TEXTE
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"Sour doesn´t always make you smile"

Effect of pH on the toxicity and bioaccumulation of ionic substances

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Effect of pH on the toxicity and bioaccumulation of ionic substances

Acronym: pHION

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Abstract: "Sour doesn´t always make you smile" – Effect of pH on the toxicity and bioaccumulation of ionic substances

Depending on the pH, ionizable substances are present in varying proportions in their neutral or charged form. The extent to which these two chemical species contribute to the toxicity of ionizable chemicals and whether intracellular ion trapping has a decisive influence in this context is controversially discussed. Against this background, we determined the acute toxicity of more than 20 ionizable substances, each at different pH values, on the embryonic development of the zebrafish, *Danio rerio*, and supplemented this dataset with additional data from the literature. Using a dataset of ten ionisable substances (the acids diclofenac, ibuprofen, naproxen and triclosan and the bases citalopram, fluoxetine, metoprolol, propranolol, tramadol and tetracaine) at four external pH levels we detected a high correlation between mortality (LC50) at 96 hpf and reduced heart rate (EC20) at 48 hours post fertilization for all compounds and all external pH levels. Moreover, the observed pH-dependent effects were strongly associated with log D and thus likely driven by differences in uptake (toxicokinetic) rather than internal (toxicodynamic) processes. To simulate the toxicity of the entire dataset comprising 12 acids and 12 bases, models were created based on different premises for the uptake and toxic effects of neutal and ionic species, and their abilities to explain the real data set were assessed. Using this approach, we were able to show that both neutral and charged species are taken up into cells according to their logD-based distribution, that both species exert basal toxicity intracellularly in a quantitatively similar manner, and that intracellular ion trapping thus plays no quantitative role in the exerted toxicity. Furthermore, it was possible to attribute differences in toxicity at different pH values for these 24 ionizable substances to the respective differences in logD with high accuracy, enabling this model to be used for predicting potential toxicities in worst-case scenarios, as required in the process of registration of ionizable chemicals and the definition of Environmental Quality Standards (EQS). The studies were accompanied by detailed chemical analyses on exposure and accumulation of the substances. This applied both to the exposure to the respective LC50s of the substances investigated in the tsts with *D. rerio* and, in parallel, to chemicals selected by the Federal Environment Agency for exemplary tests with *Daphnia magna* and *Lemna minor*.

Kurzbeschreibung: Sauer ist nicht immer lustig – Effekt des pH-Wertes auf die Toxizität und Bioakkumulation ionischer Stoffe

Je nach pH-Wert liegen ionisierbare Stoffe in unterschiedlichen Anteilen in ihrer neutralen oder geladenen Form vor. Inwieweit diese beiden chemischen Spezies zur Toxizität ionisierbarer Chemikalien beitragen und ob intrazelluläres *Ion-Trapping* in diesem Zusammenhang einen entscheidenden Einfluss hat, wird kontrovers diskutiert. Vor diesem Hintergrund haben wir die akute Toxizität von mehr als 20 ionisierbaren Substanzen, jeweils bei unterschiedlichen pH-Werten, auf die Embryonalentwicklung des Zebrabärblings *Danio rerio* bestimmt und diesen Datensatz mit zusätzlichen Daten aus der Literatur ergänzt. Unter Verwendung eines Datensatzes von zehn ionisierbaren Substanzen (die Säuren Diclofenac, Ibuprofen, Naproxen und Triclosan und die Basen Citalopram, Fluoxetin, Metoprolol, Propranolol, Tramadol und Tetracain) bei vier externen pH-Werten konnten wir eine hohe Korrelation zwischen der Sterblichkeit (LC50) bei 96 hpf und der reduzierten Herzfrequenz (EC20) bei 48 Stunden nach der Befruchtung für alle Verbindungen und alle externen pH-Werte feststellen. Darüber hinaus waren die beobachteten pH-abhängigen Effekte stark mit dem logD-Wert assoziiert und daher wahrscheinlich eher auf Unterschiede bei der Aufnahme (toxikokinetisch) als auf interne (toxikodynamische) Prozesse zurückzuführen. Um die Toxizität des gesamten Datensatzes, der 12 Säuren und 12 Basen umfasst, zu simulieren, wurden Modelle erstellt, die auf verschiedenen Prämissen für die Aufnahme und die toxischen Wirkungen von neutralen und ionischen Spezies beruhen, und ihre Fähigkeiten zur Erklärung des realen Datensatzes wurden bewertet. Mit diesem Ansatz konnten wir zeigen, dass sowohl neutrale als auch geladene Spezies entsprechend ihrer logD-basierten Verteilung in die Zellen aufgenommen werden, dass beide

Spezies in quantitativ ähnlicher Weise intrazellulär eine basale Toxizität ausüben und dass intrazelluläres *Ion-Trapping* somit keine quantitative Rolle für die resultierende Toxizität spielt. Darüber hinaus war es möglich, die Unterschiede in der Toxizität bei verschiedenen pH-Werten für diese 24 ionisierbaren Stoffe mit hoher Genauigkeit den jeweiligen Unterschieden im logD zuzuordnen, so dass dieses Modell für die Vorhersage potenzieller Toxizitäten in *Worst-Case*-Szenarien verwendet werden kann, wie sie im Prozess der Registrierung ionisierbarer Chemikalien und der Festlegung von Umweltqualitätsnormen (UQN) erforderlich sind. Die Studien wurden von detaillierten chemischen Analysen zur Exposition und Akkumulation der Substanzen begleitet. Dies galt sowohl für die Exposition gegenüber den jeweiligen LC50 der untersuchten Stoffe in den Tests mit *D. rerio* als auch parallel dazu für die vom Umweltbundesamt exemplarisch ausgewählten Chemikalien in Tests mit *Daphnia magna* und *Lemna minor*.

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CO_2	Carbon dioxide
COP	Conference of the Parties
EU-ETS	EU Emissions Trading Scheme
F-gases	Fluorinated greenhouse gases
FTIP	Federal Transport Infrastructure Plan
GHG	Greenhouse gas
HGV	Heavy goods vehicle
ICAO	International Civil Aviation Organization
IMO	International Maritime Organization
KSBV	UBA-study Klimaschutzbeitrag des Verkehrs bis 2050 [UBA, 2016a]
NDC	Nationally Determined Contributions (in Paris-Agreement)
NEDC	New European Driving Cycle
N_2O	Nitrous oxide (laughing gas)
PJ	Petajoule (energy measuring unit)
PtG	Power-to-Gas (any power-based gaseous fuels)
PtL	Power-to-Liquid (any power-based liquid fuels)
RDE	Real Driving Emissions
TWh	Terawatt hours (measuring units for energy)
UNFCCC	United Nations Framework Convention on Climate Change
WLTP	Worldwide Harmonized Light-Duty Vehicles Test Procedure

Summary

It has long been known that the quantitative uptake of organic chemicals into cells and, associated with this, their potential to be toxic, depends on the lipophilicity of these substances according to their octanol water coefficient (Pow) or lipid water coefficient (Plipw). Pow and Plipw or their logarithms describe the relationship between lipophilicity and hydrophilicity of a given substance. High logP values characterise lipophilic chemicals, low hydrophilic ones. For substances with variable lipophilicity such as ionisable chemicals, however, this relationship is much more complicated and thus requires a more differentiated view. An ionizable substance can be present in several species, neutral or ionic, by association or dissociation, and each of these species can be assigned its own logP value. The partitioning of these species, however, is pH-dependent. To account for different partition patterns of neutral and ionic species, the actual lipophilicity of an ionizable compound under given pH conditions is denoted by the partition coefficient logD.

Due to their sheer number, ionisable compounds are of great importance in the environment. Depending on environmentally relevant pH fluctuations in the medium, these substances switch their speciation and lipophilicity among different states of charge which changes their ability to be taken up by cells and to exert toxic effects in them.

In order to record and simulate the toxicity of ionisable chemicals in ecotoxicological tests at different pH values and finally to develop a model for the reliable predictability of toxicities in worst case scenarios, a stepwise approach was taken.

(1) In a first step, an extensive literature research was compiled. Substances were identified that could be relevant for the practical work on the issue addressed in this project, as well as those for which toxicity data or BCF studies at different pH values have already been reported. For these substances, the most important physicochemical properties from the point of view of this project, in particular data on pH-dependent log D, for both Kow and Klipw, were listed. Furthermore, we compiled existing models in the literature for calculating the internal concentration of chemicals in different species.

We selected about 30 substances to be tested or modelled on the basis oft he following criteria

- Stability in aquatic solution: Although our project also detects degradation products of the test chemicals and metabolites, the focus was on the parent compound.
- lWater solubility: Since the biotests were performed focus on apical endpoints of rather limited sensitivity (compared to e.g. biochemical markers), it was assumed that relatively high concentrations of chemicals had to be tested. For this, a good water solubility of the test substances was a prerequisite.
- Maximum possible differences in pH-dependent characteristics: Since our project particularly investigated the pH dependence of bioaccumulation and toxicity of ionisable chemicals, the characteristics determining these should also show the greatest possible differences in the pH spectrum.
- “Plateau”: Besides maximal differences, substances with no or only little differences (“plateaus”) in log D at the tested pH limits (between: pH 5 and 6; pH 8 and 9) were also selected and often tested at an additional fourth pH level. These log D “plateaus” reflect pH ranges in which the substance is entirely neutral or ionised. Little or no toxicity differences in these ranges would further confirm the dependence of toxicity in relation to the ionised state of the substance which is in turn dependent on pH.

- Environmental relevance of the chemical: Since this project investigated general principles, an environmental relevance of the test substances is desirable, but not absolutely necessary. If there was a choice between two otherwise equivalent candidates, the chemical with the higher environmental relevance was selected.

(2) Within the framework of this project, numerous data on the toxicity of chemicals had to be collected. This was done mostly via embryotoxicity tests with the zebrafish (*Danio rerio*) and to a lesser extent with *Daphnia* tests (*Daphnia magna*) and studies with *Lemna minor*. At the beginning of the work, these tests, which were based on OECD guidelines, were optimised for the different pH conditions to be tested, which made it necessary to use a wide variety of buffers. In the end, however, it was possible to conduct highly reproducible studies in a pH range of 5 to 9, which completely covered the environmentally relevant range.

The effect studies conducted showed a very good correlation of mortality at the end of the test and the heart rate of the animals during exposure, so that the latter parameter could be established as a good proxy for acute toxicity. Furthermore, a clear dependence of toxicity on the pH in the medium was shown for all tested substances. These effect data, supplemented to a small extent by data from the literature, formed the basis for the subsequent modelling, which led to the development of a methodology for predicting toxicities.

(3) All the exposure studies were accompanied by chemical analytics of water/medium samples and exposed biota. The analysis of the exposure water samples and biota (*D. rerio*) extracts was carried out using an LC-ESI-QTOF system: Ultrahigh-performance liquid chromatography (UHPLC) system with an HPG-3400 pump (Dionex Ultimate 3000 RSLC, Thermo Fischer Scientific, Dreieich, Germany) coupled to a QTOF mass spectrometer. The QTOF system was equipped with an electrospray ionization interface (ESI), operating in positive and negative modes. For some selected compounds the analysis of the water and fish embryo samples from the exposure experiment to an LC-MS/MS system was used. The purpose of these studies was the validation of nominal concentrations of chemicals in the exposure media and the calculation of bioconcentration factors. Across all chemicals, chemical analytics revealed in the majority of cases (93 out of 121) a recovery (i.e. nominal vs. measured concentration) rate of 30% or higher, and in about half of the cases a rate of 50% or higher. Across most of the chemicals, the measured concentration of the water samples from the start (0 h) and the end (96 h) of the exposure experiments, were at the same range if the standard deviation was taken into consideration. For this reason, all modelling conducted in the frame of this project was based on the nominal effect concentrations for each chemical.

(4) The final modelling approach was conducted for *D. rerio* acute toxicity data (LC50), determined for 24 substances. The pH dependence of embryotoxicity exerted by these 24 chemicals could be reproduced in very different quality by six different models that took different mechanisms of chemical uptake and intracellular action into account. Some models, which took into account multiple aspects of the possible uptake pathways and theoretically possible toxic effects of charged and uncharged chemical species, reproduced the observed differences of these pH dependencies very well, others, however, only insufficiently. Although the potential for reliable modelling of real data was indeed substance-specific and thus some models were suitable for selected chemicals only and less suitable for others, it became clear that particularly the model considering uptake and toxicity of not only the neutral chemical species but also that of the ionic species, i.e. a ΔlogD -based model on the basis of Pow, performed best and mirrored the effect data extremely well. It was obvious that in this model the linearised dependence of log LC50 and 'effective' log D runs almost identical to the bisector through the 1:1 origin in a nice uniformity for all substances investigated. Using this correlation, it is thus now possible, with the help of the ΔlogD (Pow-based) model, to infer very reliably from

a known LC50 for an ionisable chemical at a given pH to the LC50 of the same chemical at a different pH, e.g. within the framework of worst-case scenarios.

This model has outstanding potential for implementation in the registration and authorization of chemicals due to its exceptionally high precision to predict toxicities at pH levels under worst-case scenarios. It was already applied for the first time by the EU in December 2022 in deriving the draft EQS value for ibuprofen, taking into account a worst-case scenario for surface waters modelled in this way. It would be desirable if this approach was used also for future EQS derivations for ionizable chemicals and in the process of registration and authorization of such compounds.

Zusammenfassung

Es ist seit langem bekannt, dass die quantitative Aufnahme organischer Chemikalien in die Zellen und damit verbunden ihr toxisches Potenzial von der Lipophilie dieser Stoffe entsprechend ihrem Octanol-Wasser-Koeffizienten (Pow) oder Lipid-Wasser-Koeffizienten (Plipw) abhängt. Pow und Plipw oder ihre Logarithmen beschreiben das Verhältnis zwischen Lipophilie und Hydrophilie einer bestimmten Substanz. Hohe logP-Werte kennzeichnen lipophile Chemikalien, niedrige hydrophile Chemikalien. Bei Stoffen mit variabler Lipophilie, wie z. B. ionisierbaren Chemikalien, ist diese Beziehung jedoch viel komplizierter und erfordert daher eine differenziertere Betrachtung. Eine ionisierbare Substanz kann durch Assoziation oder Dissoziation in mehreren Spezies, neutral oder ionisch, vorliegen, und jeder dieser Spezies kann ein eigener logP-Wert zugeordnet werden. Die Verteilung dieser Spezies ist jedoch pH-abhängig. Um den unterschiedlichen Verteilungsmustern von neutralen und ionischen Spezies Rechnung zu tragen, wird die tatsächliche Lipophilie einer ionisierbaren Verbindung unter gegebenen pH-Bedingungen durch den Verteilungskoeffizienten logD angegeben.

Aufgrund ihrer schieren Anzahl sind ionisierbare Verbindungen in der Umwelt von großer Bedeutung. In Abhängigkeit von umweltrelevanten pH-Schwankungen im Medium wechseln diese Stoffe ihre Speziation und Lipophilie zwischen verschiedenen Ladungszuständen, wodurch sich ihre Fähigkeit ändert, von Zellen aufgenommen zu werden und in ihnen toxische Wirkungen zu entfalten.

Um die Toxizität von ionisierbaren Chemikalien in ökotoxikologischen Tests bei verschiedenen pH-Werten zu erfassen und zu simulieren und schließlich ein Modell zur verlässlichen Voraussagbarkeit von Toxizitäten in worst case-Szenarien zu entwickeln, wurde stufenweise vorgegangen.

(1) In einem ersten Schritt wurde eine umfangreiche Literaturrecherche durchgeführt. Es wurden Stoffe identifiziert, die für die praktische Arbeit an dem in diesem Projekt behandelten Thema relevant sein könnten, sowie solche, für die bereits Toxizitätsdaten oder BCF-Studien bei verschiedenen pH-Werten berichtet wurden. Für diese Stoffe wurden die aus Sicht dieses Projekts wichtigsten physikalisch-chemischen Eigenschaften, insbesondere Daten zu pH-abhängigem log D, sowohl für Kow als auch für Klipw, aufgelistet. Darüber hinaus haben wir in der Literatur vorhandene Modelle zur Berechnung der internen Konzentration von Chemikalien in verschiedenen Arten zusammengestellt.

Wir wählten etwa 30 Substanzen aus, die wir anhand der folgenden Kriterien testeten oder modellierten

- Stabilität in aquatischer Lösung: Obwohl in unserem Projekt auch Abbauprodukte der Testchemikalien und Metaboliten nachgewiesen werden, lag der Schwerpunkt auf der Ausgangsverbindung.
- Wasserlöslichkeit: Da sich die eingesetzten Biotests auf apikale Endpunkte von eher begrenzter Empfindlichkeit (im Vergleich zu z. B. biochemischen Markern) konzentrierten, wurde davon ausgegangen, dass relativ hohe Konzentrationen von Chemikalien getestet werden mussten. Dafür war eine gute Wasserlöslichkeit der Testsubstanzen eine Voraussetzung.
- Größtmögliche Unterschiede bei pH-abhängigen Merkmalen: Da in unserem Projekt insbesondere die pH-Abhängigkeit der Bioakkumulation und Toxizität ionisierbarer Chemikalien untersucht wurde, sollten auch die diese bestimmenden Merkmale möglichst große Unterschiede im pH-Spektrum aufweisen.

- "Plateau": Neben den maximalen Unterschieden wurden auch Stoffe ausgewählt, die bei den getesteten pH-Grenzwerten (zwischen: pH 5 und 6; pH 8 und 9) keine oder nur geringe Unterschiede ("Plateaus") in log D aufwiesen, und oft bei einem zusätzlichen vierten pH-Wert getestet. Diese log D-"Plateaus" spiegeln pH-Bereiche wider, in denen die Substanz vollständig neutral oder ionisiert ist. Geringe oder keine Toxizitätsunterschiede in diesen Bereichen würden die Abhängigkeit der Toxizität vom ionisierten Zustand der Substanz, der wiederum vom pH-Wert abhängt, weiter bestätigen.

- Umweltrelevanz der Chemikalie: Da in diesem Projekt allgemeine Grundsätze untersucht wurden, ist eine Umweltrelevanz der Prüfsubstanzen wünschenswert, aber nicht unbedingt erforderlich. Wenn die Wahl zwischen zwei ansonsten gleichwertigen Kandidaten bestand, wurde die Chemikalie mit der höheren Umweltrelevanz ausgewählt.

(2) Im Rahmen dieses Projektes mussten zahlreiche Daten zur Toxizität von Chemikalien erhoben werden. Dies erfolgte zum größten Teil über Embryotoxizitätstests mit dem Zebrabärbling (*Danio rerio*) und zum geringeren Teil mit Daphnientests (*Daphnia magna*) und Studien mit *Lemna minor*. Zu Beginn der Arbeiten wurden diese Tests, die sich an OECD-Richtlinien orientierten, für die unterschiedlichen zu testenden pH-Bedingungen optimiert, was den Einsatz unterschiedlichster Puffer notwendig machte. Letztlich war es aber möglich, hoch reproduzierbar Studien in einem pH-Bereich von 5 bis 9 durchzuführen, was den umweltrelevanten Bereich vollständig abdeckte.

Die durchgeführten Effektstudien zeigten für *Danio rerio* eine sehr gute Korrelation von Mortalität zum Ende des Tests und der Herzschlagrate der Tiere während der Exposition, so dass letzterer Parameter als guter Proxy für akute Toxizität etabliert werden konnte. Des Weiteren zeigte sich für alle getesteten Substanzen eine deutliche Abhängigkeit der Toxizität vom pH im Medium. Diese Effektdaten, zu einem geringen Teil supplementiert durch Daten aus der Literatur, bildeten die Grundlage für die anschließende Modellierung, die zur Entwicklung einer Methodologie für eine Voraussage von Toxizitäten führte.

(3) Alle Expositionsstudien wurden von chemischen Analysen von Wasser-/Mediumproben und den exponierten Biota begleitet. Die Analyse der Expositions-Wasserproben und der Extrakte der Biota (*D. rerio*) wurde mit einem LC-ESI-QTOF-System durchgeführt: Es wurde ein Ultrahochleistungs-Flüssigkeitschromatographie (UHPLC)-System mit einer HPG-3400-Pumpe (Dionex Ultimate 3000 RSLC, Thermo Fischer Scientific, Dreieich, Deutschland) gekoppelt an ein QTOF-Massenspektrometer verwendet. Das QTOF-System war mit einer Elektrospray-Ionisierungsschnittstelle (ESI) ausgestattet, die im positiven und negativen Modus arbeitete. Für einige ausgewählte Verbindungen wurde die Analyse der Wasser- und Fischembryo-Proben aus dem Expositionsversuch mit einem LC-MS/MS-System durchgeführt. Der Zweck dieser Studien war die Validierung der nominalen Konzentrationen von Chemikalien in den Expositionsmedien und die Berechnung von Biokonzentrationsfaktoren. Für alle Chemikalien ergab die chemische Analytik in der Mehrzahl der Fälle (93 von 121) eine Wiederfindungsrate (d. h. nominale gegenüber gemessener Konzentration) von 30 % oder mehr und in etwa der Hälfte der Fälle eine Rate von 50 % oder mehr. Bei den meisten Chemikalien lagen die gemessenen Konzentrationen der Wasserproben zu Beginn (0 h) und am Ende (96 h) der Expositionsversuche im gleichen Bereich, wenn man die Standardabweichung berücksichtigt. Aus diesem Grund basierten alle im Rahmen des Projektes durchgeführten Modellierungen auf den nominalen Effektkonzentrationen für jede Chemikalie.

(4) Der abschließende Modellierungsansatz wurde für Daten zur akuten Toxizität (LC50) von *D. rerio* durchgeführt, die für 24 Substanzen ermittelt wurden. Die pH-Abhängigkeit der Embryotoxizität dieser 24 Chemikalien konnte durch sechs verschiedene Modelle, die unterschiedliche Mechanismen der Aufnahme von Chemikalien und der intrazellulären Wirkung

berücksichtigten, in sehr unterschiedlicher Qualität wiedergegeben werden. Einige Modelle, die mehrere Aspekte der möglichen Aufnahmewege und theoretisch möglichen toxischen Wirkungen geladener und ungeladener chemischer Spezies berücksichtigten, reproduzierten die beobachteten Unterschiede dieser pH-Abhängigkeiten sehr gut, andere hingegen nur unzureichend. Obwohl das Potenzial für eine zuverlässige Modellierung der realen Daten in der Tat stoffspezifisch war und daher einige Modelle nur für ausgewählte Chemikalien und weniger für andere geeignet waren, wurde deutlich, dass insbesondere das Modell, das die Aufnahme und Toxizität nicht nur der neutralen chemischen Spezies, sondern auch die der ionischen Spezies berücksichtigte, d.h. ein ΔlogD -basiertes Modell auf der Grundlage von Pow, am besten abschnitt und die Wirkungsdaten äußerst gut widerspiegelte. Es war offensichtlich, dass in diesem Modell die linearisierte Abhängigkeit von log LC50 und "effektivem" log D fast identisch mit der Winkelhalbierenden durch den 1:1-Ursprung verläuft, und zwar in schöner Gleichförmigkeit für alle untersuchten Substanzen. Mit Hilfe dieses Zusammenhangs ist es nun also möglich, mit Hilfe des ΔlogD (Pow-basierten) Modells von einer bekannten LC50 für eine ionisierbare Chemikalie bei einem bestimmten pH-Wert sehr zuverlässig auf die LC50 der gleichen Chemikalie bei einem anderen pH-Wert zu schließen, z.B. im Rahmen von Worst-Case-Szenarien.

