shown in previous studies.

The development of quantification methods for genes for these systems has gained considerable importance in recent years. In particular, the assessment of entire genome or protein activities within the context of omics developments has created completely new possibilities for assessing the environmental impact of various substance classes (Fitzgerald et al., 2020; Li et al., 2019b). Thus, the more gene responses for various neuroactive drugs were available, the better a detailed description of the correlations on the molecular level could be created and used. This also means that with a subsequent quantification of the molecular effects it would be much easier to identify the main drivers for complex mixtures (Aguirre-Martínez et al., 2017). This knowledge helped for the development of a suitable biotest battery for testing neuroactive pharmaceuticals. Although the molecular effect data only indicate parts the complete gene relationships, they nevertheless provide important information about the responses at neuronal key positions (Yang et al., 2018).

Furthermore, collaborating with medicine is very useful approach to obtain information for the foresight of potentially affected genes at the experimental design stage. The comparative analysis of existing gene reactions in humans and zebrafish show similarities for different neuronal systems (Etchin et al., 2011; Lieschke and Currie, 2007; MacRae and Peterson, 2015). In addition to similarities for oxidative stress genes and genes for the control of major embryonic developmental stages, the structure of the central nervous system is also comparable between both organisms. In particular, many homologous genes can be identified for the serotonergic nervous system which controls the transmission of signals from sensory organs and subsequent processes in the brain (Herculano and Maximino, 2014). The results discussed in this thesis showed that neuronal responses caused by psychotropic drugs in humans could be identified in zebrafish embryos and *Daphnia*. A comparison of the molecular structures showed strong similarities especially for the final proteins, for all tested organisms and human. Even if some of the sequences differed significantly from each other on a gene level, strongly matching structures were identified on a protein level with more than 98-% agreement for most of the tested proteins.

In general, the significance of these data depends on an accurate test performance. As described above, an effect assessment for aquatic non-target organisms is only possible if the neurotoxicological effects can be distinguished from general stress responses. Similar to the analysis of physiological endpoints, various approaches exist for assessments on the molecular level. Even if different standard

protocols exist concerning the minimal information required for data evaluation, there are no standard procedures for a cross-species data comparison (Johnson et al., 2014).

The present work shows that the bioassay battery is a useful tool for the quantification of cross-species effects, at least for a small number of species. Although there are no clearly validated standard procedures for the implementation of behavioural data, the tests of the battery reflect the effects much more accurately than the standard procedures that have been is use for many years. These physiological data could improve the existing procedures for regulatory risk assessment and in addition, the selected biotest battery can be used for effect quantification at a much lower concentration range than conventional test systems on a morphological level. This does not mean that these conventional test systems are useless in future risk assessment, but rather that the evaluation process can be improved by physiological and morphological test systems with different species. In particular, the combination studies and a collection of information from various professional sources are basic strengths of the test design. In the following section, the stepwise evaluation process will be discussed more in detail.

## 6.2 Evaluation of mirtazapine and synthetic mixtures for process validation

The application of various biological test systems for the analysis of a complex, but even more environmentally relevant mixed wastewater sample containing a predominant proportion of neuroactive substances has shown that the individualised adjustment of the procedures to the samples is necessary (Ogungbemi et al., 2021).

Previously, the development of validated standard test methods was mostly accompanied by an increase in the ecotoxicological importance of a specific substance group (White et al., 2019). Often, the consideration of the tested substance is a retrospective evaluation of its effects. Since many new occurring impacts cannot be quantified with commonly used methods new concepts have to be developed and verified. Thus, the inhomogeneous group of micropollutants poses new challenges for the design of suitable chemical-analytical detection methods and ecotoxicological bioassays (Gosset et al., 2020; Priyan et al., 2021). Even the combination of different well-developed bioassays on various biological levels show these challenges. However, various factors can help to speed-up and simplify this design process. This includes the previously described systematic approach with various complexity stages, as well as the interpretation of as much relevant extra information as possible

during every stage. Thus, the results revealed that qualitative differences such as an increase or decrease of swimming distances could be measured easily. By comparison, a *quantification* of the effects is more resource consuming and the results could not be compared between different studies in most of the cases. This issue will be discussed separately in the next section. Nevertheless, the physiological and molecular data after a mirtazapine exposure in zebrafish and *Daphnia* indicated that the methods for single-substance analysis could be used for samples which are more complex.

A possible way of carrying out the application and validation is an analysis of the effects of selected psychotropic drugs, because this allows a comparative review with existing studies. The test systems for the analysis of behavioural alterations have a greater potential to map existing effects much more accurately than existing standard test guidelines (Basnet et al., 2019). Based on known behavioural studies with zebrafish embryos, the light-dark transition test was chosen as a test method that allows a technical effect quantification.

This method has already been used in previous studies to analyse micropollutants, and is part of a test battery of different systems for movement absorption in zebrafish embryos (Legradi et al., 2015). From an ecotoxicological point of view, a critical assessment of the test systems is nevertheless necessary in order to be able to provide a meaningful orientation for future research. The application and further development of testing to record physiological behavioural changes is a very sensible step regarding the chosen pharmaceuticals, as these primarily affect behaviour and development (Carlezon Jr and Konradi, 2004; Hancock and McKim, 2017). Since very little is known about the cognitive abilities of the aquatic test organisms, it is very difficult to assess the consequences of exposure on sensation and its resulting behavioural effects beyond detectable swimming behaviour (Jesuthasan, 2012; Miklósi and Andrew, 2006; Vernier et al., 2012). However, if a sufficiently large number of replicates are available, the collected data do allow the assessment of the sedative or enhancing effects of a psychotropic drug on zebrafish behaviour, even taking into account differences between test individuals. Quantitative movement assessment allows for a comparison between the different exposure groups and permits an estimation of possible threshold values. First, studies with mirtazapine provided important information on the neurotoxicological properties of this drug in aquatic non-target organisms. Until now, physiological and molecular-effect data has not been described for a psychotropic drug with a dual mechanism of action in zebrafish embryos in detail. In addition, none of the previous studies analysed a specific concentration range for physiological effects; most of the studies concentrated on determining the lowest effect concentration for morphological effects.

The evaluation of mirtazapine as a pure substance has provided important evidence to show that the known medical background of a drug can be applied to the retrospective investigation of its environmental effects on non-target aquatic organisms (Bambino and Chu, 2017; Feitsma and Cuppen,

2008; MacRae and Peterson, 2015). This enabled the testing of more complex mixture of substance with various mechanisms of action.

During the final evaluation step, it was possible to discover important relationships between effects in humans and effects in other organisms, such as rodents or fish. This demonstrated close relationship between different species within the context of neurotoxicological research (Barbazuk et al., 2000; Shin et al., 2002) which may also be used to apply the findings of psychopharmacological research in humans to the analysis of the effects in zebrafish (Inohara and Nunez, 2000).

In general, the mirtazapine studies demonstrated that qualitative effect-data could deliver first evidence regarding drug effects on physiological level, although in total, a more in-depth causal analysis is important for further evaluation steps towards an OECD or ISO-validated test procedure (De Oliveira et al., 2020b). Indeed, substance-specific analyses seem to be the only way to distinguish between individual differences of the organisms and effects caused by the pharmaceuticals themselves. This aspect is of crucial importance for environmental risk assessment, as such mechanistic elucidation is the final step in the complete description of the entire effect path (Calow, 2009). For example, homologous genes of the central control units for behaviour are found in both humans and zebrafish, so that these findings can be considered when designing suitable primers for gene expression analysis (Barbazuk et al., 2000). In general, the combination of effect detection at the physiological level and the clarification of causes at the genetic level has already been carried out with selected individual substances: e.g. Boehmler and coworkers (2007) have already shown in detail the effects of the atypical psychotropic clozapine on fish swimming behaviour and on gene expression rate in zebrafish embryos (Boehmler et al., 2007). Extensive studies have been conducted with zebrafish embryos with bupropion and sertraline (Franco et al., 2019; Zhang et al., 2003). Most studies are a mixture of classical OECD-validated test methods and newly developed study methods.

The optimisation of the methods used for the complex wastewater samples is possible with help of such pure substance tests, but existing effects of substances in combination cannot be clarified with this test design. A particular difficulty is the effect assessment of drug classes that partly influence different transmitter systems, and which are antagonistic to each other in their physiological reactions. One way of simplifying samples and generating important findings for effect interpretation is testing combined batches of psychotropic drugs with various modes of action.

The use of artificially produced mixtures is already well-known from research on other substance classes such as pesticides and bioassay tests follow standardised OECD-validated procedures (OECD, 1994; OECD, 2012). As the application of such standard test methods to mixtures of neuroactive substances is likely to lead to a wrong prioritisation of effects in the case of exposure concentrations

that are too high, the application of the methods already optimised for single-substance testing is suitable for clarifying the physiological and mechanistic causes of effects for mixtures.