Dieses Modell hat ein hervorragendes Potenzial für den Einsatz bei der Registrierung und Zulassung von Chemikalien aufgrund seiner außergewöhnlich hohen Genauigkeit bei der Vorhersage von Toxizitäten bei pH-Werten unter Worst-Case-Szenarien. Es wurde von der EU bereits im Dezember 2022 bei der Ableitung des Entwurfs der Umweltqualitätsnorm für Ibuprofen unter Berücksichtigung eines Worst-Case-Szenarios für Oberflächengewässer, die auf diese Weise modelliert wurden, zum ersten Mal angewendet. Es wäre wünschenswert, wenn dieser Ansatz auch für künftige UQN-Ableitungen für ionisierbare Chemikalien und für den Prozess der Registrierung und Zulassung solcher Verbindungen verwendet würde.

1 WP 1: Literature research on the subject of bioaccumulation and toxicity of ionisable substances

1.1 Preliminary remarks

Work package 1 (WP1) addresses the existing literature on the topic of "bioaccumulation and toxicity of ionisable substances". The literature research on this topic was completed. Substances were identified that could be relevant for the practical work on the issue addressed in pHION, as well as those for which toxicity data or BCF studies at different pH values have already been reported. For these substances, the most important physicochemical properties from the point of view of pHION, in particular data on pH-dependent log D, for both Kow and K_{lipw}, were compiled. This has been done for selected substances for which the practical work has been started. The toxicity data and BCF values available in the literature were then checked for their reliability in order to assess their suitability for modelling. On the basis of the accessible information, however, no serious deficiencies can be identified in any of the published studies on this topic. Only in a few cases could a more precise determination of effect concentrations probably have been made by expanding the data. Furthermore, the models proposed in the literature for calculating internal concentrations are compiled in this report in a condensed manner.

1.2 Literature research on published toxicity/effect values and bioaccumulation data for aquatic species determined for at least two different pH values

The databases "Web of Science" and "Google Scholar" were used for the literature research. The search for suitable literature was difficult due to the task, as no simple combinations of search terms could be used. Even combinations of chemical names with the search term "pH*" often did not lead to the desired result, since in most studies only one pH value was considered. Nevertheless, a database on toxicity data for a number of chemicals at more than one pH (Table 1) could be created. These results derive from work by Carolin Rieber conducted at the University of Tübingen.

The toxicity of ionisable compounds differs whenever various pHs of the medium are tested. Present studies however often investigate the toxicity only at a single specific pH value. The function of this review is to get an overview of the already existing studies where the toxicity of ionisable chemicals is specified by at least two different pH values. In total, a considerable number of different substances have been already tested and the results were published in papers. For example, for a single paper (Baumer et al. 2017), an extensive list of information on the impact of various pharmaceuticals on *Aliivibrio fischeri* is accessible in the form of electronic supplementary material. The literature was scanned for toxicity tests of ionisable substances which were tested for at least two different pH values.

The toxicity of ionisable substances in aquatic medium is measured in many studies. This measurement was taken for one specific pH value. However, the characteristics of ionisable chemicals are changing in dependence of the pH of the test medium, as the substance exists in the dissociated or in the neutral form. That change of characteristics presumably affects the absorption, accumulation and hence the toxic effects on the exposed organism.

There has been a study on the basic existent literature from Rendal et al. (2011). This study shows that acids and bases differ in their toxicity and bioconcentration factors (BCF) at different pH-values (pH 6-9) depending on the proportion of non-dissociated (neutral) and dissociated (ionic) species of the chemical at a specific pH-value. From this knowledge we can assume that neutral species are better absorbed into the cells of organisms. Within tissues, the neutral species accumulate faster and thus also are supposed to faster reach the lethal body burden (LBB) than ionic species. We thus assume that uncharged species have a higher toxicity than the charged species respectively.

There are already some approaches to determine toxic estimation from physicochemical criteria. However, generalisation of such procedure remains uncertain. Rendal et al. (2011) suggested to determine the BCFs at given pH values from the difference between pH and pk_s- values. This thought is not satisfactory for weak bases.
Determining the toxicity on the basis of LBB is suitable for neutral species, but it is not sufficiently proven for ions. A study suggests that the intracellular effect concentration for β-blocker is independent from the outer pH value (Bittner et al. 2018). The significance of ion trapping (Neuwoehner and Escher 2011) in the relation is only known for few substances.

Above illustrated approaches to estimate the toxicity only consider the individual physicochemical parameters but not the precise structure of the molecules. Therefore, the most promising procedure to predict a substance's toxicity at different conditions is the application of quantitative structure-toxicity relationship (QSTR-Models). The applicability of such models have been already proven (Aalizadeh et al. 2017).

Used databases were "Google Scholar" and "Web of Science" with keywords like "toxicity", "pH value", "ionising substances", pharmaceuticals" and "pH dependence" to get relevant search results. All substances that were found to be toxicity-tested in the published literature are listed in Table 1.

Table 1: Toxicity of ionisable chemicals at different pH values and their physicochemical parameters

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
3380-34-5	Clc2cc(Cl)ccc2Oc1ccc(Cl)cc1O	**Triclosan**	*Danio rerio embryos*	289,55	4,76	5	8		-	0,305	0,41	0,13	4,97	4,50	0,47	PHION
3380-34-6	Clc2cc(Cl)ccc2Oc1ccc(Cl)cc1O	**Triclosan**	*Danio rerio embryos*	289,55	4,76	8	9		-	0,41	0,65	0,20	4,50	3,70	0,80	PHION
525-66-6	CC(C)NCC(O)COc1cccc2ccccc12	**Propranolol**	*Danio rerio embryos*	259,35	2,59	6	9		-	436,54	0,67	-2,81	-0,66	1,83	-2,49	PHION
15307-86-5	OC(=O)Cc1ccccc1Nc2c(Cl)cccc2Cl	**Diclofenac**	*Danio rerio embryos*	296,15	4,51	5	8		-	0,067	20	2,47	3,21	0,85	2,36	PHION
59729-33-8	c12C(CCCN(C)C)(c3ccc(F)cc3)OCc1cc(C#N)cc2	**Citalopram**	*Danio rerio embryos*	324,4	3,74	6	8		-	400	20	-1,30	0,44	1,98	-1,54	PHION
59729-33-9	c12C(CCCN(C)C)(c3ccc(F)cc3)OCc1cc(C#N)cc3	**Citalopram**	*Danio rerio embryos*	324,4	3,74	6	9			400	2,34	-2,23	0,44	2,91	-2,47	PHION
525-66-6	CC(C)NCC(O)COc1cccc2ccccc12	**Propranolol**	*Danio rerio embryos*	259,35	2,59	5,5	8		2.417 - 0.023	643	6	-2,02	-0,66	0,92	-1,58	Bittner 2018

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
51384-51-1	CC(C)NCC(O)COc1ccc(CCOC)cc1	**Metropolol**	*Danio rerio embryos*	267,37	1,88	7	8.6		1.914 - 0.054	509	14	-1,55	-0,81	0,80	-1,61	Bittner 2018
29122-68-7	CC(C)NCC(COc1ccc(cc1)CC(=O)N)O	**Atenolol**	*Danio rerio embryos*	266,34	0,16	5,5	8		> 10 mM	2660	n.a.		-2,75	-1,24	-1,51	Bittner 2018
36894-69-6	CC(CCc1ccccc1)NCC(c2ccc(c(c2)C(=O)N)O)O	**Labetalol**	*Danio rerio embryos*	328,41	3,09	5,5	8		up to 6.5 mM	2132	492		-0,05	1,66	-1,71	Bittner 2018
34580-13-7	C1(\c2c(C(=O)Cc3c1cccc3)scc2)=C1\CCN(C)CC1	**Ketotifen**	*Danio rerio embryos*	309,43	3,84	5,5	8		1.98 - 0.026	613	8	-1,88	1,72	3,29	-1,57	Bittner 2019
562-10-7	CC(c1ccccc1)(c2ccccn2)OCCN(C)C	**Doxylamine**	*Danio rerio embryos*	270,369	-1,06	7	8		0.954 - 0.171	258	46	-0,75	1,10	2,04	-0,94	Bittner 2019
5636-83-9	CC(c1ccccn1)C2=C(Cc3c2cccc3)CCN(C)C	**Dimethindene**	*Danio rerio embryos*	292,34	4,98	7	8		0.168 - 0.023	49	7	-0,86	1,10	2,03	-0,93	Bittner 2019
83881-51-0	c1ccccc1C(c2ccc(Cl)cc2)N3CCN(CCOCC(=O)O)CC3	**Cetirizine**	*Danio rerio embryos*	309,43	-0,61	7	8		3.45 - 2.29	1068	709	-0,18	0,77	0,40	0,37	Bittner 2019
83881-51-1	c1ccccc1C(c2ccc(Cl)cc2)N3CCN(CCOCC(=O)O)CC4	**Cetirizine**	*Danio rerio embryos*	309,43		5,5	7		2.67 - 3.45	826	1068	0,11	0,87	0,77	0,10	Bittner 2019

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
79516-68-0	C[C@H]1[C@@](CCN(C1)[C@H]2CC[C@@](CC2)(	**Levocabastine**	*Danio rerio embryos*	420,53	2,19	5,5	8		up to 92 µM	n.a.	38	-	2,45	2,50	-0,05	Bittner 2019
79617-96-2	Clc3c(ccc(c3)C1(c2c(cccc2)C(CC1)(NC)))Cl	**Sertraline**	*D. rerio larvae*	306,24	5,29	5,8	7,0		0.00275 - 0.00078	0,8	0,2	-0,55	2,08	2,67	-0,59	Alsop & Wilson 2019
79617-96-3	Clc3c(ccc(c3)C1(c2c(cccc2)C(CC1)(NC)))Cl	**Sertraline**	*D. rerio larvae*	306,24	5,29	7,0	8,2		0.00078 - 0.00067	0,2	0,2	-0,07	2,67	3,58	-0,91	Alsop & Wilson 2019
54910-89-3	CNCCC(c2ccccc2)Oc1ccc(cc1)C(F)(F)F	**Fluoxetine**	*D. rerio larvae*	309,33	4,64	5,8 - 6,6	8,2 - 8,4		0.00859 - 0.00118	2,7	0,4	-0,86	1,30	2,38	-1,08	Alsop & Wilson 2019
15307-86-5	OC(=O)Cc1ccccc1Nc2c(Cl)cccc2Cl	**Diclofenac**	*D. rerio larvae*	296,15	4,51	5,8	7		0.015 - > 0.035	4,4	20,7	0,67	2,26	1,37	0,89	Alsop & Wilson 2019
54910-89-3	CNCCC(c2ccccc2)Oc1ccc(cc1)C(F)(F)F	**Fluoxetine**	*P. promelas*	309,33	4,64	7	9		-	5,5	0,2	-1,44	1,50	3,31	-1,81	Nakamura et al. 2007

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
79617-96-2	Clc3c(ccc(c3)C1(c2c(cccc2)C(CC1)(NC)))Cl	**Sertraline**	*P. promelas*	306,24	5,29	6,5	8,5		-	647	72	-0,95	2,45	3,90	-1,45	Valenti et al.2009
21312-10-7	N1=C(NS(=O)(=O)c2ccc(NC(=O)C)cc2)C=C(C)O1	**Acetyl-sulfamethoxazole**	*Aliivibrio fischeri*	295,31	1,21	5,5	6	-3,3 bis -2,94		148	339	0,36	0,68	0,55	0,13	Baumer et al.2017
41859-67-0	OC(=O)C(C)(C)Oc1ccc(CCNC(=O)c2ccc(Cl)cc2)cc1	**Bezafibrate**	*Aliivibrio fischeri*	361,83	4,25	5,5	7	-4,2 bis -2,59		23	930	1,61	2,30	0,97	1,33	Baumer et al.2017
882-09-7	CC(C)(Oc1ccc(Cl)cc1)C(O)=O	**Clofibric Acid**	*Aliivibrio fischeri*	214,65	2,84	5,5	8	-3,8 bis -2,00		34	2147	1,80	0,80	-0,60	1,40	Baumer et al.2017
15307-86-5	OC(=O)Cc1ccccc1Nc2c(Cl)cccc2Cl	**Diclofenac**	*Aliivibrio fischeri*	296,15	4,51	5,5	7	-5,08 bis -3,89		2	38	1,19	2,74	1,37	1,37	Baumer et al.2017
54-31-9	NS(=O)(=O)c2cc(C(=O)(O))c(NCc1ccco1)cc2Cl	**Furosemide**	*Aliivibrio fischeri*	330,74	2,31	5,5	6	-3,35 bis -2,55		148	932	0,80	0,47	0,00	0,47	Baumer et al.2017
15687-27-1	O=C(O)C(c(ccc(c1)CC(C)C)c1)C	**Ibuprofen**	*Aliivibrio fischeri*	206,29	3,79	5,5	9	-5,23 bis -2,14		1,2	1494	3,09	3,07	0,41	2,66	Baumer et al.2017
22071-15-4	O=C(c(cccc1C(C(=O)O)C)c1)c(cccc2)c2	**Ketoprofen**	*Aliivibrio fischeri*	254,29	3,00	5,5	8	-4,51 bis -2,32		7,9	1217	2,19	1,99	0,19	1,80	Baumer et al.2017

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
59729-33-8	c12C(CCCN(C)C)(c3ccc(F)cc3)OCc1cc(C#N)cc2	**Citalopram**	*Aliivibrio fischeri*		3,74	5,5	7			8100	6804	-0,08				Baumer et al. 2017
83881-51-0	c1ccccc1C(c2ccc(Cl)cc2)N3CCN(CCOCC(=O)O)CC3	**Cetirizin**	*Aliivibrio fischeri*		-0,61	7	9			193,79	172,68	-0,05				Baumer et al. 2017
469-21-6	CC(C1=CC=CC=C1)(C2=CC=CC=N2)OCCN(C)C	**Doxylamine**	*Aliivibrio fischeri*			5,5	9			81,39	162,78	0,30				Baumer et al. 2017
525-66-6	CC(C)NCC(O)COc1cccc2ccccc12	**Propranolol**	*Aliivibrio fischeri*		2,59	5,5	7			136,78	132,27	-0,01				Baumer et al. 2017
22204-53-1	COc2ccc1cc(ccc1c2)C(C)C(O)=O	**Naproxen**	*Aliivibrio fischeri*	230,27	3,10	5,5	8	-4,54 bis -2,34		7	1053	2,20	1,65	-0,36	2,01	Baumer et al.2017
103-90-2	O=C(Nc(ccc(O)c1)c1)C	**Paracetamol**	*Aliivibrio fischeri*	151,17	0,46	6	9	-1,86 bis -3,66		2087	33	-1,80	0,91	0,78		Baumer et al.2017
69-72-7	O=C(O)c(c(O)ccc1)c1	**Salicylic acid**	*Aliivibrio fischeri*	138,12	2,26	5,5	7	-3,85 bis -2,35		20	617	1,50	-0,64	-1,47	0,84	Baumer et al.2017
57-68-1	O=S(=O)(Nc(nc(cc1C)C)n1)c(ccc(N)c2)c2	**Sulfadimidine**	*Aliivibrio fischeri*	278,33	0,19	5,5	6	-2,56 bis -2,59		767	715	-0,03	0,63	0,61	0,02	Baumer et al.2017

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
57-68-2	O=S(=O)(Nc(nc(cc1C)C)n1)c(ccc(N)c2)c3	**Sulfadimidine**	*Aliivibrio fischeri*	278,33	0,19	5,5	7	-2,56 bis -2,2		767	1756	0,36	0,63	0,39	0,24	Baumer et al.2017
723-46-6	Cc1cc(NS(=O)(=O)c2ccc(N)cc2)no1	**Sulfamethoxazole**	*Aliivibrio fischeri*	253,28	0,89	5,5	7	-3,83 bis -2,82		37	383	1,01	0,68	0,14	0,54	Baumer et al.2017
58-55-9	CN1C(=O)N(C)c2ncnc2C1(=O)	**Theophylline**	*Aliivibrio fischeri*	180,17	-0,04	5,5	8	-2,04 bis $^{-1}$,98		1643	1887	0,06	-0,81	-0,93	0,12	Baumer et al.2017
56211-40-6	CC(C)NC(=O)NS(=O)(=O)c1cnccc1Nc2cccc(C)c2	**Torasemide**	*Aliivibrio fischeri*	348,42	3,37	5,5	7	-3,67 bis -2,88		74	459	0,79	1,77	1,22	0,55	Baumer et al.2017
3380-34-5	Clc2cc(Cl)ccc2Oc1ccc(Cl)cc1O	**Triclosan**	*Aliivibrio fischeri*	289,55	4,76	5,5	8	-5,12 bis -4,62		2	7	0,50	4,98	4,50	0,48	Baumer et al.2017
81-81-2	c1ccc2C(O)=C(C(c3ccccc3)CC(=O)C)C(=O)Oc2c1	**Warfarin**	*Aliivibrio fischeri*	308,34	2,70	6	8	-3,96 bis -2,89		34	397	1,07	3,00	1,18	1,82	Baumer et al.2017
65-85-0	C1=CC=C(C=C1)C(=O)O	**Benzoic acid**	*Daphnia magna*		1,65	6	9			222,3	1088,1	0,69				Rendal et al. 2011
456-22-4	C1=CC(=CC=C1C(=O)O)F	**4-F-Benzoic acid**	*Daphnia magna*		2,07	6	9			343,3	3771,8	1,04				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
535-80-8	C1=CC(=CC(=C1)Cl)C(=O)O	**3-Cl-Benzoic acid**	*Daphnia magna*		2,53	6	9			122,1	840,8	0,84				Rendal et al. 2011
74-11-3	C1=CC(=CC=C1C(=O)O)Cl	**4-Cl-Benzoic acid**	*Daphnia magna*		2,48	6	9			148,7	750	0,70				Rendal et al. 2011
585-76-2	C1=CC(=CC(=C1)Br)C(=O)O	**3-Br-Benzoic acid**	*Daphnia magna*		2,68	6	9			102,5	492,5	0,68				Rendal et al. 2011
585-76-5	C1=CC(=CC=C1C(=O)O)Br	**4-Br-Benzoic acid**	*Daphnia magna*		2,60	6	9			78,4	697,5	0,95				Rendal et al. 2011
		2-Phthalic acid	*Daphnia magna*		0,88	6	9			4369,2	6462,5	0,17				Rendal et al. 2011
		3-Phthalic acid	*Daphnia magna*		0,86	6	9			3551,9	7593,8	0,33				Rendal et al. 2011
118-92-3	C1=CC=C(C(=C1)C(=O)O)N	**2-Amino-Benzoic acid**	*Daphnia magna*		1,27	6	9			46,6	464,9	1,00				Rendal et al. 2011
99-05-8	C1=CC(=CC(=C1)N)C(=O)O	**3-Amino-Benzoic acid**	*Daphnia magna*		0,77	6	9			181	865,4	0,68				Rendal et al. 2011
150-13-0	C1=CC(=CC=C1C(=O)O)N	**4-Amino-Benzoic acid**	*Daphnia magna*		0,68	6	9			134,4	1040,9	0,89				Rendal et al. 2011
108-95-2	C1=CC=C(C=C1)O	**Phenol**	*Daphnia magna*		1,54	6	9			28,4	50,5	0,25				Rendal et al. 2011
95-48-7	CC1=CC=CC=C1O	**2-Methyl-Phenol**	*Daphnia magna*		1,96	6	9			16,7	27,3	0,21				Rendal et al. 2011
108-39-4	CC1=CC(=CC=C1)O	**3-Methyl-Phenol**	*Daphnia magna*		2,04	6	9			23,7	32,7	0,14				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
106-44-5	CC1=CC=C(C=C1)O	**4-Methyl-Phenol**	*Daphnia magna*		2,07	6	9			16,7	24,8	0,17				Rendal et al. 2011
120-83-2	C1=CC(=C(C=C1Cl)Cl)O	**2,4-Cl-Phenol**	*Daphnia magna*		3,10	6	9			2,1	3,4	0,21				Rendal et al. 2011
88-06-2	C1=C(C=C(C(=C1Cl)O)Cl)Cl	**2,4,6-Cl-Phenol**	*Daphnia magna*		3,64	6	9			0,8	11,6	1,16				Rendal et al. 2011
77-10-1	C1CCC(CC1)(C2=CC=CC=C2)N3CCCCC3	**PCP**	*Daphnia magna*		5,11	6	9			0	1,4	-				Rendal et al. 2011
88-75-5	C1=CC=C(C(=C1)[N+](=O[O-])O	**2-Nitro-Phenol**	*Daphnia magna*		1,67	6	9			24,2	43	0,25				Rendal et al. 2011
554-84-7	C1=CC(=CC(=C1)O)[N+](=O)]O-]	**3-Nitro-Phenol**	*Daphnia magna*		1,90	6	9			24,7	41,1	0,22				Rendal et al. 2011
51-28-5	C1=CC(=C(C=C1[N+](=O)[O-])[N+](=O)[O-])O	**2,4-Nitro-Phenol**	*Daphnia magna*		1,72	6	9			1,2	6	0,70				Rendal et al. 2011
108-46-3	C1=CC(=CC(=C1)O)O	**Resorcinol**	*Daphnia magna*		0,82	6	9			15,6	34,8	0,35				Rendal et al. 2011
106-48-9	C1=CC(=CC=C1O)Cl	**4-Cl-Phenol**	*Poecilia reticulata*		2,42	6	8			7,7	9,1	0,07				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
120-83-2	C1=CC(=C(C=C1Cl)Cl)O	**2,4-Cl-Phenol**	*Poecilia reticulata*		3,10	6	8			3,5	7,6	0,34				Rendal et al. 2011
87-65-0	C1=CC(=C(C(=C1)Cl)O)Cl	**2,6-Cl-Phenol**	*Poecilia reticulata*		2,90	6	8			3,9	17,9	0,66				Rendal et al. 2011
95-95-4	C1=C(C(=CC(=C1Cl)Cl)Cl)O	**2,4,5-Cl-Phenol**	*Poecilia reticulata*		3,84	6	8			1,5	3,1	0,32				Rendal et al. 2011
88-06-2	C1=C(C=C(C(C1Cl)O)Cl)Cl	**2,4,6-Cl-Phenol**	*Poecilia reticulata*		3,64	6	8			0,9	7,9	0,94				Rendal et al. 2011
58-90-2	C1=C(C(=C(C(=C1Cl)Cl)Cl)O)Cl	**2,3,4,6-Cl-Phenol**	*Poecilia reticulata*		4,44	6	8			0,3	3,7	1,09				Rendal et al. 2011
77-10-1	C1CCC(CC1)(C2=CC=CC=C2)N3CCCCC3	**PCP**	*Poecilia reticulata*		5,11	6	8			0,1	0,9	0,95				Rendal et al. 2011
		3,4,5-Cl-2,6-OCH3-Phenol	*Poecilia reticulata*		3,78	6	8			2,4	6,6	0,44				Rendal et al. 2011
554-84-7	C1=CC(=CC(=C1)O)[N+](=O)[O-]	**3-Nitro-Phenol**	*Poecilia reticulata*		1,90	6	8			11	16,7	0,18				Rendal et al. 2011
100-02-7	C1=CC(=CC=C1[N+](=O)[O-])O	**4-Nitro-Phenol**	*Poecilia reticulata*		1,67	6	8			9	39,2	0,64				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
329-71-5	C1=CC(=C(C=C1[N+](=O)[O-])O)[N+](=O)[O-]	**2,5-Nitro-Phenol**	*Poecilia reticulata*		1,79	6	8			0,5	4,8	0,98				Rendal et al. 2011
4099-73-4	CCCCC1=CC(=CC(=C1O)[N+](=O)[O-])[N+](=O)[O-]	**2-Butyl-4,6-nitro-Phenol**	*Poecilia reticulata*		3,49	6	8			0,1	1	1,00				Rendal et al. 2011
108-95-2	C1=CC=C(C=C1)O	**Phenol**	*Carassius auratus*		1,00	6	10			130	300	0,36				Rendal et al. 2011
95-57-8	OC1=CC=CC=C1Cl	**2-Cl-Phenol**	*Carassius auratus*		2,22	6	10			85	500	0,77				Rendal et al. 2011
108-43-0	OC1=CC=CC(Cl)=C1	**3-Cl-Phenol**	*Carassius auratus*		2,35	6	10			50	100	0,30				Rendal et al. 2011
106-48-9	C1=CC(=CC=C1O)Cl	**4-Cl-Phenol**	*Carassius auratus*		2,42	6	10			50	200	0,60				Rendal et al. 2011
576-24-9	C1=CC(=C(C(=C1)Cl)Cl)O	**2,3-Cl-Phenol**	*Carassius auratus*		2,96	6	10			10	100	1,00				Rendal et al. 2011
120-83-2	C1=CC(=C(C=C1Cl)Cl)O	**2,4-Cl-Phenol**	*Carassius auratus*		3,10	6	10			5	100	1,30				Rendal et al. 2011
583-78-8	C1=CC(=C(C=C1Cl)O)Cl	**2,5-Cl-Phenol**	*Carassius auratus*		3,03	6	10			5	100	1,30				Rendal et al. 2011
87-65-0	C1=CC(=C(C(=C1)Cl)O)Cl	**2,6-Cl-Phenol**	*Carassius auratus*		2,90	6	10			15	100	0,82				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
591-35-5	C1=C(C=C(C=C1Cl)Cl)O	**3,5-Cl-Phenol**	*Carassius auratus*		3,16	6	10			2	40	1,30				Rendal et al. 2011
95-95-4	C1=C(C(=CC(=C1Cl)Cl)Cl)O	**2,4,5-Cl-Phenol**	*Carassius auratus*		3,84	6	10			0,7	50	1,85				Rendal et al. 2011
88-06-2	C1=C(C=C(C(=C1Cl)O)Cl)Cl	**2,4,6-Cl-Phenol**	*Carassius auratus*		3,64	6	10			1,5	70	1,67				Rendal et al. 2011
58-90-2	C1=C(C(=C(C(=C1Cl)Cl)Cl)O)Cl	**2,3,4,6-Cl-Phenol**	*Carassius auratus*		4,44	6	10			0,2	10	1,70				Rendal et al. 2011
77-10-1	C1CCC(CC1)(C2=CC=CC=C2)N3CCCCC3	**PCP**	*Carassius auratus*		5,11	6	10			0,2	3	1,18				Rendal et al. 2011
62-53-3	C1=CC=C(C=C1)N	**Aniline**	*Daphnia magna*		1,14	6	9			13,77	7,57	-0,26				Rendal et al. 2011
95-53-4	CC1=CC=CC=C1N	**2-Methyl-Aniline**	*Daphnia magna*		1,38	6	9			8,91	3,55	-0,40				Rendal et al. 2011
108-44-1	CC1=CC(=CC=C1)N	**3-Methyl-Aniline**	*Daphnia magna*		1,55	6	9			8,32	3,09	-0,43				Rendal et al. 2011
106-49-0	CC1=CC=C(C=C1)N	**4-Methyl-Aniline**	*Daphnia magna*		1,53	6	9			4,57	2,29	-0,30				Rendal et al. 2011
95-51-2	C1=CC=C(C(=C1)N)Cl	**2-Cl-Aniline**	*Daphnia magna*		2,01	6	9			9,03	4,53	-0,30				Rendal et al. 2011
108-42-9	C1=CC(=CC(=C1)Cl)N	**3-Cl-Aniline**	*Daphnia magna*		1,99	6	9			8,05	3,2	-0,40				Rendal et al. 2011
591-19-5	C1=CC(=CC(=C1)Br)N	**3-Br-Aniline**	*Daphnia magna*		2,10	6	9			4,63	2,22	-0,32				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
608-27-5	C1=CC(=C(C(=C1)Cl)Cl)N	**2,3-Cl-Aniline**	*Daphnia magna*		2,80	6	9			3,02	2,4	-0,10				Rendal et al. 2011
554-00-7	C1=CC(=C(C=C1Cl)Cl)N	**2,4-Cl-Aniline**	*Daphnia magna*		2,82	6	9			4,26	3,23	-0,12				Rendal et al. 2011
95-82-9	C1=CC(=C(CC1Cl)N)Cl	**2,5-Cl-Aniline**	*Daphnia magna*		2,86	6	9			3,16	2,45	-0,11				Rendal et al. 2011
147-82-0	C1=C(C=C(C(=C1Br)N)Br)Br	**2,4,6-Br-Aniline**	*Daphnia magna*		4,81	6	9			4,15	3,37	-0,09				Rendal et al. 2011
88-74-4	C1=CC=C(C(=C1)N)[N+](=O)[O-]	**2-Nitro-Aniline**	*Daphnia magna*		1,67	6	9			10,48	7,08	-0,17				Rendal et al. 2011
97-02-9	C1=CC(=C(C=C1[N+](=O)[O-])[N+](=O)[O-])N	**2,4-Nitro-Aniline**	*Daphnia magna*		1,64	6	9			8,97	6,8	-0,12				Rendal et al. 2011
54910-89-3	CNCCC(c2ccccc2)Oc1ccc(cc1)C(F)(F)F	**Fluoxetine**	*Senedesmus vacuolatus*		3,93	6,5	10			0,19	0,02	-0,98				Rendal et al. 2011
83891-03-6	C1=CC=C(C=C1)C(CCN)OC2=CC=C(C=C2)C(F)(F)F	**Norfluoxetine**	*Senedesmus vacuolatus*		3,76	6,5	10			0,47	0,06	-0,89				Rendal et al. 2011
525-66-6	CC(C)NCC(O)COc1cccc2ccccc12	**Propranolol**	*Senedesmus vacuolatus*		2,90	6,5	10			20,75	0,1	-2,32				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
137-58-6	CCN(CC)CC(=O)NC1=C(C=CC=C1C)C	**Lidocain**	*Senedesmus vacuolatus*		2,20	6,5	10			134,75	107,6	-0,10				Rendal et al. 2011
739-71-9	CC(CN1C2=CC=CC=C2CCC3=CC=CC=C31)CN(C)C	**Trimipramine**	*Senedesmus vacuolatus*		4,71	6,5	10			15,6	0,53	-1,47				Rendal et al. 2011
79617-96-2	CNC1CCC(C2=CC=CC=C12)C3=CC(=C(C=C3)Cl)Cl	**Sertraline**	*Pimephales promelas*		5,08	6,5	8,5			0,54	0,05	-1,03				Rendal et al. 2011
80-32-0	C1=CC(=CC=C1N)S(=O)NC2=NN=C(C=C2)Cl	**SPZ**	*Pantoea agglomerans*		0,68	5	8			2,34	0,85	-0,44				Rendal et al. 2011
723-46-6	CC1=CC(=NO1)NS(=O)(=O)C2=CC=C(C=C2)N	**SMX**	*Pantoea agglomerans*		0,66	5	8			1,01	0,48	-0,32				Rendal et al. 2011
68-35-9	C1=CN=C(N=C1)NS(=O)(=O)C2=CC=C(C=C2)N	**SDZ**	*Pantoea agglomerans*		-0,07	5	8			2,57	0,88	-0,47				Rendal et al. 2011
122-11-2	COC1=NC(=NC(=C1)NS(=O)(=O)C2=CC=C(C=C2)N)OC	**SDT**	*Pantoea agglomerans*		0,73	5	8			19,78	2,05	-0,98				Rendal et al. 2011

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
72-14-0	C1=CC(=CC=C1N)S(=O)(=O)NC2=NC=CS2	**STZ**	*Pantoea agglomerans*		0,05	5	8			11,61	0,77	-1,18				Rendal et al. 2011
57-68-1	CC1=CC(=NC(==N1)NS(=O)(=O)C2=CC=C(C=C2)N)C	**SDM**	*Pantoea agglomerans*		0,30	5	8			20	1,14	-1,24				Rendal et al. 2011
144-83-2	C1=CC=NC(=C1)NS(=O)(=O)C2=CC=C(C=C2)N	**SPY**	*Pantoea agglomerans*		0,47	5	8			20	2,22	-0,95				Rendal et al. 2011
54910-89-3	CNCCC(c2ccccc2)Oc1ccc(cc1)C(F)(F)F	**Fluoxetine**	*Oryzias latipes*		3,93	7	9			5,5	0,2	-1,44				Rendal et al. 2011
54-05-7	CCN(CC)CCCC(C)NC1=C2C=CC(=CC2=NC=C1)Cl	**Chloroquine**	*Salix viminalis*		4,41	6	9			36	3	-1,08				Rendal et al. 2011
64902-72-3	CC1=NC(=NC(=N1)OC)NC(=O)NS(=O)(=O)C2=CC=CC=C2Cl	**Chlorosulfuron**	*Chlorella fusca*		0,74	5	8			0,02	13,9	2,84				Fahl et al. 1994
120-83-2	C1=CC(=C(C=C1Cl)Cl)O	**2,4-Cl-Phenol**	*Scenedesmus*		3,10	6,5	9			30,16	0,26	-2,06				Xing et al. 2012

CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
			vacuolatus													
88-06-2	C1=C(C=C(C(=C1Cl)O)Cl)Cl	**2,4,6-Cl-Phenol**	*Scenedesmus vacuolatus*		3,64	6,5	9			4,37	30,69	0,85				Xing et al. 2012
77-10-1	C1CCC(CC1)(C2=CC=CC=C2)N3CCCCC3	**PCP**	*Scenedesmus vacuolatus*		5,11	6,5	9			137,48	24,06	-0,76				Xing et al. 2012
137-58-6	CCN(CC)CC(=O)NC1=C(C=CC=C1C)C	**Lidocain**	*Scenedesmus vacuolatus*		2,20	7,2	9,5			134,75	107,56	-0,10				Neuwoehner & Escher 2011
54910-89-3	CNCCC(c2ccccc2)Oc1ccc(cc1)C(F)(F)F	**Fluoxetine**	*Scenedesmus vacuolatus*		3,93	6,5	10			0,001	0,55	2,74				Neuwoehner & Escher 2011
22071-15-4	CC(C1=CC=CC(=C1)C(=O)C2=CC=CC=C2)C(=O)O	**Ketoprofen**	*Daphnia magna*			6	9			45	230	0,71				Boström & Berglund 2015
22204-53-1	COc2ccc1cc(ccc1c2)C(C)C(O)=O	**Naproxen**	*Daphnia magna*		3,10	6	8			10	96	0,98				Boström &

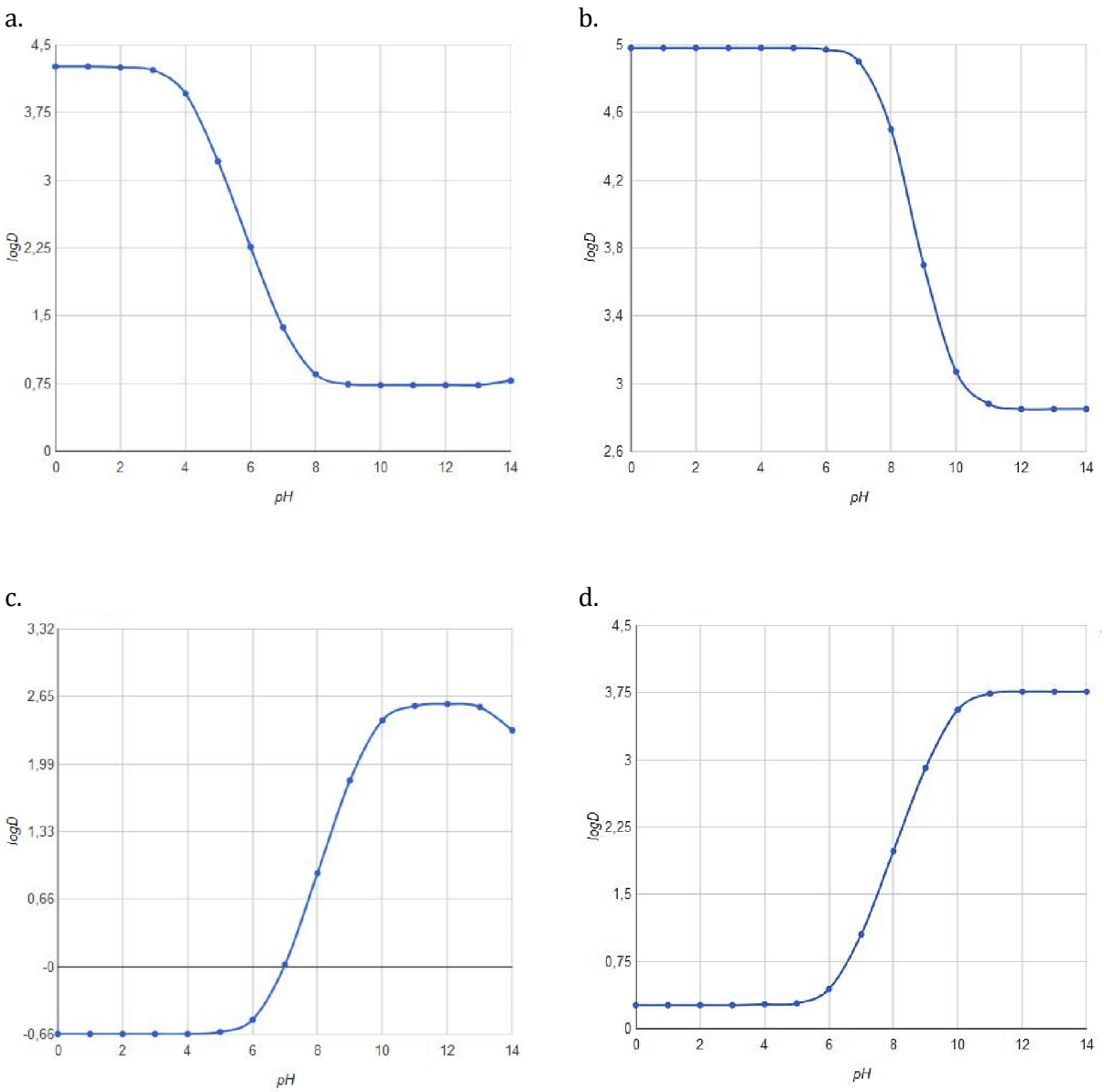
CAS	SMILES	Compound	Test organism	MW	log Kow (max)	pH min	pH max	logEC50	Tox [mM]	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	Source
																Berglund 2015
79617-96-2	CNC1CCC(C2=CC=CC=C12)C3=CC(=C(C=C3)Cl)Cl	**Sertraline**	*Daphnia magna*		5,08	6	9			8,5	0,18	-1,67				Boström & Berglund 2015
80-32-0	C1=CC(=CC=C1N)S(=O)(=O)NC2=NN=C(C=C2)Cl	**Sulfachloropyridiazine**	*Pantoea agglomerans*			5	8			2,34	0,85	-0,44				Tappe et al. 2008
68-35-9	C1=CN=C(N=C1)NS(=O)(=O)C2=CC=C(C=C2)N	**Sulfadiazine**	*Daphnia magna*			6	8,5			49,89	749,5	1,18				Liu et al. 2016
738-70-5	COC1=CC(=CC(=C1OC)OC)CC2=CN=C(N=C2N)N	**Trimethoprim**	*Salix viminalis*			4,3	8,2			100	10	-1,00				Mikes & Trapp 2010

Source: Own depiction

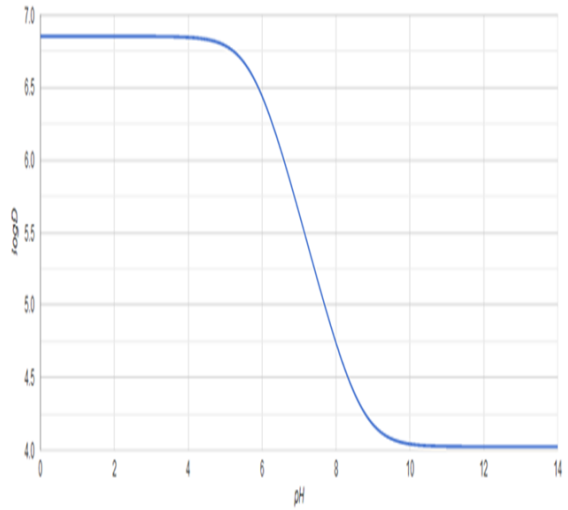
1.3 Physicochemical properties of selected ionised chemicals in dependence of pH

Due to the outstanding importance of log D at different pH values of the medium for the topic addressed in pHION, we initially concentrated on modelling log D using the software "*Log D Predictor*". So far, the modelling has been carried out for eight substances (Figure 1), which were also selected as the first chemicals to be tested experimentally in the fish embryo test (FET) with *Danio rerio*. In preliminary work, a very good correlation between the log D values modelled using this software and the actual log D values of ionisable substances at different pH values was established (see report on WP 2 and 3, part 1). In this respect, these models form the basis for the selection of the pH values to be tested for the future practical work in pHION.

Figure 1: "Log D Predictor" modelling of log D in dependence of pH for the first eight substances



e.



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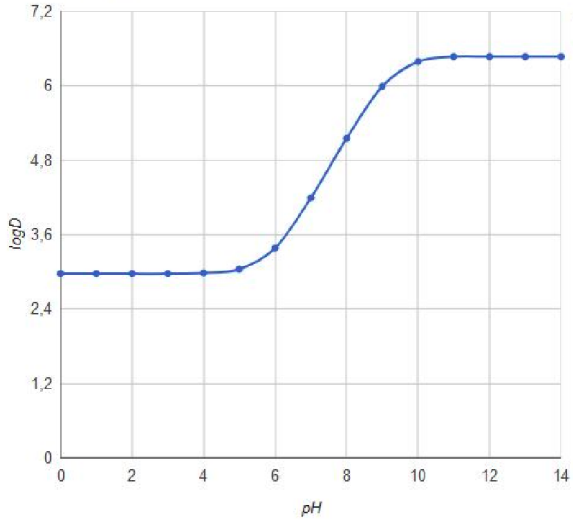
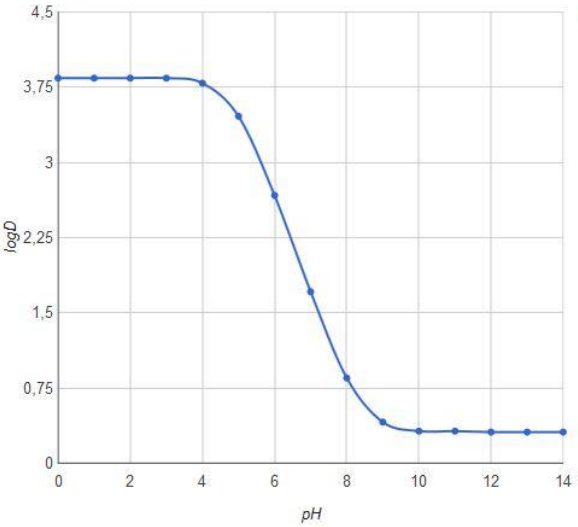
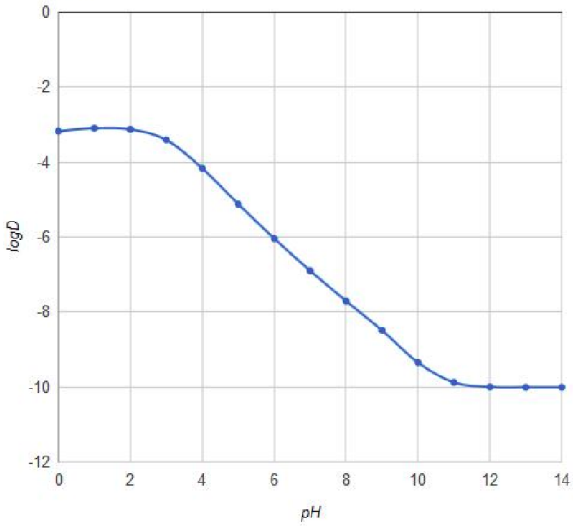


g.



h.



a. diclofenac; b. triclosan; c. propranolol; d. citalopram; e. difenacoum; f. enclomiphene; g. ibuprofen; h. glyphosate.
Source: Own depiction

1.4 Review of existing toxicity data and BCF values in the literature in terms of their reliability in order to assess their suitability for modelling

As mentioned above, on the basis of the accessible information, no serious deficiencies can be identified in all published studies on this topic. Only in a few cases a more precise determination of effect concentrations could probably have been made by expanding the data. Of particular importance are data sets such as the one in the Supplementary Information by Baumer et al. (2017).