The prioritisation of the individual substances was based on the quantities retrieved in the hospital wastewater and the selection of a high variety of substance classes. Although the results show only a part of the complex effects of the substance mixtures, they show the benefits of knowing already well-researched effects such as the serotonin syndrome, which occurs in both humans and zebrafish when the number of serotonin-altering substances is too high and significantly alters the physiological response. By attributing the observations, at least in part, to a few already known mechanistic causes of effects, it was possible to interpret the data for the complex environmental mixed sample much more precisely.

The two-step approach with first of developing a test strategy for a pure substance and then subsequently applying an artificially mixture of neuroactive substances was thus an important step for the final evaluation of the effects of the complex wastewater sample. The study results demonstrated that effect prediction with standardised models is not possible in most cases for samples whose components have various mechanisms of action.

As speculated in the introduction and then confirmed by the study results, the final effects of a mixture of neuroactive substances do not correspond to the sum of the effects for each of its components alone. Rather, they are a complex result of different substance effects that are additionally moderated by organism-specific reactions. However, the detection of retrieved neuroactive substances in the environment and their effects in aquatic systems are more complex than laboratory studies predict and therefore, the following section will discuss how the effects of an actual sample of hospital wastewater represent the final level of complexity.

## 6.3 Determination of substance and physiological effects of complex neuroactive wastewater samples

The treatment of wastewater before its final discharge into aquatic systems has improved in recent decades through significant steps in enhancing analytical detection methods and hazard assessment through biotests (Četkauskaitė et al., 2016; Wolska et al., 2007). Extensive technological advance leading to three purification stages now allows the removal of more than 80 % of all substances from wastewater (Qasim, 2017).

Nevertheless, a constantly changing society poses new challenges for current and future wastewater treatment. The potential risks of biologically active substances are not yet sufficiently investigated to allow a clear risk assessment and it has become the subject of various discussions (Kuzmanović et al., 2015; Shao et al., 2019a). Research on the ecotoxicological effects of these substances to assess their risk potential is being conducted in parallel with research into new technology solutions for micropollutant removal in the field of urban wastewater management.

The samples from the building of the main hospital in Aachen (Aix-la-Chapelle) represent a special case of a point source for neuroactive substances. Normally, psychotropic drugs are discharged from different sources such as private households and the retrieved concentrations show various circadian fluctuations (Vollmer, 2010). The selected study location selected for this study showed some suitable advantages for the analysis of the neuroactive potential of the samples in comparison to other downstream study sites.

First, the medication list provided by the hospital was a useful instrument for the development of a suitable method for the analysis of the sample components. It was demonstrated that not all of the drugs found were excretions from the patients but that persons external to the wards must have introduced some of the substances into the wastewater stream. Second, sewage analyses showed that there is a periodic loading of the sewage systems with various compositions of neuroactive substances. The effects of these mixture differed both on the physiological level and at the mechanistic level. Also, the chemical analyses and the expected qualitative physiological effects corresponded for most of the samples.

From the beginning of these investigations, it was evident that there are two different concerns regarding the substances investigated. On the one hand, there is the classical view of the effects of the drugs for the improvement of human health and on the other hand, there is the necessity to protect the aquatic environment as comprehensively as possible against the effects of these substances (Brulle and Pellow, 2006). While for other substance groups, such as pesticides, this balance has increasingly shifted towards environmental protection in recent years, this is not the case for pharmaceuticals. One reason for this is that the prescribed standard tests for the majority of prescribed psychotropic drugs do not show any reaction at the environmentally relevant concentration ranges, so that the resulting risk to various, primarily aquatic, ecosystems is often regarded as low. However, this assumption can no longer remain unchallenged with the scientific knowledge acquired in recent years. Most studies analysing predicted-effect concentrations, use either acute or less frequently, chronic studies with values from the aquatic triad or simulated data based on structural similarities (Pivetta et al., 2020). However, this partly contradicts the knowledge that is known from medical effects in humans.

Psychotropic drugs, for example, are primarily intended to cause behavioural changes at low concentrations whereby morphological, endocrine, or other effects should be excluded to avoid endangering patients' health. Some morphological effects such as odemas, cardiovascular diseases and finally the death of the organism are the result of a comparatively high exposure concentrations, which simply do not occur in the environment due to the point sources of these pollutants and high dilution factors (Rice and Westerhoff, 2017). Nevertheless, below exposure concentrations at which morphological effects do occur, other physiological effects are similar to medical treatment effects in humans (González et al., 2018; Richendrfer et al., 2012).

For a better understanding such effects, it is necessary to reduce the complexity of the real wastewater samples and to obtain a detailed estimation of the changes induced on both physiological behaviour and on the molecular effect level. The focus on pure substances and their effects has already been successful tested for various neuroactive substances and provides quantifiable effect data, particularly because of the reduced complexity (Boehmler et al., 2007; Yang et al., 2021a).

Analyses of different physiological endpoints for zebrafish larvae and *Daphnia* were successfully conducted for various hospital wastewater samples. This shows that the assembled biotest battery was a useful tool for such studies and that different test systems could be successfully optimised by a tiered approach starting with single-substance studies with mirtazapine. However, the analysis of the underlying molecular mechanisms of action was difficult and resource-consuming and even if available medical background information could disprove many uncertainties, the selection of suitable target genes could only be undertaken for a few central nodes. None of the effects could be predicted by existing standard models and a detailed analysis of the molecular causes must therefore be undertaken individually for each component. Furthermore, many physiological reactions can be traced to a complete overload of the whole neurotransmitter system (Stewart et al., 2013). This also provides additional uncertainties for the discussion of the risk potential of the various sample types, especially for the original wastewater samples. As a result, various aquatic organisms were tested at different biological levels to close this gap between physiological reaction and mechanisms of action that could not be analysed in all detail. The significance of this comparative analysis will be discussed in the following section.

## 6.4 Cross-species analysis - evaluation of the benefits for risk assessment

The relation between neuroactive substances and behavioural changes in humans and non-target aquatic organisms has been known for several decades (Dell'Omo, 2002; Ruffin, 1963). There are

different target points in higher-level neurotransmitter systems and the coding genes for these systems are found in homologous form not only in humans but also in many aquatic freshwater species (Christen et al., 2010; McRobb et al., 2014). In general, complex interactions between the various organisms without external influence are the main reason for the stability of the complete ecosystem (Pennekamp et al., 2018; Tilman, 1996). Many processes depend on strong behavioural interactions between predators and prey (Sih, 1984; Smith and Slatkin, 1973). Both groups are evolutionarily optimised which means that small changes of the system could endanger the complete populations (Schmitz, 2017). If one of the groups show anthropogenically triggered behavioural changes, the ecosystem balance could shift from a complete system to an instable one (Sih, 1984). Previous studies with low trophic consumers and top predators have demonstrated that small changes in species abundance can lead to shifts in system structure, which can also impact surrounding terrestrial systems (Guenet et al., 2010). The behavioural changes triggered by neuroactive substances such as psychotropic drugs can therefore influence interspecies interactions, which can lead to changes in population structure (Jennions and Petrie, 1997; Saaristo et al., 2018) and shifts in the entire ecosystem structure (Sih, 1984).

This shows that it is a very useful approach to consider physiological effects on various trophic levels and with different aquatic species. In this context, the available experimental data for neurotoxicological effects on higher-level predators such as fish are much more extensive than for consumers at lower trophic levels. This shows that a cross-species analysis needs a stable data basis which allows qualitative effects statements for different species. Testing physiological effects in *Danio rerio* and *Daphnia magna* with the same experimental setting and after exposure to the same substance is only one example of a cross-species comparison. It seems that behaviour is a strict individual species-specific feature, but results have also shown that it is based on some basic movement sequences (Creton, 2009). In contrast, it could be shown here that the dual mechanism of action of a mirtazapine exposure could be measured in zebrafish but not in *Daphnia*. That means, that even if it seems apparent that general responses are common in various organisms, they have to be verified for each individual separately. Nevertheless, both species showed a decreased activity for a specific threshold concentration.

Many laboratory studies allow conclusions about the impact of effects in complex ecological contexts and provide the basis for further field studies (Wong and Candolin, 2015). Nevertheless, these single-species studies cannot represent the complete complex interactions between different aquatic species. In particular, there are various knowledge gaps due to the lack of standardised methods; such gaps must be closed (Godfray et al., 2014; Goulson et al., 2015). Comparable to the various stages in the development of the biotest battery, the analysis of neuroactive substance effects, can be

undertaken with a step-wise approach employing microcosm and mesocosm experiments which help to identify the interactions between various aquatic organisms (Caquet et al., 2000; Sharma et al., 2020). With particular reference to the effect level, physiological endpoints and species abundances are important for at least partial extrapolations to natural aquatic ecosystems. However, these experiments are both cost- and time intensive and the analysis is much more complex than most of the single-species analyses carried out in the laboratory. As a result, various methods have been established on a laboratory scale to build a bridge between substance effect causes and physiological endpoints in order to increase the significance of such studies for data interpretation.