1.5 Compilation of existing models in the literature for calculating the internal concentration of chemicals in different species

Log D: More accurately termed log DOW, this coefficient (analogous to K_{OW} or log K_{OW}) describes the ratio of the solubility of an ionisable chemical in octanol or water. For ionisable substances, the (log) D_{OW} is strongly dependent on the pH, whereby it is assumed that neutral species are more capable of membrane passage than charged species, which in turn is significant for

bioconcentration and toxicity. Whether a model development based on Δ log D (= log $D_{100\ \%\ neutral\ species}$ - log $D_{50\ \%\ neutral\ species}$) is possible, is being investigated in pHION.

Log K_{lipw} / log D_{lipw}: The calculation of the ratio of the solubility of a substance in phospholipids (liposomes) or water represents a biologically relevant method for predicting the uptake (and thus bioaccumulation and toxicity) of chemicals in biological systems. The distribution coefficient K_{lipw} (or D_{lipw} for ionisable substances at a defined pH) of a substance can be determined via the COSMOmic model (Bittermann et al. 2014), but requires consideration of the membrane potential (Gaussian potential for a DMPC membrane) for accurate prediction of experimentally determined K_{lipw} values. Such predictions are also possible for ionisable substances, whereby the quality of the prediction is somewhat better for anions than for cations (Bittermann et al. 2014).

Ion trap: A simple toxicokinetic model, which assumes that only the neutral form of an ionisable substance is capable of membrane passage, was proposed by Neuwoehner and Escher (2011). This assumption is justified for situations where the pH is less than 2 units below the pK_a of a substance. Thus, for pH > pK_a -2, it can be assumed that the cell membrane acts as a barrier to external ions and that ions occurring in the cell are solely due to the pH present in the cell, thus accounting for the ion trapping effect. The proposed model also takes into account the D_{lipw}. Its suitability has been demonstrated for the substances fluoxetine, norfluoxetine, propranolol, lidocaine and trimipramine (Neuwoehner and Escher 2011).

Fish plasma model: Huggett et al. (2003) proposed the so-called "fish plasma model" (FPM) to assess chronic effects of chemicals in fish. It relates therapeutic plasma concentrations (of e.g. pharmaceuticals) in the human organism (HPC_T) to estimated steady-state concentrations in the blood plasma of fish (FPC_{SS}). Schreiber et al. (2011) extended this model for ionisable substances by calculating bioconcentrations (for estimating the FPC_{SS}) on the basis of the pH-dependent D_{OW} and thus demonstrated a potential risk with regard to long-term damage for about one third of the pharmaceuticals tested.

pH - pK_a - model: The effect that toxicity and bioconcentration factors of acids increase in the lower pH range and of bases in the higher pH range is the basis for an approach by Rendal et al. (2011). In a meta-study, they were able to show that this pH effect is greatest (more than 2 orders of magnitude) when the calculated difference pH - pK_a is in the range between -1 and 3 for acids and between -3 and 1 for bases. These correlations were shown for a data set that was based on published experimental data on the one hand and on data generated with the so-called "cell model" on the other. The "cell model" is based on the Fick-Nernst-Planck diffusion equation.

2 WP 2: Modelling bioaccumulation and toxicity as a function of pH: review of current models and formulation of alternatives

2.1 Preliminary remarks

As a central hypothesis of our own, we investigated whether a Δ log D, which represents the difference between the log D values at two specific test pH values, allows to predict the observed differences in the toxicity ratios (Δ log toxicity) at these pH values with sufficient accuracy. For this purpose, literature data were evaluated (on the microtox assay, e.g. Baumer et al. (2017)), but the toxicity data experimentally determined in pHION for the first four test substances were also integrated. A very good correlation between the two ratios was found, which supports the test hypothesis. The evaluation will be updated in pHION as knowledge is gained.

2.2 Deriving own models and hypotheses

As explained above, a Δ log D was calculated in a novel approach by the German Environment Agency (UBA). It describes the difference between the log D of a pH value at which ideally only the neutral species of a substance occurs and the log D for another pH at which 50 % of the molecules are uncharged (Figure 2.1). On the basis of available data, it was examined, whether the parameter Δ log D is related to the ratio of toxicities generated by a substance at different pH values. The background to this approach is to develop a methodology in future to reliably predict toxicities for other, worst-case pH values, if only one toxicity value is present at a certain pH value.
Indeed, preliminary studies based on literature data showed a linear dependence between the ratio of observed toxicity at pH 6 and 8, respectively, and the Δ log D calculated on the basis of these two pH values. In pHION, toxicity data in FET with *Danio rerio* (LC_{50} after 72 and 96 hpf) were preliminarily obtained for four substances: propranolol, diclofenac, citalopram and triclosan (for detailed data see chapter 5.X a-d). The LC_{50} values (96 hpf) determined for different pH values were calculated in such a way that a Δ Tox = log (LC_{50} [pH_{max}] / LC_{50} [pH_{min}]) was determined for two pH values in each case, whereby pH_{min} referred to the smaller pH and pH_{max} to the larger pH of the pair of values to be compared. These values were compared with the corresponding Δ log D = log D_{min} - log D_{max} for all pH comparisons, as well as two other physicochemical parameters used in the literature for other modelling approaches: the log ($f_{neutral\ max}$ / $f_{neutral\ min}$), which relates the percentages of a substance at two given pH values, and a Δ log K_{lipw} = log K_{lipw} max - log $K_{lipw\ min}$ (Table 2). In the following, linear correlation analyses were carried out for the following data:

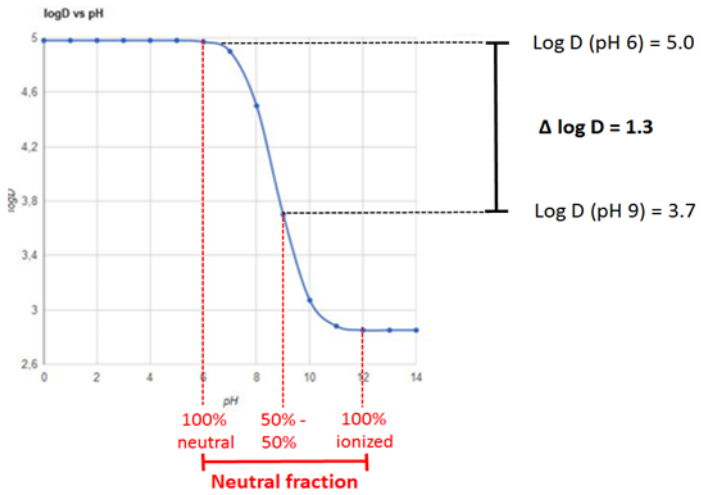
a. Δ Tox vs. Δ log D for propranolol, diclofenac, citalopram and triclosan,

b. Δ Tox vs. Δ log D for propranolol, diclofenac and citalopram, as log ($f_{neutral\ max}$ / $f_{neutral\ min}$)- and Δ log K_{lipw}-values could currently only be calculated for these three of the tested chemicals,

c. Δ Tox vs. log ($f_{neutral\ max}$ / $f_{neutral\ min}$) for propranolol, diclofenac and citalopram, and

d. Δ Tox vs. Δ log K_{lipw} for propranolol, diclofenac and citalopram.

Figure 2.1: Schematic illustration of the Δ log D concept for an acid



Shown is the difference between the log D values at pH 6 and pH 9. For further explanation, refer to the text.
Source: Own depiction

Highly significant correlations were found in the analyses a., b. and c. On the one hand, the hypothesis that the Δ Tox can be reliably predicted via the Δ log D (or the associated distribution of neutral and charged species of an ionisable substance log ($f_{neutral\ max}$ / $f_{neutral\ min}$)) was supported. On the other hand, the much poorer correlation in analysis d. showed that - despite weak significance - already with only three test substances, the Δ log K_{lipw} hardly offers any possibilities for reliable toxicity estimation (Figure 2.2).

In addition, a correlation calculation was carried out for a series of literature data and the Δ log D data determined experimentally in pHION (FET with *Danio rerio* over 96 h for propranolol, diclofenac, citalopram and triclosan). For this purpose, the effect concentrations at different pH values were first determined for selected chemicals and a Δ log of these concentrations was calculated. For the same pH values and substances, the Δ log D was determined according to the above procedure. A positive linear correlation between these parameters was also found here (Figure 2.3), which supports the hypothesis that in future modulations of the toxicity of ionisable substances at different pH values can be estimated more reliably on the basis of Δ log D simulations based on octanol-water than is possible via Δ log K_{lipw}.

Table 2: Δ Tox analysis, physicochemical parameters and calculation of data relating these physicochemical parameters to different pH combinations

Compound	Test organism	MW	log Kow (max)	pH min	pH max	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	fn min	fn max	log (fn max/fn min)	Δ log Klip w
Triclosan	*Danio rerio embryos*	189,55	4,76	5	8	0,305	0,41	0,13	4,97	4,50	0,47				
Triclosan	*Danio rerio embryos*	189,55	4,76	8	9	0,41	0,65	0,20	4,50	3,70	0,80				
Triclosan	*Danio rerio embryos*	189,55	4,76	5	9	0,305	0,65	0,33	4,97	3,70	1,27				
Propranolol	*Danio rerio embryos*	259,35	2,59	8	9	9,06	0,67	-1,13	0,95	1,83	-0,88	2,87	22,79	0,89986243	0,28
Propranolol	*Danio rerio embryos*	259,35	2,59	6	8	436,54	9,06	-1,68	-0,66	0,95	-1,61	0,03	2,87	1,98076064	0,06
Propranolol	*Danio rerio embryos*	259,35	2,59	6	9	436,54	0,67	-2,81	-0,66	1,83	-2,49	0,03	22,79	2,88062307	0,34
Diclofenac	*Danio rerio embryos*	296,15	4,51	5	6	0,067	0,64	0,98	3,21	2,25	0,96	3,00	1,00	-0,47712125	-0,25
Diclofenac	*Danio rerio embryos*	296,15	4,51	6	8	0,64	19,60	1,49	2,25	0,85	1,40	1,00	0,01	-2	-0,11

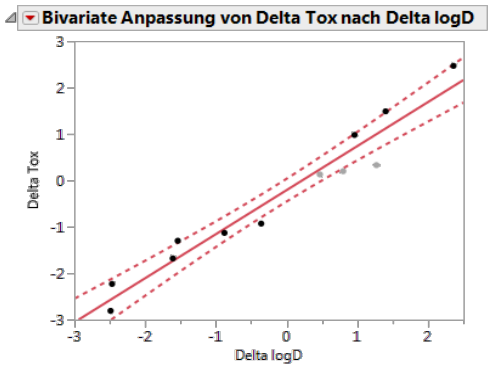
Compound	Test organism	MW	log Kow (max)	pH min	pH max	tox pH min [mg/L]	tox pH max [mg/L]	Δ Tox	log D min	log D max	Δ log D	fn min	fn max	log (fn max/fn min)	Δ log Klip w
Diclofenac	*Danio rerio embryos*	296,15	4,51	5	8	0,067	20	2,47	3,21	0,85	2,36	3,00	0,01	-2,47712125	-0,46
Citalopram	*Danio rerio embryos*	324,4	3,74	6	8	400	20	-1,30	0,44	1,98	-1,54	0,03	2,51	1,92255247	0,52
Citalopram	*Danio rerio embryos*	324,4	3,74	6	9	400	2,34	-2,23	0,44	2,91	-2,47	0,03	20,45	2,83357206	1,31
Citalopram	*Danio rerio embryos*	324,4	3,74	8	9	20	2,34	-0,93	1,98	2,34	-0,36	2,51	20,45	0,91101959	0,59

Further explanation are provided in the text.

Source: Own depiction

Figure 2.2: Linear regression analyses

a.



Lineare Anpassung

Lineare Anpassung

Delta Tox = -0,208095 + 0,9487374*Delta logD

Übersicht der Anpassung

r^2	0,947315
r^2 korrigiert	0,942046
Wurzel der mittleren quadratischen Abweichung	0,379235
Mittelwert der Zielgröße	-0,37333
Beobachtungen (oder Summe Gewichte)	12

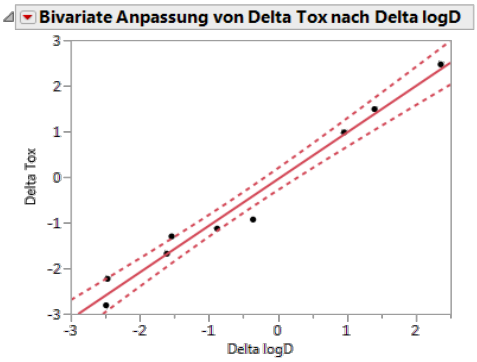
Varianzanalyse

Quelle	Freiheitsgrade	Summe Quadrate	Mittlere Quadrate	F-Wert
Modell	1	25,859672	25,8597	179,8065
Fehler	10	1,438194	0,1438	Wahrsch. > F
K. Summe	11	27,297867		<,0001*

Parameterschätzer

Term	Schätzer	Std.-Fehler	t-Wert	Wahrsch. > |t|
Achsenabschnitt	-0,208095	0,110167	-1,89	0,0882
Delta logD	0,9487374	0,070753	13,41	<,0001*

b.



Lineare Anpassung

Lineare Anpassung

Delta Tox = -0,045742 + 1,0212366*Delta logD

Übersicht der Anpassung

r^2	0,977167
r^2 korrigiert	0,973905
Wurzel der mittleren quadratischen Abweichung	0,290485
Mittelwert der Zielgröße	-0,57111
Beobachtungen (oder Summe Gewichte)	9

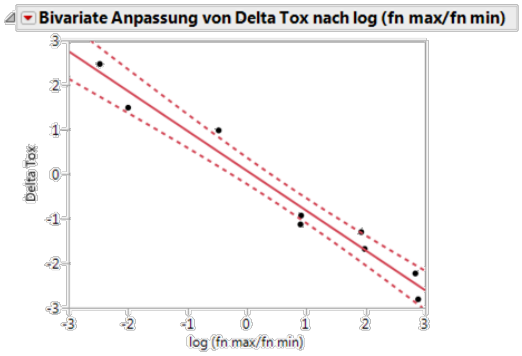
Varianzanalyse

Quelle	Freiheitsgrade	Summe Quadrate	Mittlere Quadrate	F-Wert
Modell	1	25,278420	25,2784	299,5739
Fehler	7	0,590669	0,0844	Wahrsch. > F
K. Summe	8	25,869089		<,0001*

Parameterschätzer

Term	Schätzer	Std.-Fehler	t-Wert	Wahrsch. > |t|
Achsenabschnitt	-0,045742	0,101474	-0,45	0,6658
Delta logD	1,0212366	0,059003	17,31	<,0001*

c.



Lineare Anpassung

Lineare Anpassung

Delta Tox = 0,0719216 - 0,8939083*log (fn max/fn min)

Übersicht der Anpassung

r^2	0,966613
r^2 korrigiert	0,961844
Wurzel der mittleren quadratischen Abweichung	0,35126
Mittelwert der Zielgröße	-0,57111
Beobachtungen (oder Summe Gewichte)	9

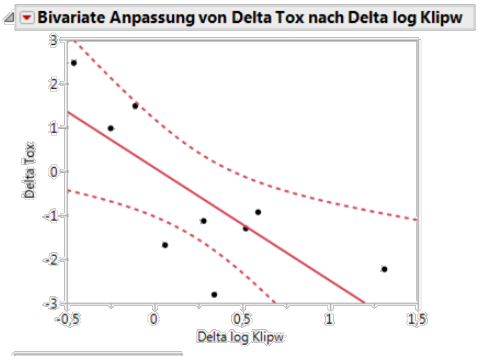
Varianzanalyse

Quelle	Freiheitsgrade	Summe Quadrate	Mittlere Quadrate	F-Wert
Modell	1	25,005402	25,0054	202,6634
Fehler	7	0,863687	0,1234	Wahrsch. > F
K. Summe	8	25,869089		<,0001*

Parameterschätzer

Term	Schätzer	Std.-Fehler	t-Wert	Wahrsch. > |t|
Achsenabschnitt	0,0719216	0,125497	0,57	0,5845
log (fn max/fn min)	-0,893908	0,062792	-14,24	<,0001*

d.



Lineare Anpassung

Lineare Anpassung

Delta Tox = 0,080262 - 2,5712097*Delta log Klipw

Übersicht der Anpassung

r^2	0,57266
r^2 korrigiert	0,511612
Wurzel der mittleren quadratischen Abweichung	1,25669
Mittelwert der Zielgröße	-0,57111
Beobachtungen (oder Summe Gewichte)	9

Varianzanalyse

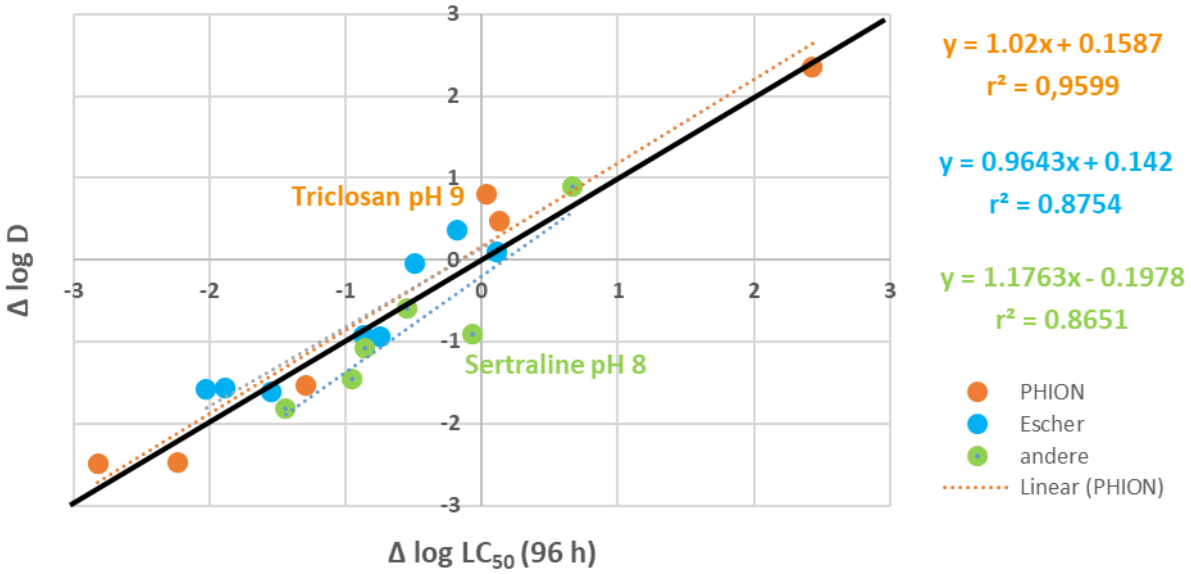
Quelle	Freiheitsgrade	Summe Quadrate	Mittlere Quadrate	F-Wert
Modell	1	14,814196	14,8142	9,3804
Fehler	7	11,054893	1,5793	Wahrsch. > F
K. Summe	8	25,869089		0,0183*

Parameterschätzer

Term	Schätzer	Std.-Fehler	t-Wert	Wahrsch. > |t|
Achsenabschnitt	0,080262	0,469793	0,17	0,8692
Delta log Klipw	-2,57121	0,839512	-3,06	0,0183*

Linear regression analyses of a. Δ Tox vs. Δ log D for propranolol, diclofenac, citalopram [black dots] and triclosan [grey dots]; b. Δ Tox vs. Δ log D for propranolol, diclofenac and citalopram; c. Δ Tox vs. log ($f_{neutral\ max}$ / $f_{neutral\ min}$) for propranolol, diclofenac and citalopram; and d. Δ Tox vs. Δ log K_{lipw} for propranolol, diclofenac and citalopram.
Source: Own depiction

Figure 2.3: Correlation between the Δ log LC_{50} and the corresponding Δ log D



Correlation between the Δ log LC_{50}, determined for different pH values of a given substance (FET with *Danio rerio*, 96 h), and the corresponding Δ log D, calculated on the basis of the same pH values for this substance. Data basis: Experimental from pHION (orange) and taken from literature (blue: data from AG Escher [UFZ Leipzig and Tübingen University], green: other literature sources). The values determined for triclosan at pH 9 and sertraline at pH 8 are slightly off trend.
Source: Own depiction

3 WP 3: Selection of test substances

3.1 Preliminary remarks

A total of up to 30 substances are to be tested experimentally within the framework of pHION. Thus, at a first meeting on 8th November 2018 at UBA Dessau, the criteria for substance selection were jointly determined and then six substances were initially selected with which the investigations began.

3.2 Criteria for substance selection

The pH values to be tested in pHION are intended, on the one hand, to have a certain relevance for the environment, but on the other hand, also to represent the range in which a strong change in log D is to be expected for ionisable chemicals. This applies to the range pH 5 to pH 9 (OECD Guidance Document 23 indicates a relevance of substances with a pK_a value of 4 to 10). The main criterion for the selection of chemicals to be tested is therefore a pK_a value between > 4 and < 10, whereby the pK_a values of the selected test substances should be evenly distributed around pK_a 7, i.e. there should be no imbalance between acids and bases. This should apply to about 25 substances, which should cover a high spectrum of activity and a broad chemical domain. However, from the point of view of basic research (to cover broad a pK_a range), about 5 substances with more extreme pK_a should also be investigated in pHION. These substances should either have a lower pK_a than 4 (and then also be tested at a correspondingly low pH) or have a higher pK_a value than 10 (and then also be tested at a correspondingly high pH).

Further criteria for substance selection are the following:

- **Stability** in aquatic solution: Although pHION also detects degradation products of the test chemicals and metabolites, the focus is on the parent compound.
- Water **solubility**: Since the biotests to be performed focus on apical endpoints of rather limited sensitivity (compared to e.g. biochemical markers), it can be assumed that relatively high concentrations of chemicals have to be tested. For this, a good water solubility of the test substances is a prerequisite. Should solubilisers have to be used, nevertheless, their use should be limited. In these cases, DMSO or isopropanol should preferably be used in the lowest possible concentration (≤ 0.01 %).
- Maximum possible **differences** in pH-dependent characteristics: Since pHION particularly investigates the pH dependence of bioaccumulation and toxicity of ionisable chemicals, the characteristics determining these should also show the greatest possible differences in the pH spectrum. Central here is the log D (preferably between 10 % -100 % neutral species).
- **"Plateau"**: Besides maximal differences, substances with no or only little differences ("plateaus") in log D at the tested pH limits (between: pH 5 and 6; pH 8 and 9) were also selected and often tested at an additional fourth pH level. These log D "plateaus" reflect pH ranges in which the substance is entirely neutral or ionised. Little or no toxicity differences in these ranges would further confirm the dependence of toxicity in relation to the ionised state of the substance which is in turn dependent on pH.
- Environmental **relevance** of the chemical: Since pHION investigates general principles, an environmental relevance of the test substances is desirable, but not absolutely necessary. If there is a choice between two otherwise equivalent candidates, the chemical with the higher environmental relevance should be selected.

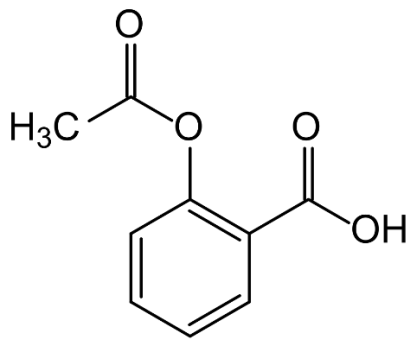
These criteria primarily concern the substance selection for the FET with *Danio rerio*, but can also be used for the substance selection for the tests with *Daphnia magna* and *Lemna minor* (conducted by the UBA Marienfelde).

3.3 Selected substances

3.3.1 Pharmaceuticals

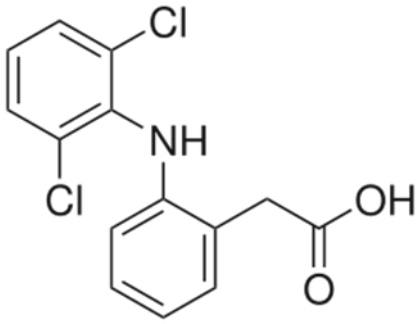
3.3.1.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

a. Acetylsalicylic acid [ASA]



Formula:	$C_9H_8O_4$
Substance group:	NSAID (non-steroidal anti-inflammatory drug)/cox inhibitor
Usage:	Analgesic drug
CAS:	50-78-2
Molecular weight:	180.16 g/mol
Acid/base	Acid
pK_a:	3.49
Water solubility:	2.5 g/L
Solubiliser used:	Isopropanol (only pH 8)
Tested pH-levels:	5, 6 and 8
Notes:	-
Supplier:	*Sigma-Aldrich*

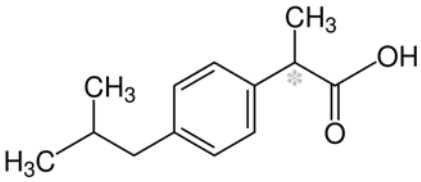
b. Diclofenac (sodium salt)



Formula:	$C_{14}H_{11}Cl_2NO_2$ $C_{14}H_{11}Cl_2NNaO_2$ (sodium salt)
Substance group:	NSAID (non-steroidal anti-inflammatory drug)/cox inhibitor
Usage:	Analgesic drug
CAS:	15307-86-5 15307-79-6 (sodium salt)
Molecular weight:	296.15 g/mol 318.10 g/mol (sodium salt)
Acid/base	Acid
pK_a:	4.15
Water solubility:	2.37 mg/L

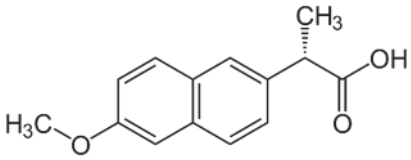
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

c. Ibuprofen (sodium salt)



Formula:	$C_{13}H_{18}O_2$ $C_{13}H_{17}NaO_2$ (sodium salt)
Substance group:	NSAID/cox inhibitor
Usage:	Analgesic/anti-rheumatic drug
CAS:	15687-27-1 31121-93-4 (sodium salt)
Molecular weight:	206.28 g/mol 228.26 g/mol (sodium salt)
Acid/base	Acid
pK_a:	5.3
Water solubility:	21 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

d. Naproxen (sodium salt)

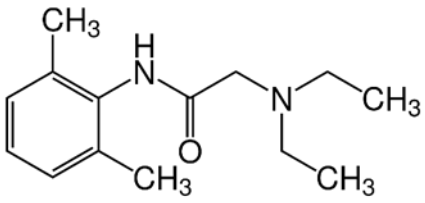


Formula:	$C_{14}H_{14}O_2$ $C_{14}H_{13}NaO_3$ (sodium salt)
Substance group:	NSAID/cox inhibitor
Usage:	Analgesic/anti-rheumatic drug
CAS:	22204-53-1 26159-34-2 (sodium salt)
Molecular weight:	230.26 g/mol 252.24 g/mol (sodium salt)
Acid/base	Acid

pK_a:	4.15
Water solubility:	153 mg/L
Solubiliser used:	-
Tested pH-levels:	6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

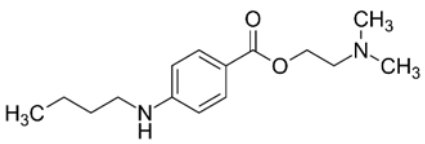
3.3.1.2 Anaesthetics

a. Lidocaine



Formula:	$C_{14}H_{22}N_2O$
Substance group:	Sodium channel blocker
Usage:	Anaesthetic drug
CAS:	137-58-6
Molecular weight:	234.34 g/mol
Acid/base	Base
pK_a:	8.01
Water solubility:	4.1 g/L
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*TCI Deutschland GmbH*

b. Tetracaine

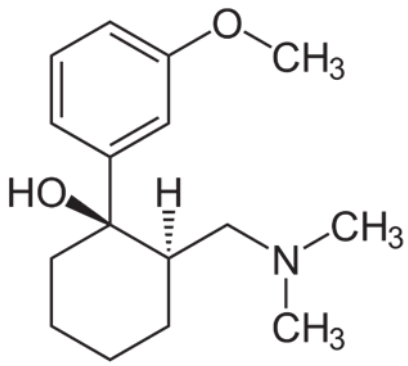


Formula:	$C_{15}H_{24}N_2O$
Substance group:	Allosteric blocker of calcium release channels
Usage:	Anaesthetic drug
CAS:	94-24-6
Molecular weight:	264.37 g/mol
Acid/base	Base
pK_a:	8.42
Water solubility:	n. a.
Solubiliser used:	-

Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*TCI Deutschland GmbH*

3.3.1.3 Opioids

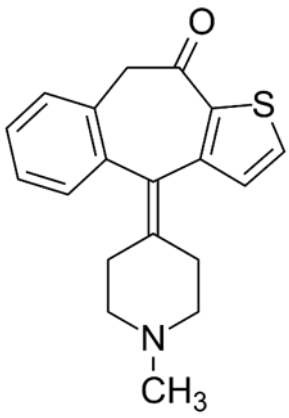
a. Tramadol (hydrochloride)



Formula:	$C_{16}H_{25}NO_2$ $C_{16}H_{26}ClNO_2$ (hydrochloride)
Substance group:	Opioid
Usage:	Analgesic drug
CAS:	27203-92-5 36282-47-0 (hydrochloride)
Molecular weight:	263.38 g/mol 299.83 g/mol (hydrochloride)
Acid/base	Base
pK_a:	9.41
Water solubility:	10 mg/L
Solubiliser used:	-
Tested pH-levels:	6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

3.3.1.4 Antihistamines

a. Ketotifen (fumarate)

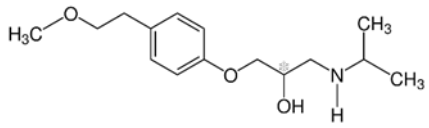


Formula:	$C_{19}H_{19}NOS$ $C_{23}H_{23}NO_5S$ (fumarate)
Substance group:	Histamine H1 receptor antagonist
Usage:	Antihistaminic drug
CAS:	34580-13-7 34580-14-8 (fumarate)
Molecular weight:	309.4 g/mol 425.5 g/mol (fumarate)
Acid/base	Base

	pK_a:	8.43
	Water solubility:	15.3 mg/L
	Solubiliser used:	-
	Tested pH-levels:	9
	Notes:	-
	Supplier:	*TCI Deutschland GmbH*

3.3.1.5 Beta blockers

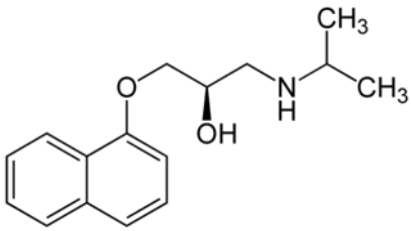
a. Metoprolol (tartrate)



Formula:	$C_{15}H_{25}NO_3$ $C_{34}H_{56}N_2O_{12}$ (tartrate)
Substance group:	Beta blocker
Usage:	High blood pressure, cardiac arrhythmia
CAS:	51384-51-1 56392-17-7 (tartrate)
Molecular weight:	267.37 g/mol 684.8 g/mol (tartrate)
Acid/base	Base
pK_a:	9.7
Water solubility:	n. a.
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	Due to solubility issues, pH 5 was tested to a limited extent.
Supplier:	*Sigma-Aldrich*

b. Propranolol (hydrochloride)

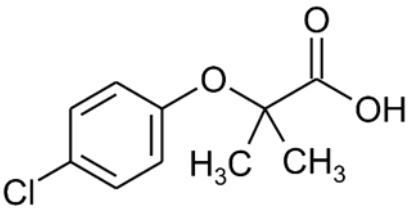
Formula:	$C_{16}H_{21}NO_2$ $C_{16}H_{22}ClNO_2$ (hydrochloride)
Substance group:	Beta blocker
Usage:	High blood pressure, cardiac arrhythmia
CAS:	525-66-6



	318-98-9 (hydrochloride)
Molecular weight:	259.34 g/mol 295.80 g/mol (hydrochloride)
Acid/base	Base
pK_a:	9.46
Water solubility:	61.7 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

3.3.1.6 Cholesterol-lowering agents

a. Clofibric acid

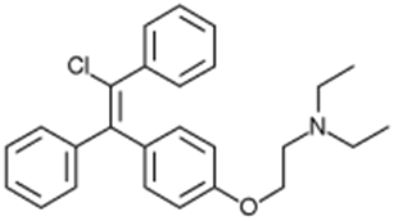


Formula:	$C_{10}H_{11}ClO_3$
Substance group:	Aryloxyalkanecarboxylic acid
Usage:	Cholesterol-lowering agent
CAS:	882-09-7
Molecular weight:	214.65 g/mol
Acid/base	Acid
pK_a:	3.2
Water solubility:	580 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 8 and 9
Notes:	Terminated due to low toxicity and limited solubility.
Supplier:	*TCI Deutschland GmbH*

3.3.1.7 Estrogen receptor modulators

a. Enclomiphene (hydrochloride)

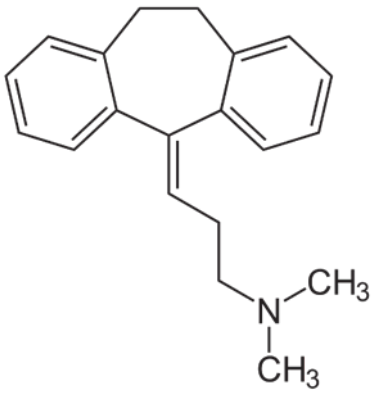
Formula:	$C_{26}H_{28}ClNO$ $C_{26}H_{29}Cl_2NO$ (hydrochloride)
Substance group:	Estrogen receptor modulator
Usage:	Infertility agent for women
CAS:	15690-57-0



	14158-65-7 (hydrochloride)
Molecular weight:	405.97 g/mol 442.40 g/mol (hydrochloride)
Acid/base	Base
pK_a:	9.31
Water solubility:	1.5 mg/L
Solubiliser used:	-
Tested pH-levels:	8 and 9
Notes:	Only tested to a reduced extent due to effort and costs.
Supplier:	*Sigma-Aldrich*

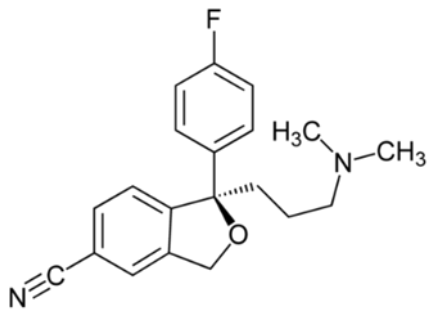
3.3.1.8 Anti-depressants

a. Amitriptyline (hydrochloride)



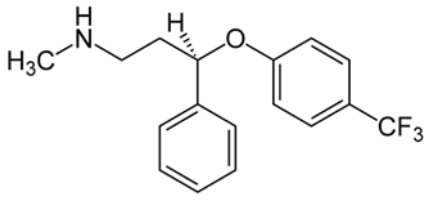
Formula:	$C_{20}H_{23}N$ $C_{20}H_{24}ClN$ (hydrochloride)
Substance group:	TCA (tricyclic anti-depressants) /NSMRI (non-selective monoamine reuptake inhibitor)
Usage:	Anti-depressant
CAS:	50-48-6 549-18-8 (hydrochloride)
Molecular weight:	277.40 g/mol 313.87 g/mol (hydrochloride)
Acid/base	Base
pK_a:	9.4
Water solubility:	9.71 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

b. Citalopram (hydrobromide)



Formula:	$C_{20}H_{21}FN_2O$ $C_{20}H_{22}BrFN_2O$ (hydrobromide)
Substance group:	SSRI (selective serotonin uptake inhibitor)
Usage:	Anti-depressant
CAS:	59729-33-8 59729-32-7 (hydrobromide)
Molecular weight:	324.39 g/mol 405.03 g/mol (hydrobromide)
Acid/base	Base
pK_a:	9.78
Water solubility:	31.09 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

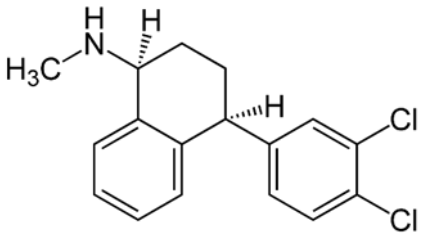
c. Fluoxetine (hydrobromide)



Formula:	$C_{17}H_{18}F_3NO$ $C_{17}H_{19}ClF_3NO$ (hydrobromide)
Substance group:	SSRI (selective serotonin uptake inhibitor)
Usage:	Anti-depressant
CAS:	54910-89-3 56296-78-7 (hydrobromide)
Molecular weight:	309.33 g/mol 345.80 g/mol (hydrobromide)
Acid/base	Base
pK_a:	9.8
Water solubility:	14 g/L (hydrochloride)
Solubiliser used:	-

	Tested pH-levels:	5, 6, 8 and 9
	Notes:	-
	Supplier:	*TIC Deutschland GmbH*

d. Sertraline (hydrobromide)

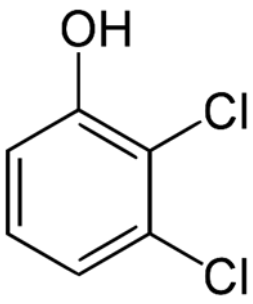


Formula:	$C_{17}H_{17}Cl_2N$ $C_{17}H_{18}Cl_3N$ (hydrobromide)
Substance group:	SSRI (selective serotonin uptake inhibitor)
Usage:	Anti-depressant
CAS:	79617-96-2 79559-97-0 (hydrobromide)
Molecular weight:	306.23 g/mol 342.69 g/mol (hydrobromide)
Acid/base	Base
pK_a:	9.16
Water solubility:	107 mg/L
Solubiliser used:	DMSO
Tested pH-levels:	5, 6, 8 and 9
Notes:	Due to solubility issues, pH 5 was tested to a limited extent.
Supplier:	*TIC Deutschland GmbH*

3.3.2 Pesticides

3.3.2.1 Chlorophenols

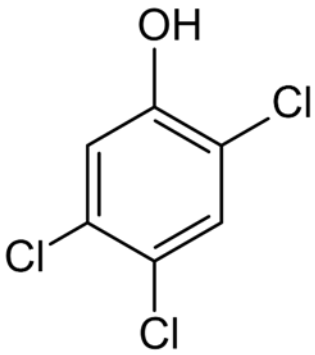
a. 2,3-Dichlorophenol [DCP]



Formula:	$C_6H_4Cl_2O$
Substance group:	Chlorophenols
Usage:	Research chemical
CAS:	576-24-9
Molecular weight:	163.00 g/mol
Acid/base	Acid
pK_a:	7.70
Water solubility:	3.6 mg/L

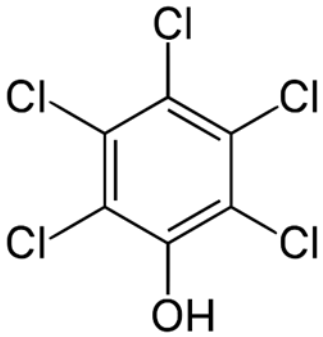
Solubiliser used:	Isopropanol
Tested pH-levels:	5, 6 and 8
Notes:	-
Supplier:	*Sigma-Aldrich*

b. 2,4,5-Trichlorophenol [TCP]



Formula:	$C_6H_3Cl_3O$
Substance group:	Chlorophenols
Usage:	Herbicide, fungicide
CAS:	95-95-4
Molecular weight:	197.45 g/mol
Acid/base	Acid
pK_a:	7.43
Water solubility:	1.19 g/L
Solubiliser used:	Isopropanol
Tested pH-levels:	5, 6 and 8
Notes:	-
Supplier:	*Sigma-Aldrich*

c. Pentachlorophenol [PCP] (sodium salt)

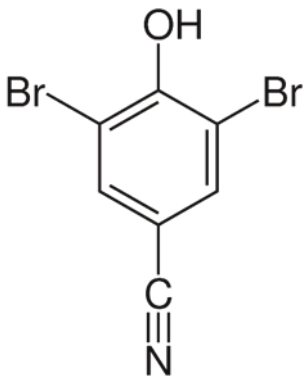


Formula:	C_6HCl_5O C_6Cl_5NaO (sodium salt)
Substance group:	Chlorophenols
Usage:	Pesticide
CAS:	87-86-5 131-52-2 (sodium salt)
Molecular weight:	266.35 g/mol 288.30 g/mol (sodium salt)
Acid/base	Acid
pK_a:	4.7
Water solubility:	14 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 6 and 8

Notes:	-
Supplier:	*abcr GmbH*

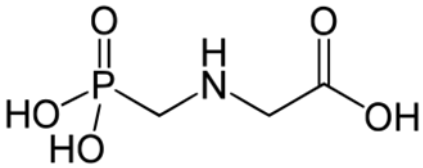
3.3.2.2 Herbicides

a. Bromoxynil



Formula:	$C_7H_3Br_2NO$
Substance group:	Benzonitriles
Usage:	Herbicide
CAS:	1689-84-5
Molecular weight:	276.92 g/mol
Acid/base	Acid
pK_a:	3.86
Water solubility:	130 mg/L
Solubiliser used:	Isopropanol
Tested pH-levels:	5, 6 and 8
Notes:	-
Supplier:	*Sigma-Aldrich*

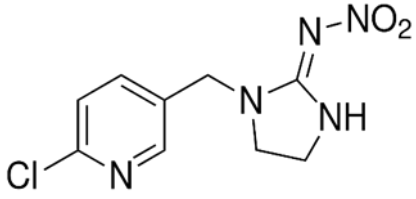
b. Glyphosate



Formula:	$C_3H_8NO_5P$
Substance group:	Shikimate pathway inhibitor
Usage:	Total herbicide
CAS:	1071-83-6
Molecular weight:	169.07 g/mol
Acid/base	Acid
pK_a:	< 2; 2.6; 5.6; 10.6
Water solubility:	10.1 g/L
Solubiliser used:	-
Tested pH-levels:	5 and 6
Notes:	Terminated due to low toxicity and limited solubility.
Supplier:	*Sigma-Aldrich*

3.3.2.3 Insecticides

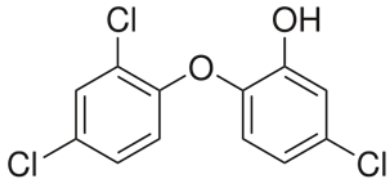
a. Imidacloprid



Formula:	$C_9H_{10}ClN_5O_2$
Substance group:	Neonicotinoids
Usage:	Insecticide
CAS:	138261-41-3
Molecular weight:	255.66 g/mol
Acid/base	Base
pK_a:	1.56; 11.12
Water solubility:	510 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 6 and 8
Notes:	Terminated early due to low toxicity and limited solubility.
Supplier:	*Sigma-Aldrich*

3.3.2.4 Antimicrobial agents

a. Triclosan



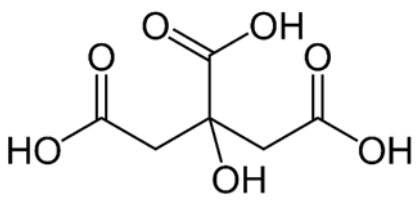
Formula:	$C_{12}H_7Cl_3O_2$
Substance group:	Antimicrobial agent
Usage:	Disinfectant, biocide
CAS:	3380-34-5
Molecular weight:	289.54 g/mol
Acid/base	Acid
pK_a:	7.90
Water solubility:	10 mg/L
Solubiliser used:	-
Tested pH-levels:	5, 6, 8 and 9
Notes:	-
Supplier:	*Sigma-Aldrich*

3.3.3 Other substances

3.3.3.1 Tricarboxylic acids

a. Citric acid

Formula:	$C_6H_8O_7$



Substance group:	Tricarboxylic acid
Usage:	Citric acid cycle
CAS:	77-92-9
Molecular weight:	192.13 g/mol
Acid/base	Acid
pK_a:	3.13; 4.76; 6.40
Water solubility:	592 g/L
Solubiliser used:	-
Tested pH-levels:	5, 6 and 8
Notes:	-
Supplier:	*Sigma-Aldrich*

3.4 Substances that did not work or were rejected beforehand

During the course of the project, many substances were discussed and investigated according to the essential criteria stated in Chapter 3.2. Substances that were shortlisted but ultimately not used due to individual characteristics, or that were tested but turned out to be unsuitable, are summarised in Table 3.