Overall, it has to be considered, that the nervous system has many different target points for neuroactive substances (Sehonova et al., 2019). This means that for cross-species discussions in effect analyses, it is important to identify the most important changes. As such, cell-level studies closed an important knowledge gap between visible physiological visible effects and the underlying molecular modes of action. At the same time, the cross-species use of the same basic structures and components on a gene regulatory level allowed a discussion of the results without any extrapolation steps.

To summarise, it was concluded that different species are affected by exposure to neuroactive psychotropic drugs on various biological levels. Physiological effects are measurable above a species-dependent individual threshold concentration and the intensity depends on various external factors. This also means, that the complex interactions between various species can shift in the process and that in some special cases the ecosystem will not return to its original structure (Richmond et al., 2019). In many cases, it seems that a decrease of species abundance is only a small factor but from a holistic point of view small changes can shift the structure of the complete system. This poses new challenges for future environmental risk assessment: the already well-established test methods should be augmented by additional physiological and molecular endpoints for different species. Since this is associated with large experimental and administrative challenges, this suggestion will be critically discussed in the following section.

## 6.5 Evaluation of the conclusions within the context of environmental risk assessment

These studies were performed with the overall objective to optimise existing standard regulatory tests and to establish new test methods for the effect analysis of neuroactive substances in aquatic ecosystems. The primary objective was to reduce uncertainties in the regulatory assessment of these substances, because even though organism behaviour is significantly influenced by such substances, a systematic regulatory assessment is currently not conducted (Ågerstrand et al., 2020; Scott and

Sloman, 2004). For both, single substance studies and complex mixtures with substances of different mechanisms of action, a specific species-dependent threshold concentration could be measured for morphological effects in various aquatic species such as *Daphnia* or fish. Compared to the effect analysis of other substance classes (neuroendocrine or neurotoxic), the aquatic triad of algae, *Daphnia* and fish is often used for first effect observations because it covers three different biological levels (Wei et al., 2006). Nevertheless, for neuroactive substances an analysis of physiological effects and the underlying molecular causes is essential in order to obtain a complete overview about possible effects in the environment. As already described in previous sections, neuroactive drugs primary used for the treatment of mental illnesses are designed for triggering changes in behaviour and complex neuronal actions. In this context, psychotropic drugs that could not be prioritised in the studies also show the same neuroactive potential and other substance classes such as pesticides can alter the behaviour of aquatic non-target species (Glaberman et al., 2017; Tierney et al., 2007).

One reason why these findings are not used for regulatory assessment is a lack of standard test methods to establish physiological endpoints without extrapolation. Scanning existing literature data, it is nearly impossible to find the same method for data recording and evaluation twice: nearly every institution has its individual protocol for the measurement of physiological data which depend on many different external factors (Legradi et al., 2018). This means that whereas it is possible to get a qualitative assessment of the data, it is usually not possible to arrive at a detailed quantitative comparisons for most of the data. This problem can be solved by the cross-species analysis of the underlying molecular mechanisms of action. Therefore, the scientific focus of the research was orientated towards a holistic linking of molecular modes of action, physiological reactions, and recommendations for a future environmental risk assessment of neuroactive psychotropic drugs. A central component of all sections of this study was the discussion of how existing regulations concerning the assessment of the environmental risk potential of psychotropic drugs can be expanded and the current procedures can be made more meaningful in their informative value by employing methods that have yet to be validated in OECD or ISO-guidelines. A final overview about the overall results of the thesis and an outlook for further investigations is given in Figure 6.1.

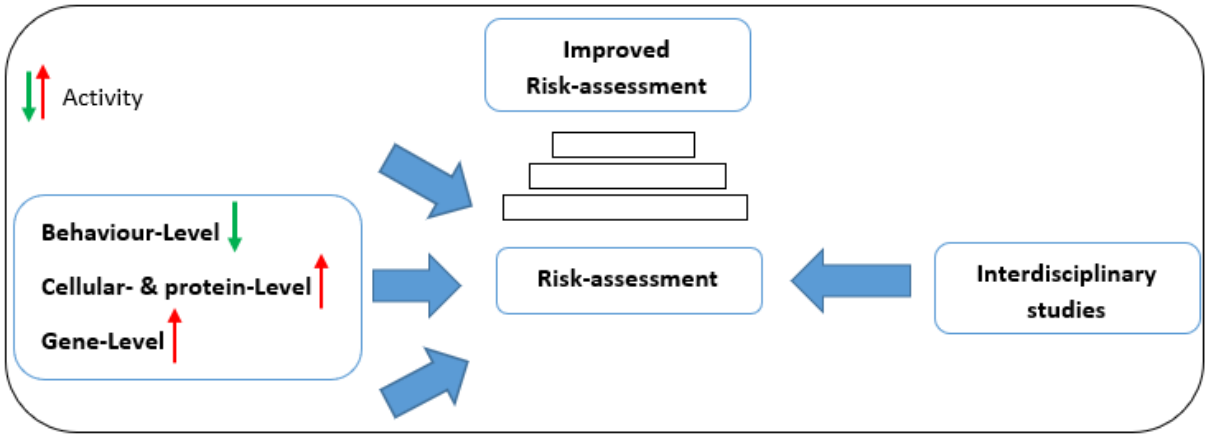



**Figure 6.1: Final summary of the studies and outlook for further investigations**

A central element for the planning of the bioassays and the interpretation of the data is a close exchange between experts from the fields of medicine, chemical residue analysis, water management systems and ecotoxicologic effect data. The test approach was designed following the principle of ”intelligent testing“ which means that as few test animals were used as possible, but ensuring that the data quality was as good as possible (Białk-Bielińska et al., 2017). Such a design was deliberately chosen to reduce the complexity of the samples to be examined, enduring that the strength of the project, namely the combination of various data, could develop its full potential. Otherwise chemical analyses with an even larger number of drug classes would have been much more difficult and the interpretation of bioassay data would only have been possible with relatively large uncertainties.

In conclusion, a critical look at the study objectives and research hypotheses shows that various progresses have been made with the study design. Not only were qualitative effect data used for the interpretation of the results, but a quantitative effect analysis could also be made for various samples and for various concentration levels. This represents a first step towards an holistic view of behavioural effects and the underlying molecular causes (Ford et al., 2021). Linking different biological levels provides evidence for the future assessment of these substances, which can already be found in standardised bioassays for other endpoints (Legradi et al., 2018). The future regulation of the substances under consideration shows a strong need for the consideration of physiological and molecular effect data. In the following and final section, findings will be discussed in more detail compared to the original objectives and, data gaps requiring further research will be outlined.

## 6.6 Outlook

The effects analysis of neuroactive psychotropic drugs at different biological levels of aquatic non-target organisms provided important information about how these substances should be considered in future regulatory assessments. The long-established evidence from human research, that behavioural effects play an important role in recognition and brain development change, is evident in current studies on non-target aquatic organisms (Calderón-Garcidueñas et al., 2014; Lu et al., 2018). Three biological levels were considered in the test design, with different subgoals that were evaluated after each experimental section with new samples. On the molecular level, the studies identified different effect lines with key changes in the functionality of higher-order neurotransmitter systems. Consequently, the genes possibly involved were studied, with a specific focus on such systems in zebrafish embryos and in *Daphnia*. That meant that only a small sub-section of the various molecular target points could be considered in the studies. Nevertheless, the results showed different effect lines which are suitable for a quantitative analysis. In addition, the cellular studies also showed the strong connections between the molecular and protein level. Finally, physiological changes could be determined for various sample types at the lowest complexity level. The analysis of the physiological effects was difficult, and the individual characteristics of each organism masked some of these effects, especially at very low testing concentrations. Therefore, different complexity levels of artificial mixtures and various concentration ranges were used for the evaluation of the complete process. Such a tiered approach is a basic procedure in environmental science studies and as seen from the results, it could be effectively used for the study of neuroactive substances.

The study aim to *quantify* the physiological effects at least for parts of the concentration range was achieved, although it was not possible to determine the lowest effect concentration for the physiological endpoints. As the determination of such values is time and resource consuming, a holistic view was chosen for the experiments and these specific endpoints were not further pursued.

The major challenge of investigations such as these is the incorporation of toxicological effects for the final evaluation of environmental risk. A survey of the literature and an analysis of current regulatory assessment show that the risk of incompletely detecting all physiological effects is high for most of the present testing concepts. This shows, that morphological effects are suitable indicators especially for higher concentration ranges, but that they cannot replace the detailed assessment of physiological effects on the molecular level. A complete assessment of the risk potential of neuroactive psychotropic drugs has not yet been completed for aquatic non-target organisms because the molecular studies of the biotest battery are resource consuming. Results have shown that a very large data basis is necessary in order to distinguish between individual differences between organisms and actual effects

caused by the drug tested. In addition, aquatic systems are complex systems with diverse groups of organisms with different interactions which complicate risk assessment. Although this problem can be reduced by the selection of suitable model organisms for effect evaluation, the final risk potential evaluated will have various uncertainties. Various advances were attained for the aquatic risk assessment of neuroactive pharmaceuticals in hospital wastewater within the scope of the work; advances which can be used as a suitable data basis for further studies. Significant success was achieved in establishing a biotest battery to help understand the physiological effects in an environmental context.

The use of zebrafish embryos in the neurotoxicological analysis of psychotropic drugs is suitable for detecting physiological changes and elucidating variations at the gene regulatory level. By strictly following the principle of maximum knowledge gain with the minimum use of experimental organisms, developments in zebrafish research allow conclusions to be made about effect expression at different biological levels. Despite large efforts, there are currently still no standardised test specifications, so that a complete comparison of findings is still not possible between different laboratories. Data generated data are very complex compared to other classical test systems and a targeted experimental design and additional multidisciplinary information on composition and modes of action in humans allow for an improved effect interpretation, even on a comparatively small scale and with limited resources. Future experimental designs should take up and deploy these findings to gain further knowledge at different levels. In particular, priorities should be placed on expanding research areas and on the further development of test methods at different biological levels.

It therefore remains for future studies to continue the process of methods optimization and there is an urgent need to develop standard test protocols and standard evaluation methods. In all, the assessment of the environmental impact of neuroactive substances is a highly complex but also intriguing topic, with many aspects already developed but many issues requiring much closer attention in the future. Recent developments and discussions concerning the management of neuroactive substances within the context of environmental risk assessment have revealed major uncertainties in specific assessment areas, some of which were able to be addressed through this work. Major efforts are required to close the conceptual and technical gaps in both standardised testing and regulatory assessment. These are important steps respond to the challenges posed by micro-pollutants and to ensure both biodiversity and ecosystem stability. Such efforts are not only called for and funded regionally and, nationally, but also internationally, up to and including the central goals within the framework of the European Union's Green Deal and the United Nations’ 2030 Agenda for Sustainable Development

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# Annex 1 – Primer sequences of the tested genes

**Table Annex 1.1**: **Primer sequences used for the qRT-PCR in the different studies.** The table shows the PCR primers for amplification and the map positions in zebrafish genome

| Gene | Accession No. | Forward primer (5' to 3') | Reverse primer (5' to 3') | Tm (°C) | Zebrafish Chromosome |
|---|---|---|---|---|---|
| *slc6a3a* | NC_007126.7 | GCTGGAAATTTGTGAGCCCG | AGGAACCGTACTTGGGAGGA | 59.4 | 15 |
| *slc6a4a* | XM_009291671.3 | AGAATCTCGCCGGAAATGGG | TCAAGGCTCGTCTGTTGGAC | 60.2 | 15 |
| *slc12a5b* | NC_007119.7 | CCTGATGATGGAGCAGCGAT | GCTCATGGTGTAGGTAG GGG | 59.4 | 8 |
| *slc17a6a* | NM_001009982.1 | AGCATGCCATGGGATTTGGA | TACCAGAAAGTGTGCCGACC | 60.1 | 25 |
| *adra1aa* | NC_007119.7 | TGCATGACAGCCAACGTAGT | CCCAAAACGCCAAACACGAT | 57.3 | 8 |
| *hrh4* | NC_007133.7.10 | CTGTTGCTGCTTCGCCAAAT | GACCACCAGAGTTGCCATCA | 59.4 | 22 |
| *eef1a* | NM_131263.1 | TGGAGACAGCAAGAACGACC | GAGGTTGGGAAGAACACGCC | 60.0 | 19 |
| *slc2a2* | NM_001042721.1 | TCCGTGGAAGGATCAAAGGC | TAGCCAGGCCCATGAGTAGT | 60.0 | 2 |
| *cyp1a* | NM_131879.2 | AACCAGTGGCAAGTCAACCA | TTCAGTTCAGTACCGTCCGC | 60.0 | 18 |
| *cyp2a* | NM_001025557.2 | CCCATTGCAGGGTGAGGATT | GCGGCACACAAGCTTACATC | 60.0 | 23 |
| β-actin | NC_007114.7 | GAAGATCAAGATCATTGCCCC | TCGGCGTGAAGTGGTAACAG | 59.6 | 3 |
| *tubb2* | NC_007113.7 | GAGGCACTGGATCCGGTA | AGGTCTCGTCGGTGTTTT | 60.0 | 2 |

**Table Annex 1.2: Primer sequences used for the qRT-PCR in the different studies.** The table shows the PCR primers for amplification and the map positions in mouse and drosophila genome

| Gene | Accession No. | Forward primer (5' to 3') | Reverse primer (5' to 3') | Tm (°C) | Mouse Chromosome |
|---|---|---|---|---|---|
| *mRPL13a* | NC_000073.7 | GGGCAGGTTCTGGTATTGGAT | GGGCAGCAAATGGTATTGGAT | 60.0 | 7 |
| *miFNg* | NM_010511.3 | GCTACACACTGCATCTTGGC | CATGTCACCATCTTGCCAG | 60.0 | 10 |
| *mus18s* | NR_003278.3 | AAGAATTTCACCTCTAGCGGCG | GAATAATGGAATAGGACCGCGG | 59.9 | 6 |
| **Gene** | **Accession No.** | **Forward primer (5' to 3')** | **Reverse primer (5' to 3')** | **Tm (°C)** | **Drosophila Chromosome** |
| *DDX54* | XM_002092960.3 | AAGGTTTCCGTGCTGATCGT | CGTCACTGTGAGGTGCTCTT | 60.0 | 3 |
| *CG6836* | NM_140822.3 | TTTCACGATGGACCCCACAG | TCCACAGTGGGATGTGTTCG | 60.0 | **3** |

# Annex 2 – Supplementary materials to chapter 2

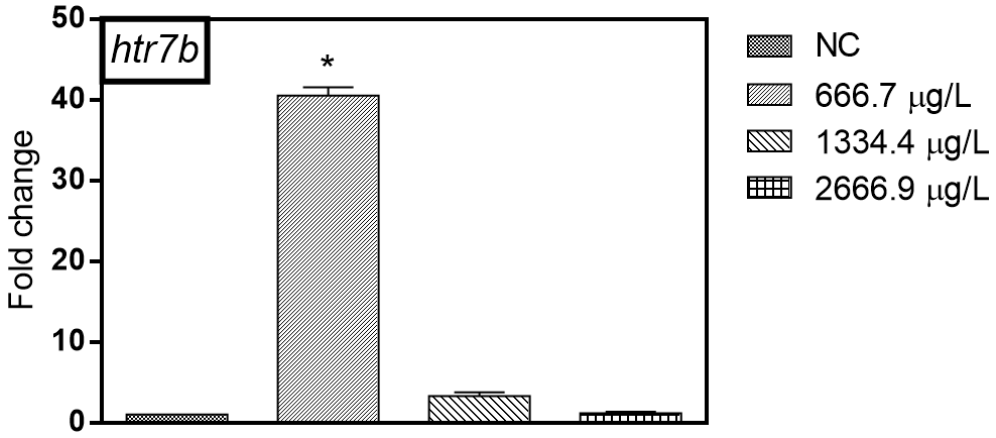


**Figure Annex 2.1: Gene transcription profiles in zebrafish embryos after 116 hpf exposure for htr7b.** Each bar represents 3 replicates (each replicate contained 20 embryos) and expresses as with a standard deviation of $p < 0.05$. NC = Negative control. An asterisk represents a significant difference to the control.