Table 3: Attempted or rejected substances during the course of the pHION project due to the listed issues

Substance	Attempted/ rejected	Issue
2,4-D	attempted	solubility issues; tested solubilisers: DMSO, methanol, isopropanol, acetone
Amisulprid	rejected	little information; expensive
Amitrol	rejected	log D slope < pH 5
Atenolol	rejected	low toxicity
Atrazine	rejected	log D slope < pH 5
Bedaquiline	rejected	solubility issue
Bezafibrat	rejected	solubility issue
Bisoprolol	rejected	expensive; no information
Bromadiolon	rejected	solubility issue
Buprenorphine	rejected	restricted purchase → documentation obligation

Substance	Attempted/ rejected	Issue
Cetrizine	rejected	log D curve unsuitable
Cimetidin	rejected	poor membrane permeability
Diltiazem	rejected	expensive
Dimethindene	rejected	solubility issue
Doxylamine	rejected	solubility issues at pH 5 and pH 6 expected (LC50 [pH 5.5] > 1400 mg/L)
Fenofibric acid	attempted	solubility issues; tested solubilisers: DMSO, methanol, isopropanol, acetone
Genistein	rejected	solubility issue; low toxicity expected at pH 9
Ketoprofen	rejected	solubility issue
Lumacaftor	rejected	no information; expensive
Mepivacaine	rejected	no information
Octenidine	rejected	neutral plateau only
Paroxetine	rejected	solubility issue; expensive
Quinmerac	attempted	solubility issues; tested solubilisers: DMSO, methanol, isopropanol, acetone
Simazine	rejected	log D slope < pH 5
Sulfamethoxazol	rejected	little differences between pH levels; no information about toxicity
Sulfamidine	rejected	little differences between pH levels
Tamoxifen	rejected	solubility issue; expensive; no mortality < 1.85 mg/L
Terbuthylazine	rejected	log D slope < pH 5
Theophylline	rejected	plateau between pH 3 and 8
Thifensulfuron	rejected	solubility issue
Valsartan	rejected	solubility issue
Verapamil	rejected	solubility issue
Warfarin	rejected	solubility issue; flat microtoxicity

Source: Own depiction

4 WP 4: Establishment of the embryo test with *Danio rerio* at different pH levels

4.1 Preliminary experience on the tolerance of *Danio rerio* embryos towards low pH levels

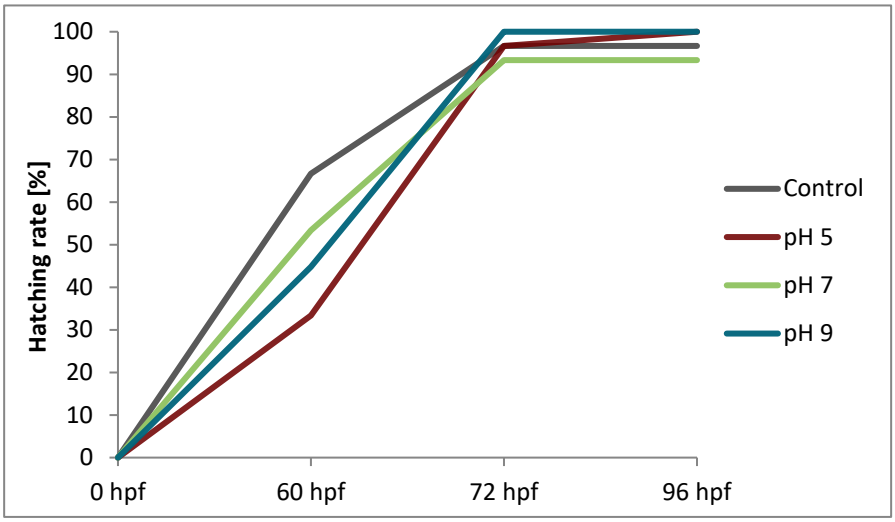
In the course of another project, zebrafish embryos were exposed to unbuffed glyphosate (Schweizer et al. 2019). Since glyphosate as an acid significantly lowers the pH value, pH values down to < 3 were measured depending on the concentration. It was shown, also in the accompanying pH controls, that pH values around pH 3 in particular induce mortality rates within the first days of exposure of almost consistently 100 %. At pH values between 3 and 3.5, mortality was generally still significantly increased. This decreased significantly from a pH above 3.5 and was no longer present at pH 4.

4.2 FET with *Danio rerio*: pre-tests at pH 5, pH 7 and pH 9

To ensure the suitability of the pH range considered in the project, preliminary tests were carried out with embryos of the zebrafish (*Danio rerio*). The fish embryo test (FET) was conducted mainly on the basis of OECD Guideline 236. Ten individuals per batch were used and the test was repeated three times. The pH values 5, 7 and 9 were tested as well as a negative control with artificial water (approximately pH 7.3), whose pH was not artificially adjusted with 1M HCl to lower the pH or 1M NaOH to increase the pH.

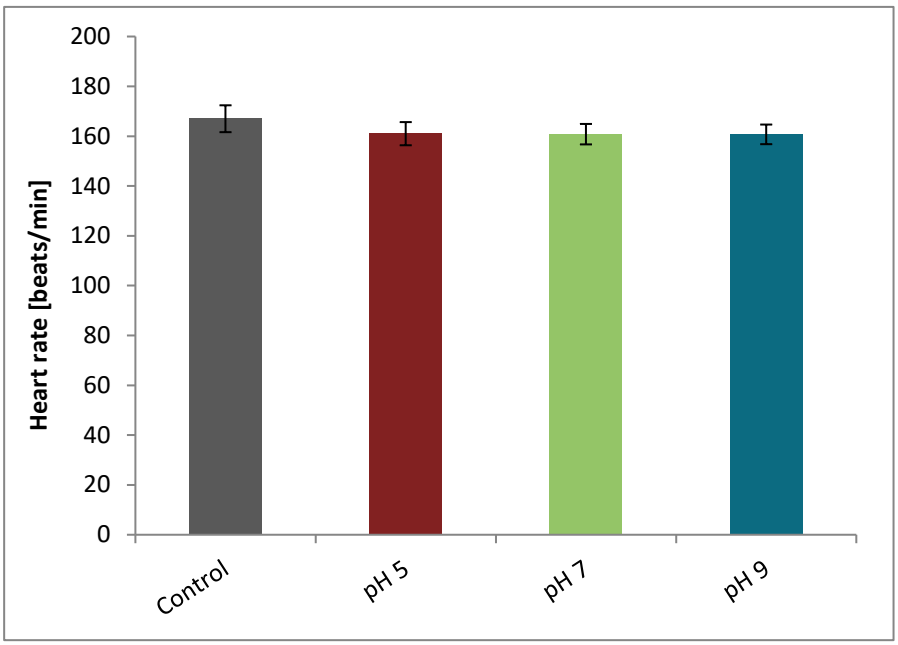
The results did not show any impairment of the embryos, neither by increasing nor decreasing the pH value. No mortality was recorded, except in the pH 9 approach, which showed a mortality of 3.33 %, due to one dead individual in the entire trial. Neither developmental delays at 24 hpf (*hours post fertilisation*) nor malformations at 96 hpf occurred. The hatching success after 96 hpf was between 93 and 100 % for all approaches including the negative controls. The hatching process (see Figure 4.1) over time also showed a consistent pattern. No significant differences were found between the pH approaches and the negative control and pH approaches. A similar pattern was observed for the heart rates at the 48 hpf. While the mean heart rate differed by less than one beat per minute between the pH approaches (pH 5: 161.0 beats/min; pH 7: 160.8 beats/min; pH 9: 160.7 beats/min), 167.0 beats/min were measured in the negative control, which can be attributed to the somewhat larger scatter of the individual values (including some outliers). Overall, however, the measured heart rates were extremely consistent (see Figure 4.2).

Figure 4.1: Mean hatching rate of *Danio rerio* embryos



Mean hatching rate of *Danio rerio* embryos over time when exposed to different pH levels (pH 5, 7, 9) and the negative control (pH approx. 7.3).
Source: Own depiction

Figure 4.2: Mean heart rates of *Danio rerio* embryos



Mean heart rates in beats per minute (including standard deviations) of *Danio rerio* embryos at 48 hpf at different pH levels (pH 5, 7, 9) and within the negative control (pH approx. 7.3).
Source: Own depiction

4.3 Stability of pH and suitable buffer systems

Since the focus of the pHION project is on the pH-dependent toxicity of ionisable substances, the stability of the adjusted pH in the test solutions used must be guaranteed. The artificial water already used in the preliminary tests, consisting of double-distilled water (*aqua bidest.*) to which different salts were added (for further details see paragraph 4.3.1). That artificial water has proven to be easy to handle with regard to setting the desired pH with HCl and NaOH. However, it indicated a low buffer capacity and thus, a rather low stability of the artificial water, although in other experiments at the University of Tübingen, no problems had occurred in larger volumes.

To test the pH stability, stock solutions of artificial water and diclofenac were prepared with different pH values (pH 5, 6 and 8) and placed into vessels of different sizes. The pH value was then measured at several time points over four days. Using a small electrode (*Seven Compact Duo S213* with *InLab Micro*® electrode, *Mettler Toledo,* Gießen), the pH could also be measured within the small exposure petri dishes with a filling volume of approx. 3-4 mL. It was found that the pH value only remained stable within large volumes (500 mL, 1 L) and deviated from the originally set pH by a maximum of ΔpH = 0.3. In small volumes (50 mL, 3 mL), on the other hand, significant fluctuations of the pH value were observed. The solutions in the range of pH 8 showed the smallest deviations, whereas at pH 5 and pH 6, a clear increase of the pH was recorded after 96 h. Basically, it can be stated with regard to the pH stability of the artificial water that the smaller the volume, the more unstable the pH.

For the pHION experiments, this means that the use of suitable buffers is essential. These were currently selected in consultation with colleagues at the UBA in Berlin/Marienfelde, who have the relevant previous experience.

4.3.1 Artificial water

Artificial water was prepared by using four different stock solutions. Each stock solution consisted of one component diluted in ad 1000 mL *aqua bidest*. Finally, for 1000 mL artificial water, 25 mL of each stock solution was added to 900 mL *aqua bidest*.

Component A: 0.23 g KCl

Component B: 2.59 g $NaHCO_3$

Component C: 4.93 g $MgS_4O \cdot 7\ H_2O$

Component D: 11.76 g $CaCl_2 \cdot 2\ H_2O$

4.3.2 Potassium hydrogen phthalate buffer (pH 5)

Recipe according to Clark and Lubs (Clark and Lubs 1916).

Component A: 20.418 g C8H5KO4 + 500 mL ad H2O (*aqua bidest*.)

Component B: 0.1 M NaOH

→ 250 mL component A + 119.25 mL component B + ad 1000 mL *aqua bidest*.

Finally, dilute stock solution with artificial water in the ratio 1:10.

4.3.3 Phosphate buffer (pH 6)

Component A: Sodium phosphate dibasic (Na_2HPO_4)

Component B: Sodium phosphate monobasic (NaH_2PO_4)

→ 900 mL artificial water + 0.533 g component A + 1.19 g component B

➔ Adjust solution to pH 6 using HCl/NaOH.

➔ Add artificial water to 1000 mL.

4.3.4 Tris buffer [0.1 M] (pH 8)

Component A: Tris buffer

Component B: HCl (32 %)

→ 12.114 g component A + 5.57 mL component B + ad 1000 mL *aqua bidest*.

➔ Before adding the acid, solve component A with some of the *aqua bidest*.

Finally, dilute stock solution with artificial water to 0.01 M.

4.3.5 Glycine buffer (pH 9)

Recipe according to Sörensen.

Component A: 7.505 g glycine + 5.85 g NaCl + 1000 mL ad *aqua bidest*.

Component B: 0.1 M NaOH

→ 876 mL component A + 124 mL component B

Finally, dilute stock solution with artificial water in a ratio of 1:1.

4.4 Issues with embryo health at the pH limits and adjustment of buffer systems

Although the preliminary FET tests were encouraging, in the subsequent substance testing, results for mortality were highly variable emphasising that we operated at embryos' tolerable limits in terms of pH.

4.4.1 Mortality issues at pH 5 and adjustment of the sodium hydrogen phthalate buffer

In the course of the first series of experiments, it became evident that the exposure of the embryos under constant conditions in the range of pH 5 also led to increased mortalities in the control towards the end of the 96h experiment. While there was basically no mortality in the buffer control up to 72 h, there were repeated increases in mortality of varying degrees in the time window between 72 and 96 h, so that some runs had to be repeated. Due to the high potassium concentration of the buffer, we decided to reduce the potential salt stress by diluting the original buffer solution. During additional test with the potassium hydrogen phthalate buffer, different dilutions were investigated considering (1) their impact on the embryos and (2) their buffering capacity. With a dilution of 1:10, we were able to reduce embryo mortality to almost 0 %, while the full buffering capacity was still provided. Thus, the potassium hydrogen phthalate buffer was subsequently only used in a 1:10 dilution.

4.4.2 Mortality and hatching issues at pH 9 and adjustment of the glycine buffer

Due to the fluctuating control mortality and the low to no hatching success at pH 9, test series on the possible dilution of the glycine buffer were carried out again. Based on data from the UBA (provided by Mr Kluttig), the use of a 1:15 dilution was investigated. However, since this did not result in sufficient buffer capacity in our test series, further dilutions in the range of 1:1, 1:2, 1:3 and 1:4 were applied. In all cases, the mortality rates could be significantly reduced and the hatching success increased. While the pH in the preparation vessels, i.e. in larger volumes, remained constant, a drop in pH of 0.3 to 0.5 units was observed after 24 h in the exposure dishes with small volumes of 2-3 mL. However, after the first decrease, the pH remained largely constant in the further course of the experiment until the end of the FET at 96 hpf. Based on these results, it was decided to dilute the glycine buffer according to Sörensen 1:1 in the future (starting with the test series with sertraline and subsequently) in order to keep the mortality rates of the control constantly low. To counteract the drop in pH at the beginning of the experiments, the glycine buffer (including the exposure substance concentrations) was adjusted to about pH 9.3 before being filled into small test Petri dishes. Thus, the pH in the test Petri dishes levelled off at about pH 9 and remained practically constant throughout the entire test.

5 WP 5 – Part A: Embryo tests with 30 selected ionisable substances

5.1 General information on the performance of the fish embryo test (FET) with *Danio rerio*

The general procedure of FETs with *Danio rerio*, including the husbandry parameters of the adult zebrafish, can be retrieved from Schweizer et al. (2019) and was conducted according to OECD TG 236 (2013).

During the 96 hpf (*hours post fertilisation*) FETs, mortalities were checked every 12 to 24 h and hatching rates were recorded from 60 hpf onwards. Depending on the substance and the possibility, additional sublethal endpoints (mainly heart rate) were recorded. On the basis of the mortality data for 72 and 96 hpf, non-linear regressions were performed using the *TableCurve 2D* programme (*v5.01, Systat Software, Inc.*) and the LC_{50} values for 72 hpf and 96 hpf were determined on the basis of the curves obtained. An overview of the LC_{50} values determined so far is shown in Table A1 (*Appendix*).

In order to test whether the course of the log D curve is also reflected in the pH-dependent change in mortality in areas of an ionic plateau, an additional fourth pH value (5 or 9) was tested for some substances.

5.2 Results of the embryo tests with *Danio rerio* for the tested substances

5.2.1 Pharmaceuticals

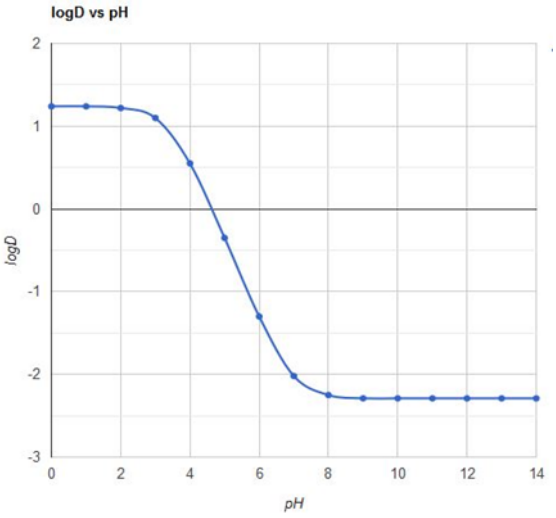
5.2.1.1 Non-steroidal anti-inflammatory drugs (NSAIDs)

a. Acetylsalicylic acid

The NSAID drug acetylsalicylic acid (ASA) was tested at pH 5, 6 and 8. The tests included two range findings as well as the main test. The log D curve for ASA as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.1. The corresponding regressions are shown in Figure 5.1a.

Table 5.1: Overview of the applied ASA concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for ASA as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.10	30.86	19.63	6	10.0	248.42	97.20
	1.00				40.0		
	10.0				70.0		
	12.0				80.0		
	14.0				90.0		
	16.0				100.0		
	18.0				110.0		
	20.0				120.0		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	25.0				150.0		
	40.0				200.0		
	60.0				300.0		
	80.0				500.0		
	100.0				750.0		
8	1500	(3865.4)	3018.7				
	1750						
	2000						
	2500						
	2750						
	2800						
	2900						
	3000						
	3250						
	3300						
	3400						
	3500						
	3750						

Source: Own depiction

As expected, ASA showed a decrease in toxicity with increasing pH, which is reflected in the increasing LC_{50} values. At 96 hpf, the LC_{50} for pH 5 was 19.36 mg/L, for pH 6 97.20 mg/L and for pH 8 3018.7 mg/L. In general, it was noticeable that the lethal effect of ASA set in rather late, which led to significantly lower mortalities and, in the case of pH 8, even to hardly any mortalities. For pH 8, the LC_{50} at 72 hpf could therefore only be extrapolated. In addition, the mortality fluctuated more at pH 8 and even decreased again slightly at the highest concentrations. Despite the solubiliser used, the higher concentrations in particular were at the solubility limit, which is why it cannot be completely ruled out that a small proportion of the ASA was present undissolved and thus not bioavailable, and that concentrations > 3400 mg/L, thus again, triggered lower mortalities.

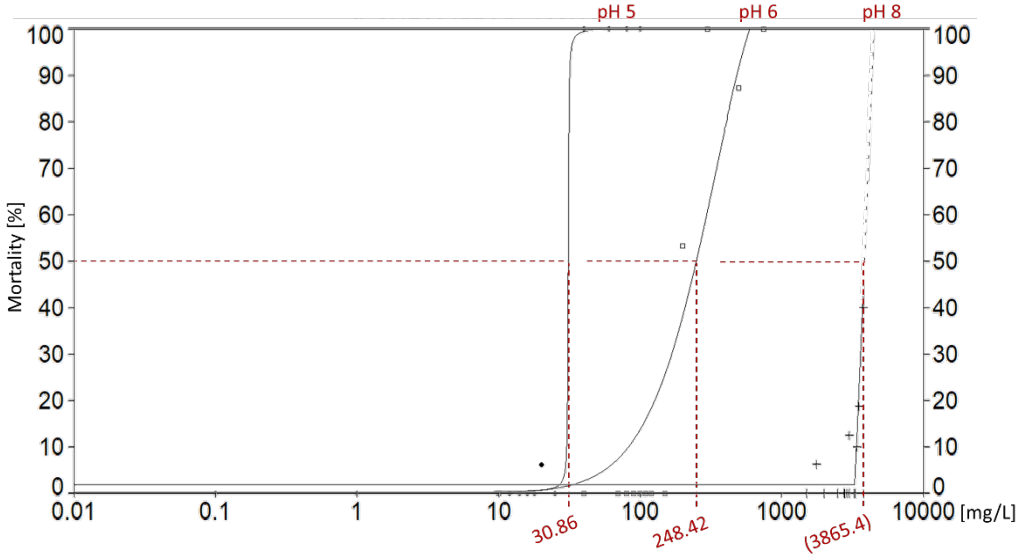
The influence on the heart rate was only evident at relatively high concentrations for all pH values; in the case of pH 5 and 6, only at concentrations that triggered 100 % mortality after 96 hpf (Figure 5.1b). At pH 5, only at a concentration of 60 mg/L did the heart rate drop below the control level of 153 beats per minute on average, to about 130 beats per minute. A comparable decrease occurred at pH 6 in embryos exposed to 200 mg/L ASA. At pH 8, however, there was hardly any effect on the heart rate. The heart rate was largely at the control level, rather with a tendency towards a slight acceleration. The lowest mean heart rate was observed in the control

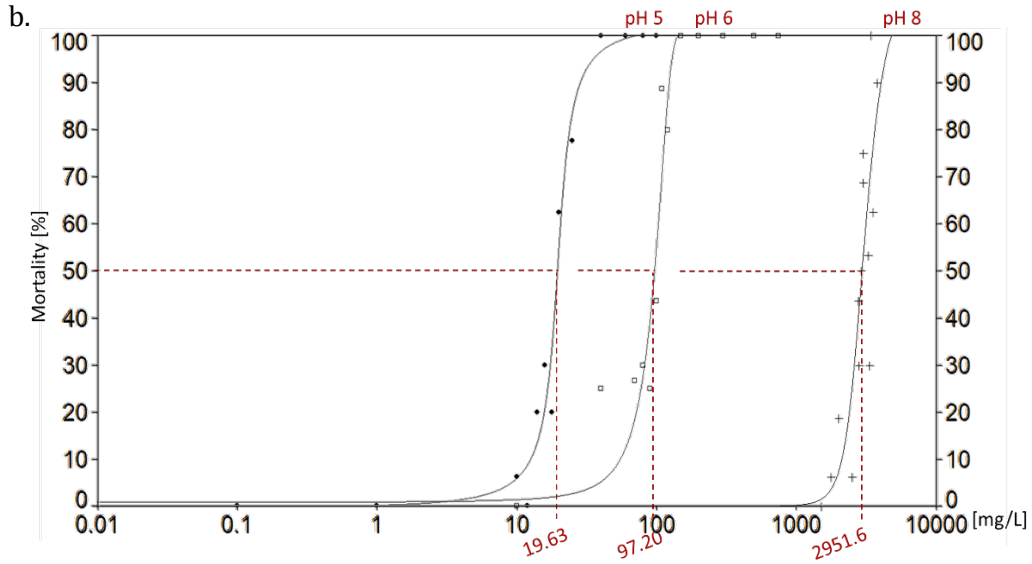
with about 128 beats per minute. Basically, the relatively low influence on the heart rate (measured at 48 hpf) was reflected in the rather later onset of the effect of ASA on the embryos, which had already been shown in the mortality (hardly any mortalities before 72 hpf).
With regard to hatching rate, the collapse was particularly marked at pH 5. Up to concentrations of 25 mg/L (with the exception of 20 mg/L), hatching success at 96 hpf was consistently > 90 %. Only at concentrations ≥ 40 mg/L did the hatching rate decrease to below 20 %, which, however, also correlated with a high mortality. At pH 8, the hatching rate was more mixed, although relatively high hatching rates were observed even at higher mortalities, with at least 20 % hatching over time even at 100 % mortality after 96 hpf. In the case of pH 6, the high concentrations showed a similar picture to pH 5 with no hatching, presumably due to impending mortality. In the low to medium concentrations, hatching success was similarly mixed comparable to pH 8, but this was already reflected in the controls with hatching rate ranging from 12.50 to 81.25 %.