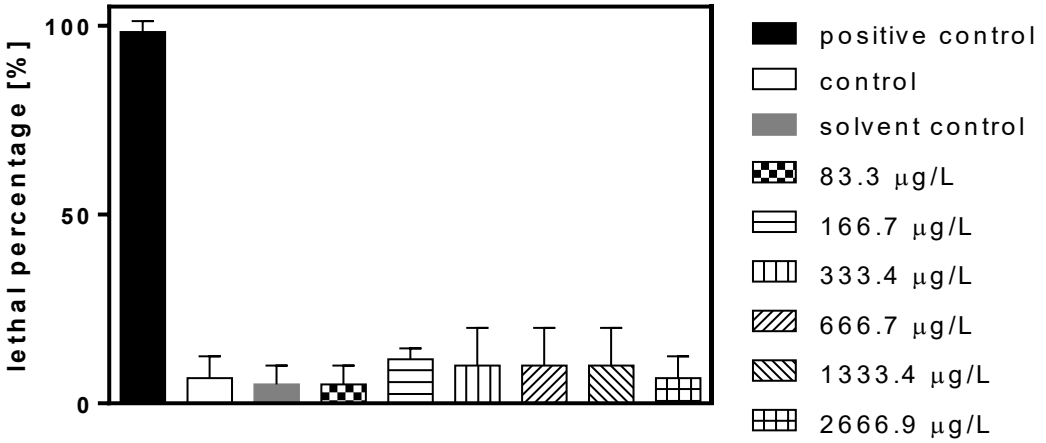


**Figure Annex 2.2: Results of the fish embryo toxicity (FET) test.** The groups are positive control [p control], the negative control [control], the solvent control [s control] and six different exposure concentrations

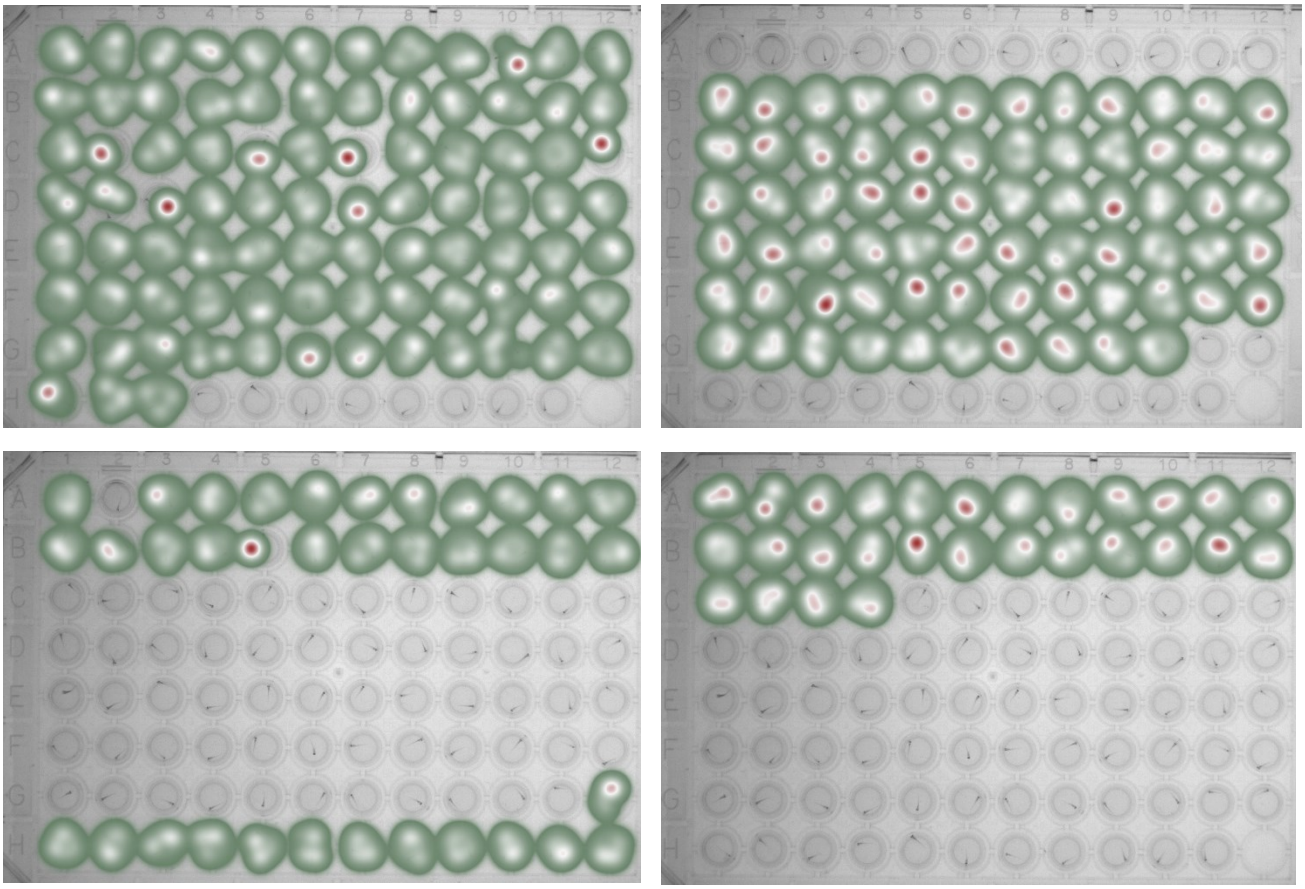

**Figure Annex 2.3: Heat-map analysis for the negative control and the three highest exposure concentrations (666.7 µg/L, 13334.4 µg/L and 2666.9 µg/L).** The figures show the retention times of the zebrafish embryos over a time period of 5 min in the dark.

# Annex 3 – Supplementary material to chapter 3

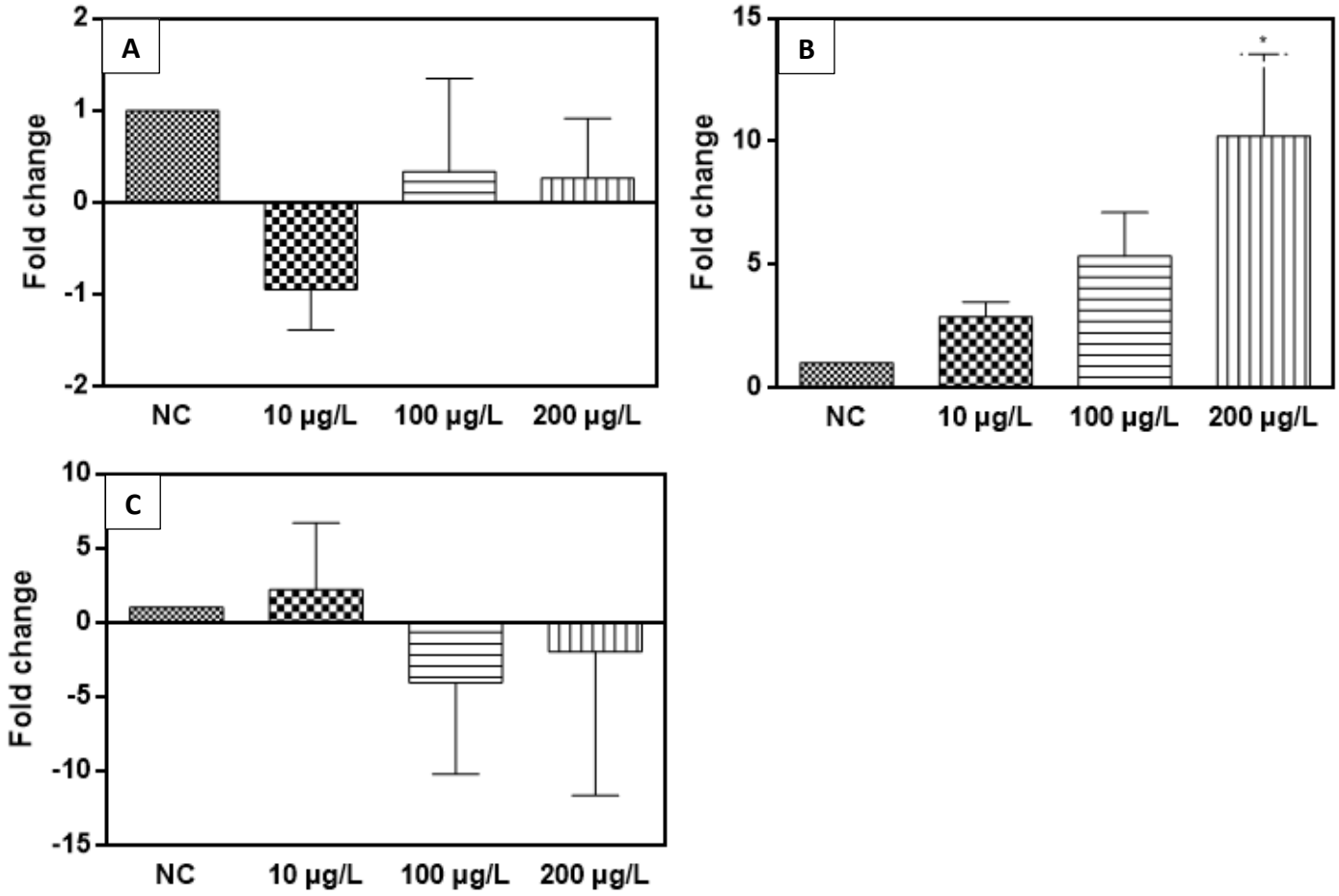


**Figure Annex 3.1: Quantification of the gene expression rate of (Annex 2.1 A) *SerT*, (Annex 2.1 B) *Trh* and (Annex 2.1 C) *DAT* after an exposure to three different mirtazapine treatments (10 µg/L, 100 µg/L, and 200 µg/L) and one control group (NC).** Each bar represents 3 replicates (with 30 individuals per replicate, n= 90) and a cDNA concentration of 3 µg. The bars show the average fold change (mean ± standard deviation) of the expression of the genes