Overall, there was a clear correlation between pH and toxicity in ASA, with toxicity decreasing with increasing pH, as expected for an acid. Despite the solubility difficulties at pH 8, the observed toxicity shift deviated from that predicted by log D by less than a factor of four.

Figure 5.2a: Regression curves of mortality upon exposure of embryos to ASA

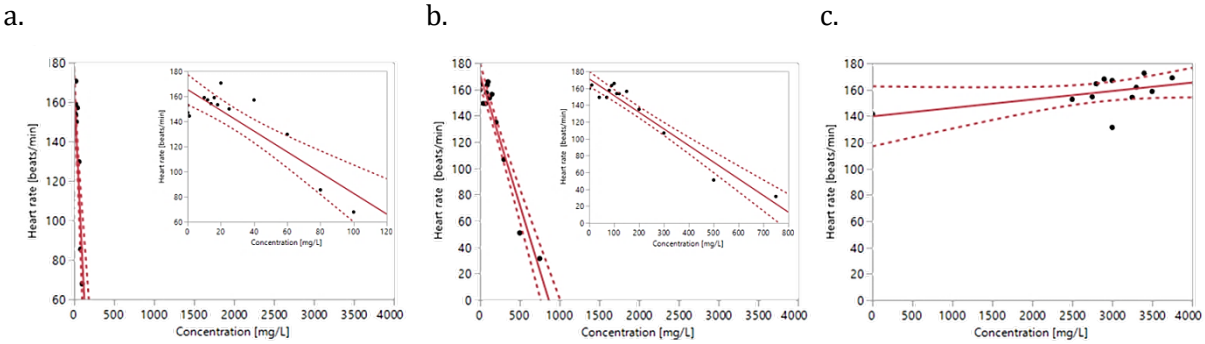
a.





Regression curves of mortality upon exposure of embryos to ASA, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.
Source: Own depiction

Figure 5.3b: Linear regression of heart rate as a function of ASA concentration



Linear regression of heart rate [beats/min] at time 48 hpf as a function of ASA concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8.
Source: Own depiction

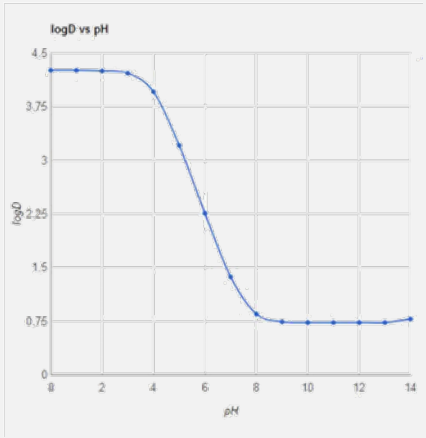
b. Diclofenac

The acid diclofenac was tested at pH 5, 6, 8 and 9. The tests included two range-findings as well as the main test. The log D curve for diclofenac as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.2. The corresponding regressions are shown in Figure 5.2a.

Table 5.2: Overview of the prepared diclofenac concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for diclofenac as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.047	0.22	0.067	6	0.093	1.18	0.64
	0.093				0.233		
	0.140				0.465		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	0.190				0.558		
	0.233				0.605		
	0.280				0.698		
	0.326				0.791		
	0.327				0.930		
	0.558				1.860		
	0.744				2.790		
	0.930				3.720		
	2.325				4.650		
	4.650				9.300		
8	0.930	57.40	19.61	9	10.00	(172.4)	74.0
	4.650				25.00		
	9.300				45.00		
	12.090				50.00		
	15.810				60.00		
	17.205				75.00		
	18.600				76.50		
	23.250				77.50		
	27.900				78.50		
	32.550				80.00		
	37.200				90.00		
	46.500				100.00		
	93.000				150.00		



Source: Own depiction

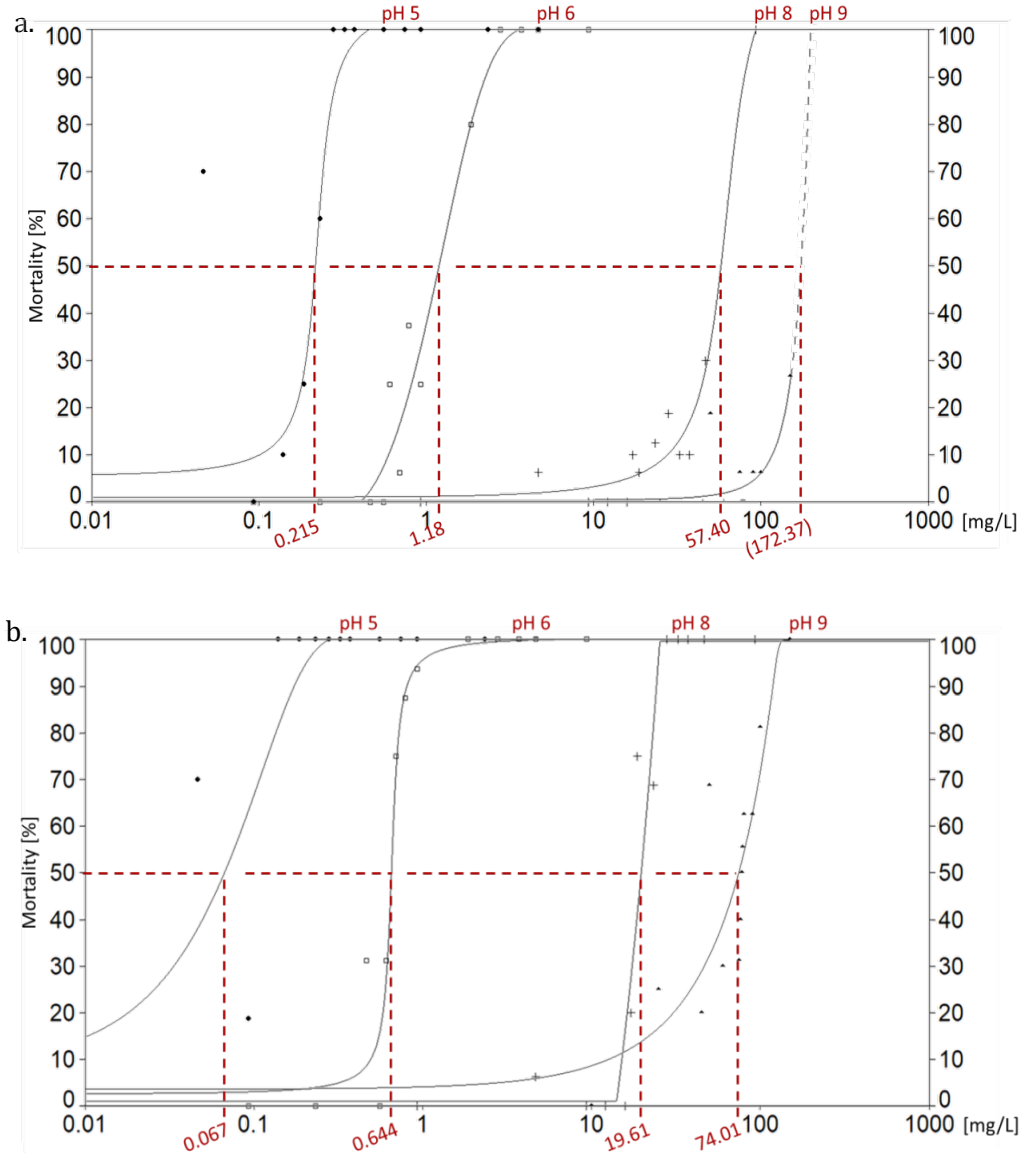
The results showed increasing mortality rates with decreasing pH. At pH 5, therefore, the lowest concentrations were necessary to achieve corresponding mortalities. A comparison of the three pH values showed that a complete mortality of 100 % after 96 hpf at pH 5 was already achieved at 0.14 mg/L diclofenac, while these values shifted to 1.86 mg/L for pH 6, 27.9 mg/L for pH 8 and 150 mg/L for pH 9. The corresponding LC_{50} values for 96 hpf were 0.067 mg/L, 0.64 mg/L, 19.61 mg/L and 74.0 mg/L for pH 5, 6, 8 and 9, respectively. Since for diclofenac mortalities were induced especially in the last 24 h of the test, the LC_{50} values for 72 hpf were significantly higher at 0.22 mg/L (pH 5), 1.18 mg/L (pH 6) and 57.40 mg/L (pH 8). At pH 9, even at the highest concentration tested (150 mg/L), only 26.67 % mortality could be obtained at the 72 hpf time point, so the LC_{50} for pH 9 (172.4 mg/L) could only be extrapolated.

A similar picture emerged for the hatching rates collected. While at pH 5 no embryos hatched at concentrations as low as 0.186 mg/L, this was only the case at pH 6 for 0.605 mg/L and at pH 8 for 46.50 mg/L. At pH 9, one individual hatched even at the highest concentration. Basically, hatching success was significantly increased at pH 8 compared to the other two pH values, whereas it was not > 50 % at any concentration at pH 9. However, even the control at pH 9 only had hatching rates between 43.75 and 68.75 %.

The heart beat rates at 48 hpf (see Figure 5.2b), especially at pH 5, showed a slowing of the beat rate with increasing concentrations of diclofenac. While the heart beat rate of the control embryos still averaged 164 beats per minute, it was already only 131 beats per minute at 0.134 mg/L and decreased continuously to 20 beats per minute up to 0.372 mg/L. At higher concentrations, no more heartbeats could be detected at pH 5. At pH 6, the decrease in heart rates was less marked. From an average of 152 beats per minute in the control, the heart rate dropped only to 127 beats per minute up to 0.930 mg/L. At 1.860 mg/L it then dropped to 64 beats per minute. Above 1.860 mg/L, there was no heartbeat due to mortality. At pH 8, the heartbeat varied between 145 and 155 beats per minute in the range of 0.930 and 17.205 mg/L and between 130 and 140 beats per minute in the range of 18.60 and 37.20 mg/L, thus decreasing only slightly to moderately in most of the concentration range compared to the control of 161 beats per minute on average. Only at 46.50 mg/L did the heart rate drop to 120, and at 93.0 mg/L it even dropped to just under 30 beats per minute. However, heartbeat could be measured in each of the pH 8 concentrations. A similar picture was seen at pH 9. While the control heart rates varied between 167 and 181 beats per minute, the heart rates were significantly lower in all diclofenac preparations, but decreased only relatively little with increasing concentrations. At concentrations between 10 and 80 mg/L diclofenac, the heart rate usually varied between about 140 and 155 beats per minute, independent of concentration, before dropping to 130, or 120 beats per minute at the highest concentrations between 90 and 150 mg/L.

Overall, it was shown for diclofenac that in the range of low pH values, in which the molecules are present in an increasingly neutral form, the toxicity of the substance increased. An increase in lethality from pH 5 to pH 6 by a factor of ten and from pH 6 to pH 8 by a factor of thirty could be demonstrated.

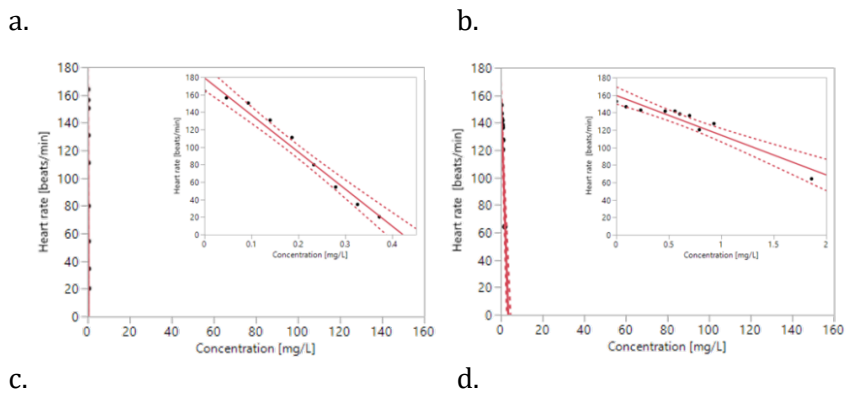
Figure 5.2a: Regression curves of mortality upon exposure of embryos to diclofenac

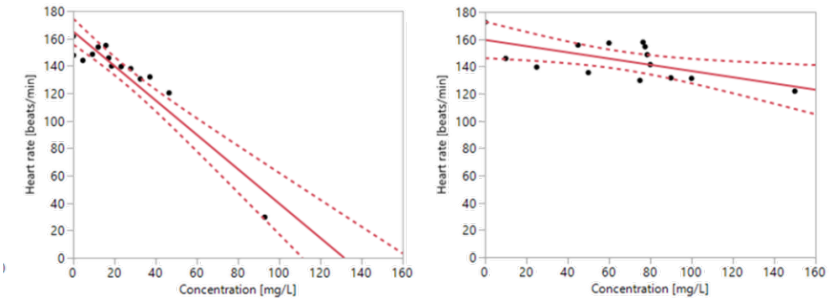


Regression curves of mortality upon exposure of embryos to diclofenac indicating LC50 values; a. after 72 hpf; b. after 96 hpf

Source: Own depiction

Figure 5.2b: Linear regression of heart rate as a function of diclofenac concentration





Linear regression of heart rate [beats/min] at time 48 hpf as a function of diclofenac concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

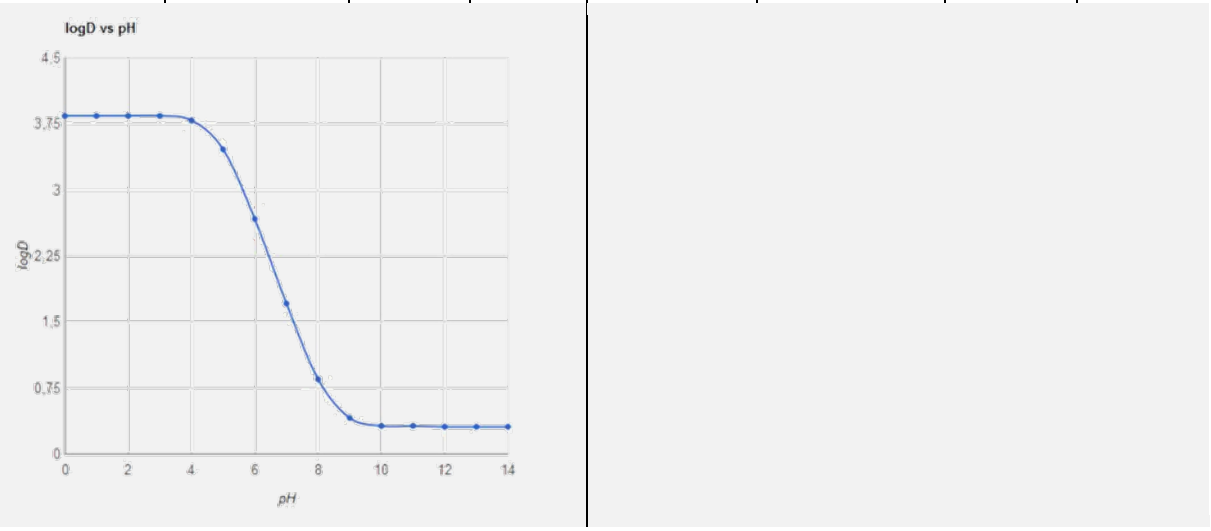
c. Ibuprofen

The arylpropionic acid ibuprofen was tested at pH 5, 6, 8 and 9. The tests included two range-findings as well as the main test. Due to problems with the pH 5 control, all concentrations were tested twice at pH 5 and only the replicates were used for evaluation. After problems with the containment of mortality in several runs at pH 8, the complete batch was repeated with new concentrations. The log D curve for ibuprofen as a function of pH and the concentrations used in each case as well as the LC_{50} values determined are noted in Table 5.3. The corresponding regressions are shown in Figure 5.3a. For pH 8, only the results of the new approach are listed and discussed.

Table 5.3: Overview of the applied ibuprofen concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for ibuprofen as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.01	13.85	8.71	6	0.05	60.28	45.20
	0.02				0.50		
	0.05				5.00		
	0.10				15.0		
	0.20				25.0		
	0.50				30.0		
	1.00				35.0		
	2.00				40.0		
	5.00				42.5		
	15.0				45.0		
	25.0				50.0		
	35.0				75.0		
	50.0				100.0		
8	100.0	249.39	187.12	9	100.0	-	352.23
	125.0				250.0		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	150.0				265.0		
	175.0				285.0		
	200.0				300.0		
	225.0				400.0		
	250.0				450.0		
	400.0				500.0		
	500.0				535.0		
	525.0				570.0		
	540.0				600.0		
	560.0				700.0		
	570.0				750.0		



Source: Own depiction

pH-dependent toxicity differences were also found for ibuprofen. With LC_{50} values at time 96 hpf of 8.71 mg/L (pH 5), 45.20 mg/L (pH 6), 187.12 mg/L (pH 8) and 352.23 mg/L (pH 9), these differences were not as pronounced as for diclofenac, citalopram and propranolol, but still much more marked than for triclosan.

A closer look at the concentration-dependent mortalities at time 96 hpf showed that at pH 5, relatively high mortality rates already occurred at low levels. At concentrations between 0.01 and 5 mg/L, these fluctuated at a level between 45 - 65 % without rising or falling as a function of concentration. Only from 5 to 15 mg/L did mortality jump from just under 45 % to 100 %. At pH 6, only single mortalities occurred in the range from 0.05 to 35 mg/L. Between 40 mg/L and 45 mg/L, mortality was in the middle range between 30 and 50 %, and from 50 mg/L it then reached almost 100 %. In the pH 8 exposures, initial mortalities occurred from 125 mg/L and fluctuated at a similar level between 40 and 60 % up to 225 mg/L. At concentrations of 250 mg/L and 400 mg/L, 100 % mortality was not reached until 96 hpf, while at all concentrations above this, complete mortality was observed at 72 hpf. At pH 9, mortality increased successively

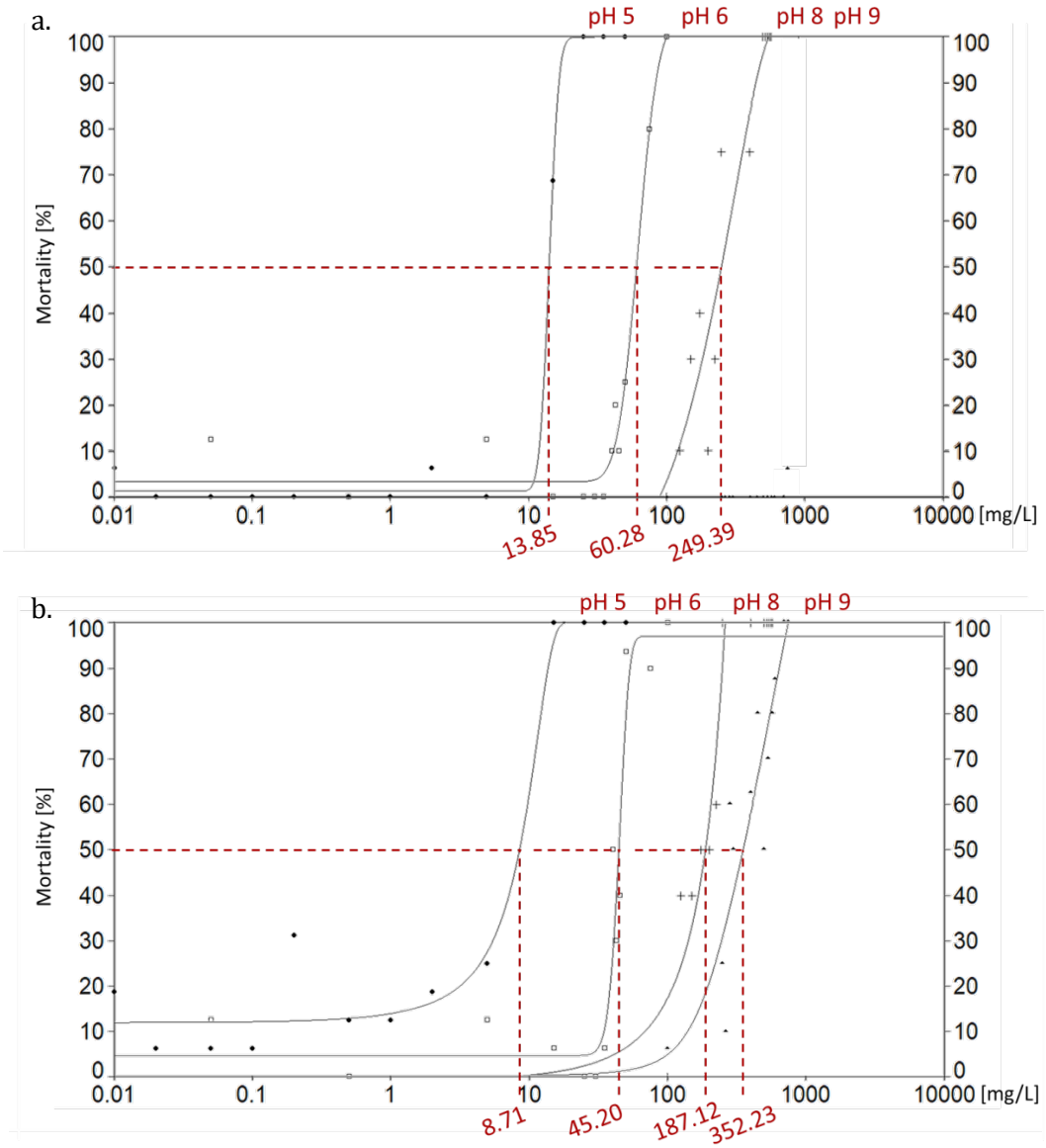
for the most part and reached 100 % at 700 mg/L. It was striking that the mortalities at pH 9 occurred exclusively between 72 and 96 hpf.