# Annex 4 – Supplementary materials to chapter 5

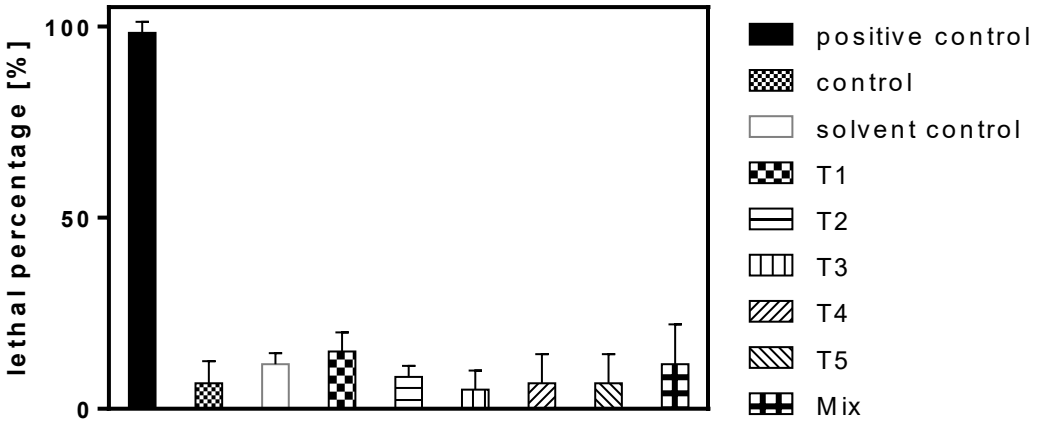


**Figure Annex 4.1: Results of the fish embryo toxicity (FET) test.** The groups are positive control [p control], the negative control [control], the solvent control [s control] and six different exposure groups.

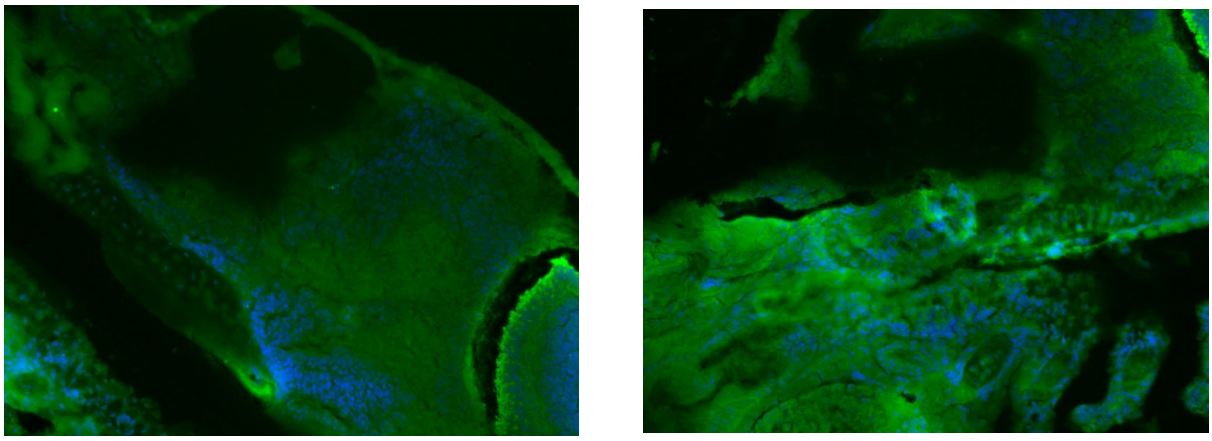

**Figure Annex 4.2: The images show the central nervous system of 116 h old and unexposed zebrafish embryos** after antibody staining with Rat monoclonal anti-5-HT (IgG) and 4',6-Diamidin-2-phenylindol. The images were taken at a magnification of 20x.

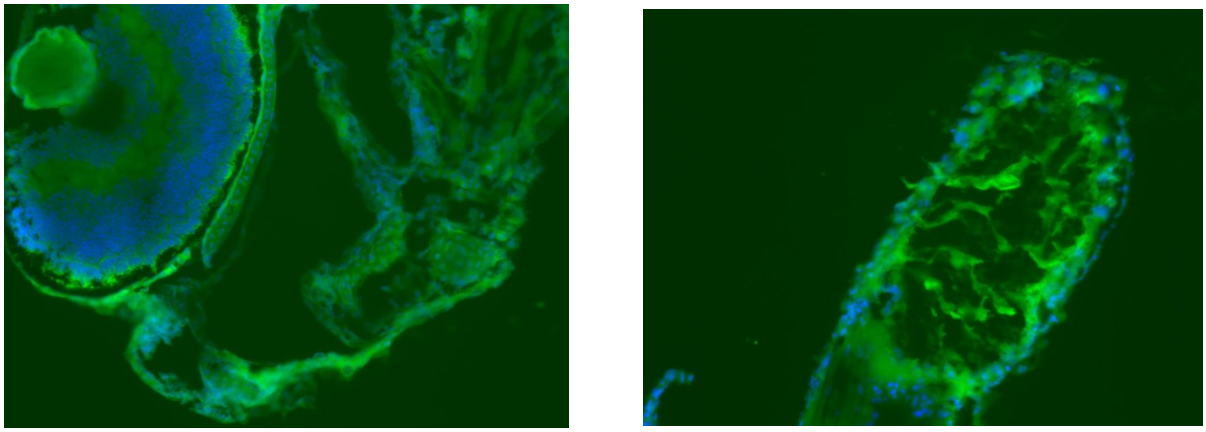

**Figure Annex 4.3**: **The images show the central nervous system and the olfactory bulb of 116 h old zebrafish embryos exposed with a native environmental concentration of sample T1.** The slices were stained with Rat monoclonal anti-5-HT (IgG) and 4',6-Diamidin-2-phenylindol. The images were taken at a magnification of 20x.

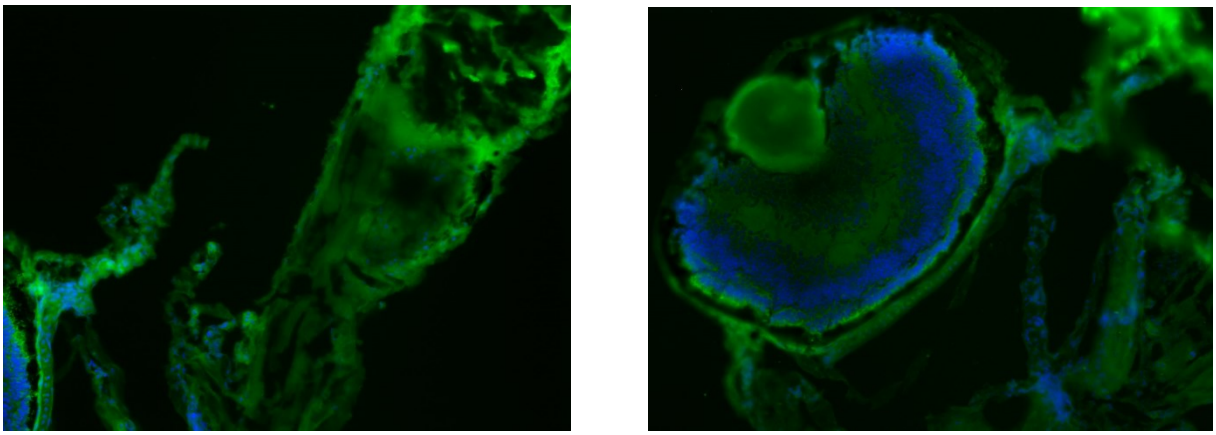

**Figure Annex 4.4: The images show the central nervous system and the olfactory bulb of 116 h old zebrafish embryos exposed with a native environmental concentration of sample T2.** The slices were stained with Rat monoclonal anti-5-HT (IgG) and 4',6-Diamidin-2-phenylindol. The images were taken at a magnification of 20x.

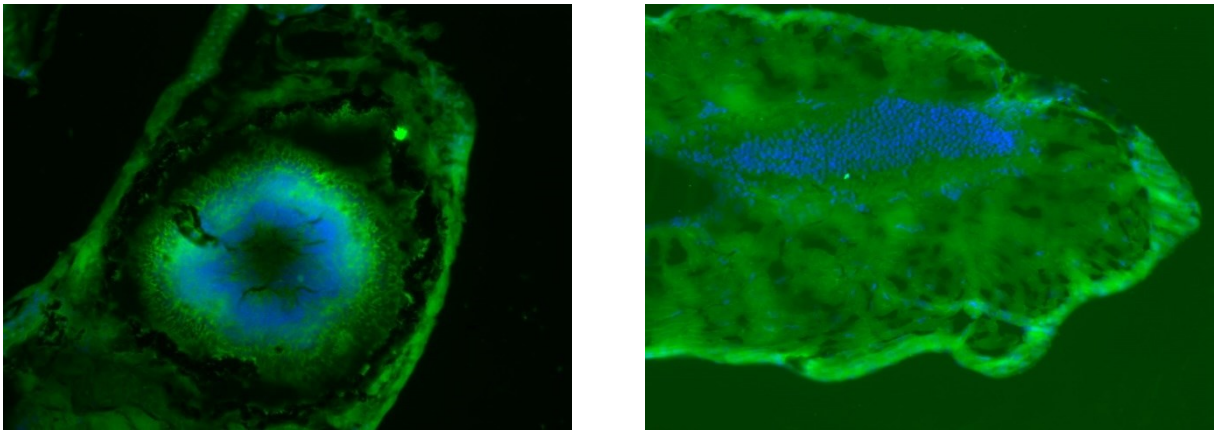

**Figure Annex 4.5: The images show the central nervous system of 116 h old zebrafish embryos exposed with a native environmental concentration of sample T3.** The slices were stained with Rat monoclonal anti-5-HT (IgG) and 4',6-Diamidin-2-phenylindol. The image was taken at a magnification of 20x.

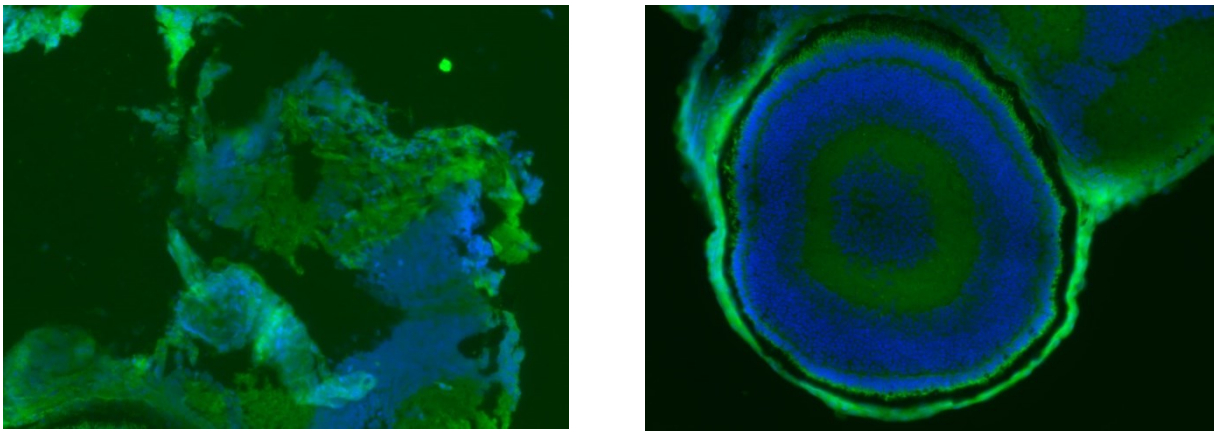

**Figure Annex 4.6: The images show the central nervous system of 116 h old zebrafish embryos exposed with a native environmental concentration of sample T4.** The slices were stained with Rat monoclonal anti-5-HT (IgG) and 4',6-Diamidin-2-phenylindol. The image was taken at a magnification of 20x.

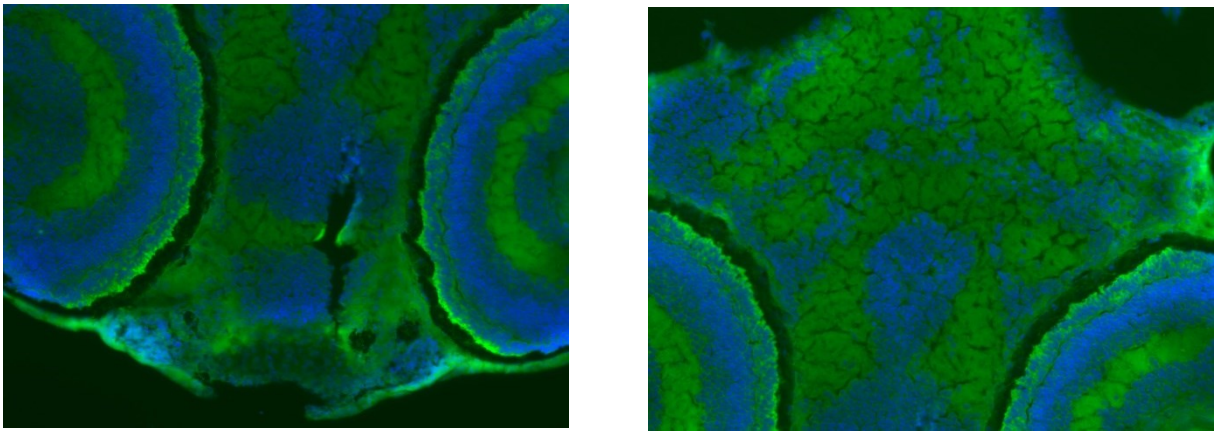

**Figure Annex 4.7: The images show the central nervous system of 116 h old zebrafish embryos exposed with a native environmental concentration of sample T5.** The slices were stained with Rat monoclonal anti-5-HT (IgG) and 4',6-Diamidin-2-phenylindol. The image was taken at a magnification of 20x.

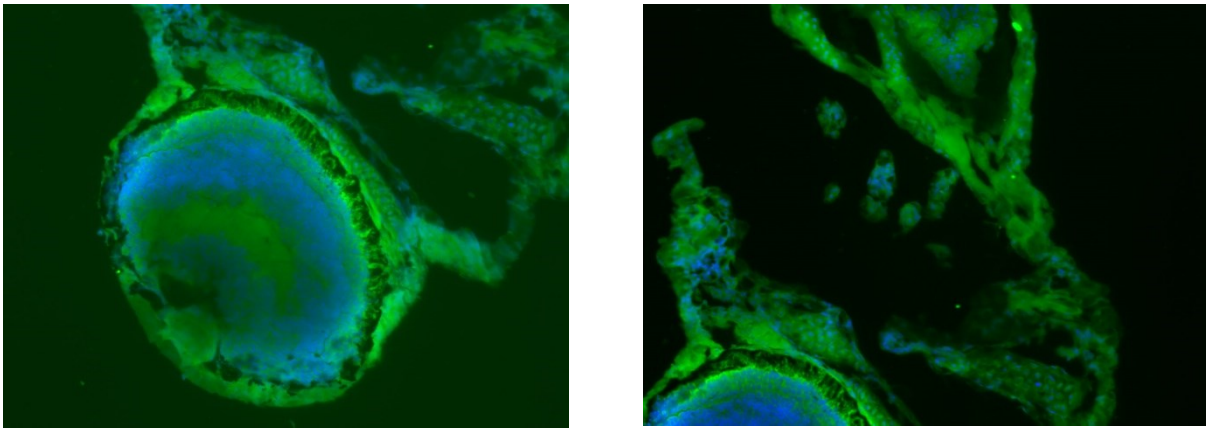

**Figure Annex 4.8: The images show the central nervous system of 116 h old zebrafish embryos exposed with a native mixture sample of T1 to T5.** The slices were stained with Rat monoclonal anti-5-HT (IgG) and 4',6-Diamidin-2-phenylindol. The image was taken at a magnification of 20x.

**Table Annex 4: Summary of the patient numbers, as well as the prescribed drugs in the period from 04.01.2020 to 06.01.2020 (04.01 / 05.01 / 06.01).** The table show the prescribed amount (in mg) over three days.

| Neuroactive substance | Number of patients | Prescribed quantities [mg] |
|---|---|---|
| **Antidepressants** | | |
| Bupropion | 3 / 3 / 3 | 900 / 900 / 900 |
| Duloxetine | 3 / 3 / 3 | 240 / 240 / 240 |
| Fluvoxamine | 0 / 0 / 1 | 0 / 0 / 12.5 |
| Mirtazapine | 9 / 9 / 8 | 172.5 / 172.5 / 172.5 |
| Sertraline | 8 / 7 / 7 | 925 / 825 / 850 |
| Venlafaxine | 7 / 7 / 8 | 1162.5 / 1162.5 / 1200 |
| **Antiepileptic drugs** | | |
| Valproic acid | 2 / 2 / 2 | 1500 / 3000 / 3000 |
| Lithium | 3 / 2 / 2 | 42.7 / 36.6 / 36.6 |
| **Antipsychotics** | | |
| Clozapine | 3 / 3 / 3 | 700 / 700 / 700 |
| Haloperidol | 2 / 2 / 2 | 6 / 6 / 6 |
| **Other** | | |
| Bisoprolol | 5 / 5 / 5 | 30 / 30 / 30 |
| Metoprolol | 3 / 3 / 3 | 237.5 / 237.5 / 237.5 |
| Diazepam | 2 / 2 / 2 | 26 / 21 / 21 |
| Estradiol | 1 / 1 / 1 | 1 / 1 / 1 |
| Ethinylestradiol | 2 / 2 / 2 | 0.06 / 0.06 / 0.06 |
| Metamizole | 2 / 1 / 1 | 2000 / 1000 / 1000 |
| Metformin | 3 / 3 / 3 | 5000 / 5000 / 5000 |
| Pantoprazole | 12 / 12 / 12 | 400 / 440 / 440 |

# Contributions to the published articles and chapters

**Chapter 1: MG:** Conceptualization, Writing – original draft and Visualization. **MRS**: Supervision. **HH:** Conceptualization, Supervision.

**Chapter 2: MG:** Conceptualization, Methodology, Investigation, Writing - original draft, Data curation, Formal analysis, Visualization. **MA**: Methodology, Resources, Formal analysis; Writing - review & editing. **DK**: Methodology, Data curation, Writing – review & editing. **KS:** Methodology, Data curation, Writing – review & editing. **MP**: Resources, Writing - review & editing. **HH:** Conceptualization, Resources, Supervision, Writing - review & editing.

**Chapter 3: MG:** Conceptualization, Data curation, Methodology, Investigation, Writing - original draft, Formal analysis, Visualization. **VS**: Methodology, Investigation, Data curation, Formal analysis. **SC:** Writing – review and editing. **MRS:** Conceptualization, Funding acquisition, Writing – review and editing. **HH:** Conceptualization, Project administration, Funding acquisition, Writing – review and editing, Supervision.

**Chapter 4: MG:** Conceptualization, Methodology, Investigation, Writing - original draft, Data curation, Formal analysis, Visualization. **MA**: Methodology, Resources, Writing - original draft, Formal analysis; Writing - review & editing. **MP**: Resources, Writing - review & editing. **HH:** Conceptualization, Resources, Supervision, Writing - review & editing.

**Chapter 5: MG:** Conceptualization, Methodology, Investigation, Writing - original draft, Formal analysis, Visualization. **MA**: Methodology, Resources, Formal analysis, Writing - review & editing. **SF:** Methodology, Data curation, Investigation, Formal analysis, Visualization, Writing - review & editing. **MK:** Methodology, Investigation, Formal analysis. **MRS:** Investigation, Formal analysis, Writing – review & editing. **WB:** Methodology, Investigation, Formal analysis, Visualization, Writing - review & editing. **VL:** Resources, Writing - review & editing. **MP**: Resources, Writing - review & editing. **FK:** Methodology, Resources. **HH:** Conceptualization, Resources, Supervision, Writing - review & editing.

**Chapter 6: MG:** Conceptualization, Writing – original draft and Visualization. **MRS**: Supervision. **HH:** Supervision.

**Chapter 7:** Conceptualization, Writing – original draft and Visualization. **MRS**: Supervision. **HH:** Supervision.

MG: Michael Gundlach[1]; DK: David Kämpfer[1]; FK: Fabian Kiessling[9]; HH: Henner Hollert[1,4]; K.E.C.S: Kilian Smith[1,8]; MA: Marc Augustin[2,3]; MK: Martin Kraus[5]; MP: Michael Paulzen[3,7]; MRS: Martina Roß-Nickoll[1]; SF: Saskia Finckh[5]; SC: Sarah E. Crawford[4]; VL: Volker Linnemann[6]; VS: Verena Schönfelder[1]; WB: Werner Brack[4,5]

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# Curriculum Vitae

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**Core competencies:** creativity, communication skills, empathy, diligence

### Career:

**05/2018 – 12/2020 Research assistant at the Institute for Environmental Research at RWTH Aachen University (PhD student for Dr.rer.nat.)**

- Scholarship-funded doctoral student at RWTH Aachen University
- Analysis of the ecotoxicological risk potential of neuroactive substances in aquatic systems
- Development of water management and regulatory solutions at the interface with ecotoxicology and medicine
- Teaching of ecological topics to students of the Master's programme in Ecotoxicology
- Acquisition of research funding and wording of ecotoxicological expert reports

**10/2016 – 04/2018 Master's programme in ecotoxicology at RWTH Aachen University**

- Interdisciplinary cooperation in the analysis of neuroactive substances in aquatic systems
- Modules on aquatic, terrestrial and regulatory ecology

**10/2013 - 10/2016 Bachelor's degree in Biology at RWTH Aachen University**

- Elective modules in the field of environmental sciences
- Bachelor thesis focusing on the impact of microplastics on the aquatic environment

**10/2012 – 10/2013 Bachelor studies in physics at RWTH Aachen University**

**07/2009 – 06/2012 Abitur at the Städt. Gymnasium Herzogenrath**

### Practical experience accompanying studies:

**10/2016 – 05/2018 Research assistant at the Institute for Environmental Research Aachen**

**04/2013 – 05/2018 Student and research assistant at the Helmholtz Institute Aachen**

# Scientific contributions

* Publications contributing to this thesis are highlighted with an asterisk:

## Research articles in international peer-reviewed journals

* Gundlach, M., Augustin, M., Smith, K.E.C., Kämpfer, D., Paulzen, M., Hollert, H. (2021): "Effects of the antidepressant mirtazapine on the swimming behaviour and gene expression rate of Danio rerio embryos – Is the sedating effect seen in humans also evident for fish?" Science of The Total Environment. 148368. doi: 10.1016/j.scitotenv.2021.148368

Gundlach, M., Di Paolo C., Chen Q., Majewski K., Haigis, A.-C., Werner, I., Hollert, H. (2021). "Clozapine modulation of zebrafish swimming behavior and gene expression as a case study to investigate effects of atypical drugs on aquatic organisms", Science of the Total Environment: 152621. https://doi.org/10.1016/j.scitotenv.2021.152621

Kase, R., Javurkova, B., Simon, E., Swart, K., Buchinger, S., Könemann, S., Escher, B.I., Carere, M., Dulio, V., Ait-Aissa, S., Holert, H., Valsecchi, S., Polesello, S., Behnisch, P., di Paolo, C., Olbrich, D., Sychrova, E., Gundlach, M., Schlichting, R., Leborgne, L., Clara, M., Scheffkneckt, C., Marneffe, Y., Chalon, C., Tusil, P., Soldan, P., von Danwitz, B., Schwaiger, J., Moran Palao, A., Bersani, F., Perceval, O., Kienle, C., Vermeirssen, E., Hilscherova, K., Reifferscheid, G., Werner, I. (2018): "Screening and risk management solutions for steroidal estrogens in surface and wastewater"´. TrAC Trends in Analytical Chemistry. 102: 343:358. doi: 10.1016/j.trac.2018.02.013

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## Platform presentations

Gundlach, M., Di Paolo, C., Haigis, A.C., Schiwy, A.H., Hollert, H. (2018): "Neuromodulatorische Effekte des atypischen Neuroleptikums Clozapin auf das Schwimmverhalten und die Genexpressionsrate von *Danio rerio* Embryonen" SETAC GLB, 09.09.2018 – 12.09.2018, Münster, Germany

Gundlach, M., Haigis, A.C., Schiwy, A.H., Hollert, H. (2019): "Neuromodulatorische Effekte von Venlafaxine auf das Schwimmverhalten und die Genexpressionsrate von *Danio rerio* Embryonen" 11th BioDetectors Confernce, 13.09.2018 – 14.09-2018, Aachen, Germany

Gundlach, M., Haigis, A-C., Schiwy, A.H., Hollert, H. (2018): "Neurotoxicological analysis of psychiatric drugs in hospital wastewater with special focus on behavior and gene expression tests with *Danio rerio*" 2. Young Water Researchers Symposium, 26.11.2018, Aachen Germany

Gundlach, M., Haigis, A-C., Hollert, H. (2018): "Adverse outcome pathways - A tool for the ecotoxicological analysis of antidepressants in hospital wastewater" Fish and Amphibian Embryos as Alternative Models in Toxicology and Teratology, 28.11.2018 – 30.11.2018, Paris, France

## Poster presentations

Gundlach, M., Chen, Q., Schiwy, S., Yang, S., Jiang, J., Velki, M., Yin, D., Hollert, H. (2017): „Coexposure effects of 17α-ethinylestradiol in the presence of micro- and nanoplastic on embryo development and larvae behavior of Danio rerio" Setac Europe, 07.05.2017-11.05.2017, Brussels, Belgium

Gundlach, M., Chen, Q., Schiwy, S., Hollert, H. (2018): "Coexposure effects of 17α-ethinylestradiol in the presence of micro- and nanoplastic on embryo development and larvae behavior of Danio rerio" SETAC GLB, 09.09.2018 – 12.09.2018, Münster, Germany

Gundlach, M., Di Paolo, C., Hollert, H. (2019): "The principle of the adverse outcome Pathway in the context of testing the antidepressant venlafaxine" Setac Europe, 26 – 30.05.2019, Helsinki, Finland

Gundlach, M., Di Paolo, C., Hollert, H. (2019): "The neuroactive cocktail: How to analyse the effects of the antidepressant clozapine in an AOP context" Setac Europe, 26 – 30.05.2019, Helsinki, Finland