With regard to the sublethal endpoints, a marked decrease in heart rate was evident at the lowest two concentrations of pH 5, before it gradually increased again to almost the control level in the range of 0.05 to 1 mg/L, only to drop again to 85 beats per minute at 15 mg/L thereafter (Figure 5.3b). At pH 6, the heart rate fluctuated between 121 and 154 beats per minute between 0.05 and 45 mg/L, relatively independent of the concentration, and thus on average slightly below the control range. Only at 75 mg/L did the heart rate decrease significantly. At pH 8, only the embryos of the 100 mg/L concentration were in the control range (167 beats per minute) with just under 160 beats per minute. Already at 125 mg/L, the frequency dropped significantly to 140 beats per minute. At concentrations up to 250 mg/L, the heart rate decreased significantly to about 80 beats per minute. At 400 mg/L, no more heartbeat could be detected, while at concentrations above this, the embryos were already completely coagulated at this point. At pH 9, the heart rate was significantly lower than in the control with 155 to 175 beats per minute, especially in the medium and high concentrations, but never dropped below 107 beats per minute.

The hatching rate at pH 5 varied between 50 and 90 % up to 1 mg/L ibuprofen, independent of concentration (Figure 5.3c). From a concentration of 5 mg/L, only single individuals hatched. At pH 6, a constantly high rate of between 88 and 94 % was observed in concentration ranges between 0.05 and 5 mg/L. After that, hatching success reduced drastically and from concentrations of 40 mg/L ibuprofen onwards, hatching failed completely. There were also clear effects on hatching behaviour at pH 8. Although hatching success at 100 mg/L was still 100 % after 96 hpf, a hatching delay was already evident. While in the control more than 50 % of the embryos had already hatched after 60 hpf, the hatching rate at 100 mg/L was 0 %. With increasing ibuprofen concentrations, the hatching rate decreased further. At concentrations of 200 mg/L and above, hatching could no longer be observed. At pH 9, hatching success varied between 12.5 and 70 % at concentrations between 100 and 300 mg/L. At concentrations ≥ 400 mg/L, only single individuals hatched. However, hatching success in the control was also relatively variable at 37.5 to 90 %.

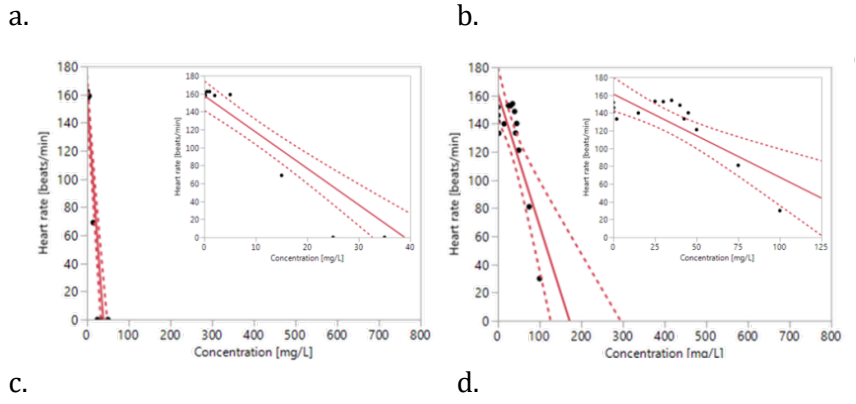
Overall, the toxicity of ibuprofen showed a clear dependence on the pH value, although the differences in toxicity between the pH values were smaller than for diclofenac, for example. Furthermore, the sublethal endpoints determined only reflected the mortality results to a limited extent.

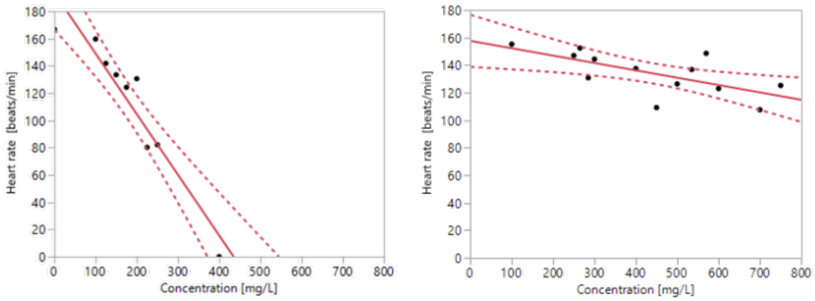
Figure 5.3a: Regression curves of mortality upon exposure of embryos to ibuprofen



Regression curves of mortality upon exposure of embryos to ibuprofen showing LC_{50} values; a. after 72 hpf; b. after 96 hpf.
Source: Own depiction

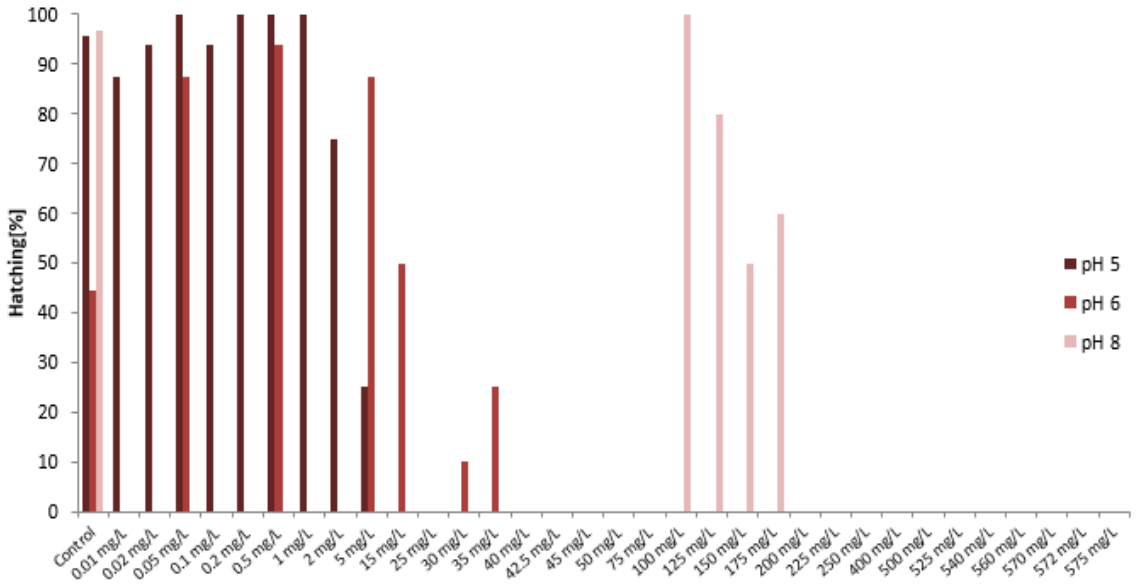
Figure 5.3b: Linear regression of heart rate as a function of ibuprofen concentration





Linear regression of heart rate [beats/min] at time 48 hpf as a function of ibuprofen concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

Figure 5.3c: Hatching rate of embryos depending on ibuprofen concentration



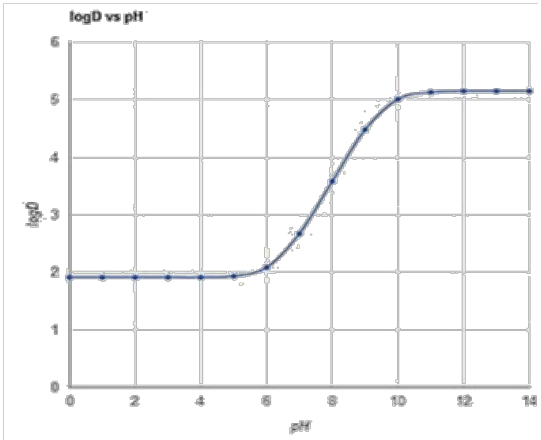
Hatching rate of embryos at time 96 hpf in percent depending on ibuprofen concentration and pH.
Source: Own depiction

d. Naproxen

The acid naproxen was tested at pH 6, 8 and 9. The tests included two range-findings as well as the main test. The log D curve for naproxen as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.4. The corresponding regressions are shown in Figure 5.4a.

Table 5.4: Overview of the applied naproxen concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for naproxen as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
6	0.20	9.36	8.27	8	10.00	358.46	327.25
	2.00				50.00		
	5.00				100.0		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	7.50				250.0		
	8.50				275.0		
	9.50				300.0		
	10.00				320.0		
	11.00				340.0		
	12.00				360.0		
	13.00				380.0		
	15.00				400.0		
	20.00				500.0		
	100.0				750.0		
9	10.00	1594.50	1261.60				
	100.0						
	250.0						
	500.0						
	750.0						
	800.0						
	900.0						
	1000.0						
	1150.0						
	1300.0						
	1450.0						
	1500.0						
	2000.0						

Source: Own depiction

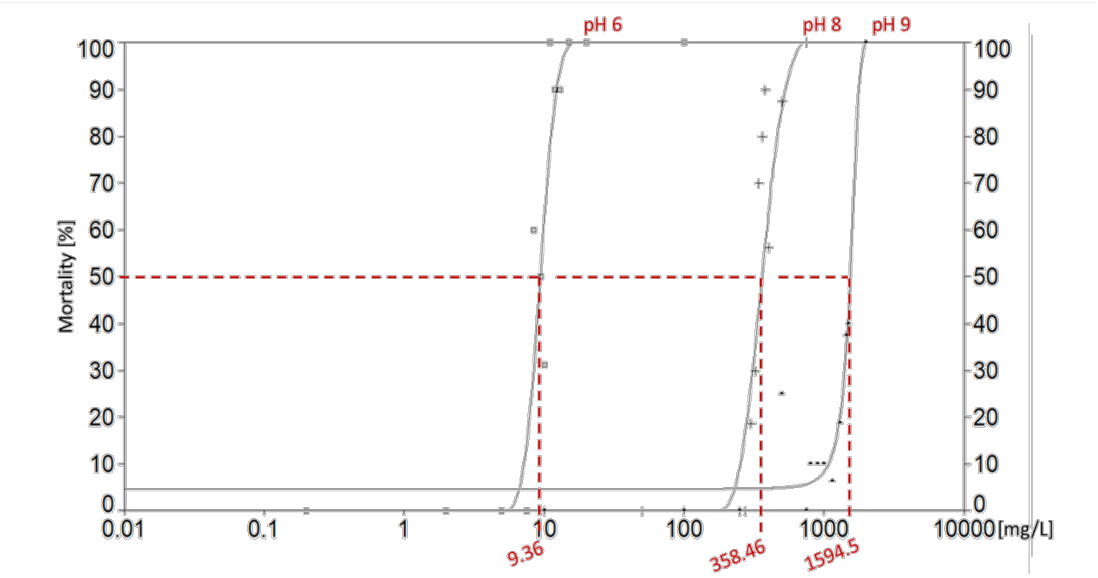
Naproxen showed a clearly increasing toxicity with decreasing pH. The LC_{50} for pH 6 at the 96 hpf time point was 8.27 mg/L, increasing to 327.25 mg/L at pH 8 and even to 1261.60 mg/L at pH 9. The LC_{50} values at time 72 hpf were in similar concentration ranges with 9.36 mg/L (pH 6), 358.46 mg/L (pH 8) and 1594.50 mg/L (pH 9). Naproxen often affected embryos at very early time points and induced significant mortalities in the first hours. However, these mortality rates then remained relatively constant in many cases during the rest of the test.

Heart rate was little affected by naproxen and was in the extended control range between 140 and 165 beats per minute at most concentrations (Figure 5.4b). At pH 8, there was even a tendency for a slight increase in heart beat rate compared to the control at concentrations between 275 and 400 mg/L. Heart beat rates ≤ 140 beats per minute only occurred with concurrent mortalities of at least 80 % at time 48 hpf.

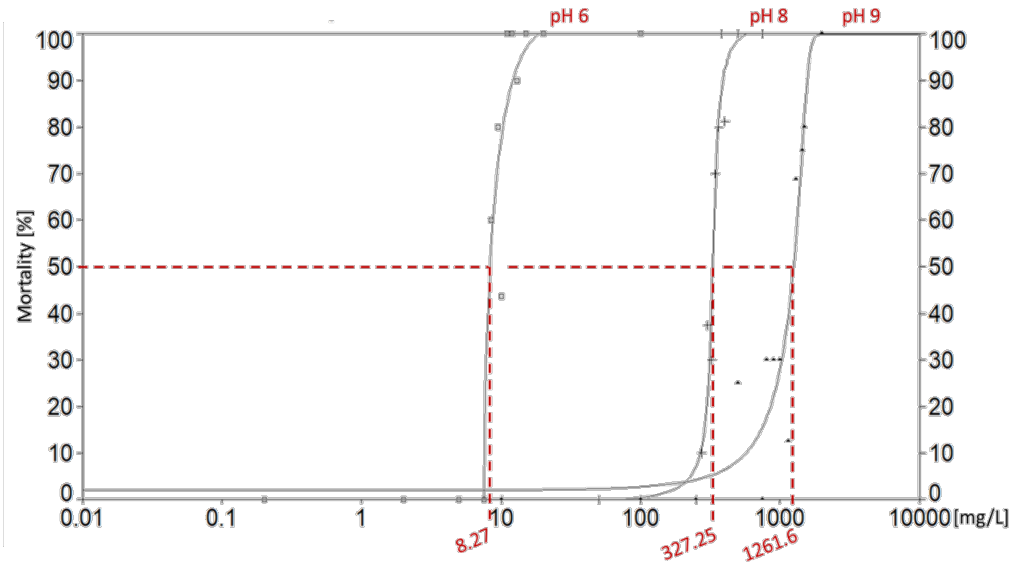
The hatching rate was clearly dependent on pH. While at pH 8 in concentrations up to and including 275 mg/L hatching success was at least 90 % (96 hpf) and thereafter successively decreased with increasing concentrations, at pH 6 only single individuals hatched in the low concentrations and from 8.5 mg/L onwards hatching events failed completely. However, the control hatching rate was also significantly lower than expected, with a maximum of 60 %. At pH 9, hatching success was > 80 % in the two lowest concentrations, fluctuated between 25 and 75 % in the middle concentrations from 250 to 1300 mg/L, before completely failing from 1450 mg/L onwards.

Figure 5.4a: Regression curves of mortality upon exposure of embryos to naproxen

a.



b.

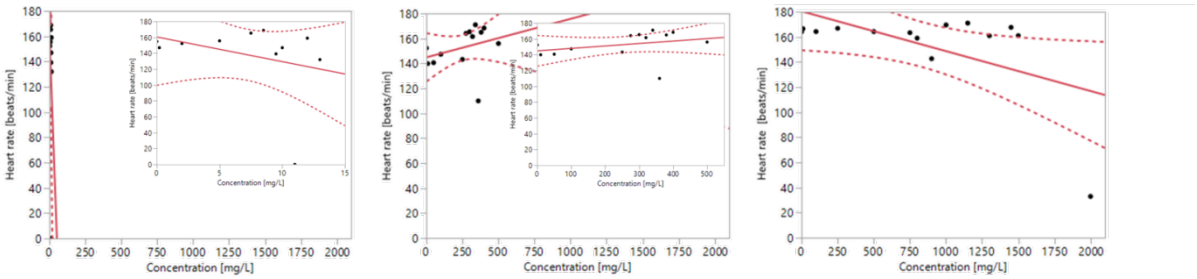


Regression curves of mortality upon exposure of embryos to naproxen showing LC_{50} values; a. after 72 hpf; b. after 96 hpf.
Source: Own depiction

Overall, naproxen showed a clear pH-dependent toxicity, which, as expected for an acid, decreased with increasing pH. The pH-related differences in toxicity are somewhat more pronounced than the Log D suggested, but are very close to the prediction.

Figure 5.4b: Linear regression of heart rate as a function of naproxen concentration

a. b. c.



Linear regression of heart rate [beats/min] at time 48 hpf as a function of naproxen concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 6; b. pH 8; c. pH 9.

5.2.1.2 Anaesthetics

a. Lidocaine

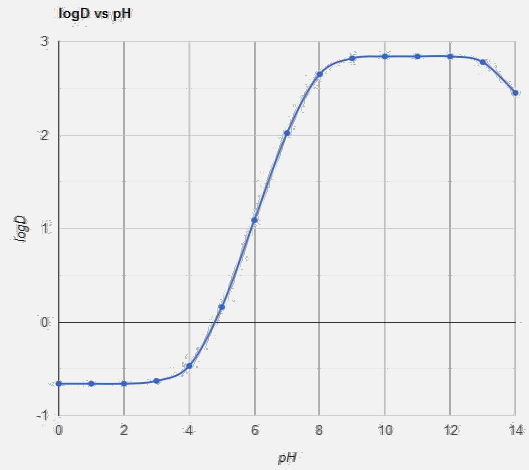
Lidocaine was tested at pH 5, 6, 8 and 9. The tests included two range findings as well as the main test. Due to low mortality with simultaneous solubility problems, pH 6 was not pursued further after two range findings. Solubility problems also occurred at pH 5 above 2000 mg/L, which is why no LC_{50} for 72 hpf could be determined and the LC_{50} for 96 hpf was only extrapolated.

The log D curve for lidocaine as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.5. The corresponding regressions are shown in Figure 5.5a.

Table 5.5: Overview of the prepared lidocaine concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for lidocaine as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	25.00	-	(2079.6)	6	10.00	-	-
	100.0				100.0		
	250.0				250.0		
	500.0				500.0		
	625.0				1000.0		
	750.0				1150.0		
	875.0				1350.0		
	1000.0				1500.0		
	1200.0						
	1400.0						
	1600.0						
	1800.0						

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	2000.0						
8	1.00	69.32	67.47	9	1.00	41.72	26.99
	10.00				10.00		
	50.00				14.00		
	65.00				18.00		
	66.00				22.00		
	68.00				24.00		
	70.00				25.00		
	72.00				26.00		
	74.00				30.00		
	75.00				35.00		
	85.00				40.00		
	100.0				45.00		
	125.0				50.00		

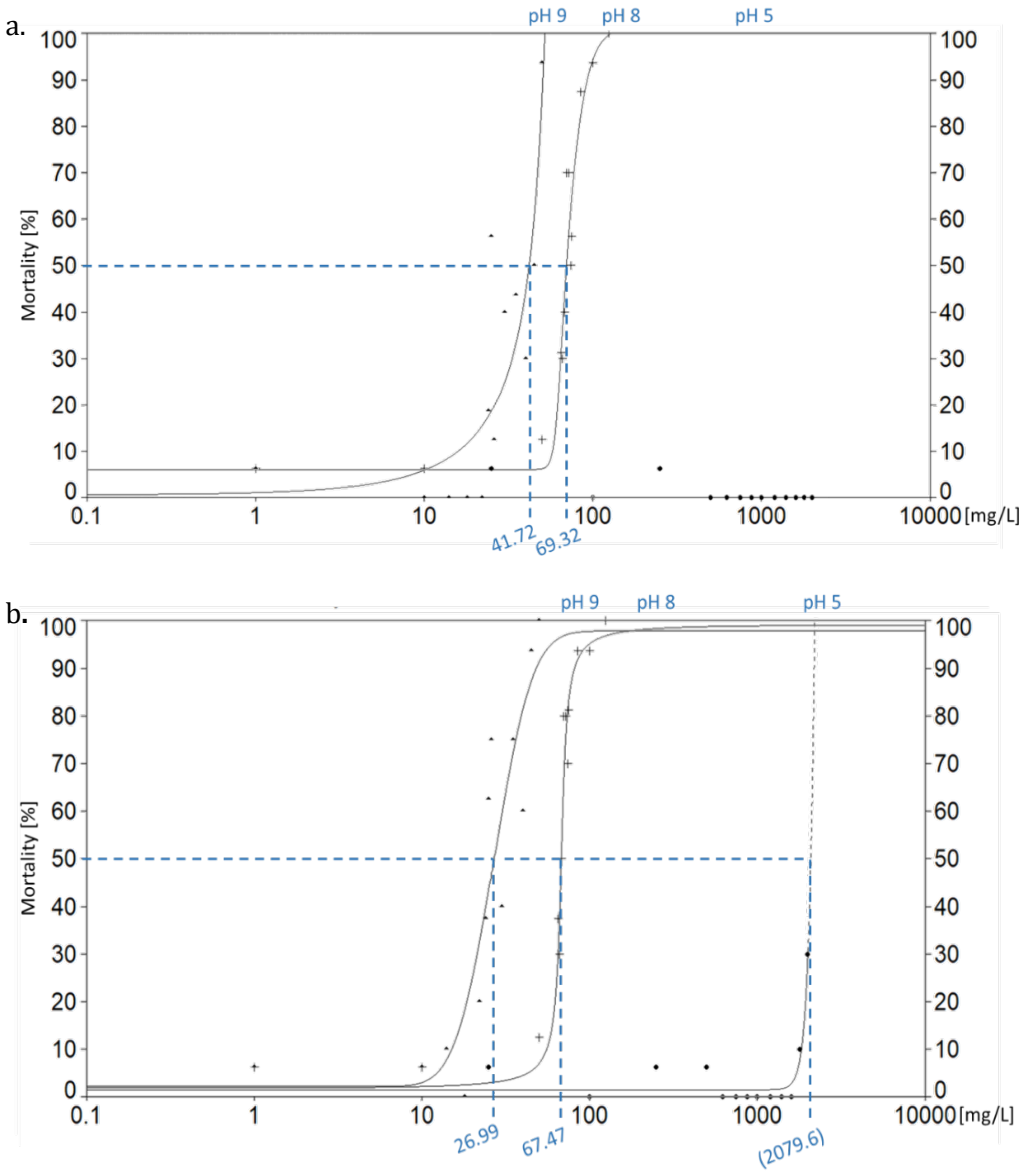


Source: Own depiction

The toxicity of the anaesthetic lidocaine increased markedly with increasing pH, although no LC_{50} (> 1500 mg/L) could be determined for pH 6 due to the high concentrations required and the associated solubility problems, and an LC_{50} of 2079 mg/L (96 hpf) could only be extrapolated for pH 5. Although it was possible to induce lethal effects also at pH 6, the lidocaine precipitated again during the test at the higher concentrations (≥ 1350 mg/L), which was also reflected in the decreasing mortality at 1500 mg/L compared to 1350 mg/L (Figure 5.5b). Since it was possible to induce at least sufficiently high mortalities (≤ 30 %) at pH 5 without any crystallisation of lidocaine occurring, this means that water solubility decreased faster with increasing pH than toxicity increased in the same range. For pH 8 and 9, LC_{50} values could be determined for 72 as well as for 96 hpf. At pH 8, these differed only slightly at 69.32 mg/L (72

hpf) and 67.47 mg/L (96 hpf), while the difference between the two time points was more pronounced at 41.72 mg/L (72 hpf) and 29.99 mg/L (96 hpf).
Compared to tetracaine, lidocaine affected the heart rate of the embryos more moderately (Figure 5.5c). At pH 8, the heart rate was still in the control range up to 10 mg/L and then dropped to 80 to 100 beats per minute, especially from 65 mg/L. From 75 mg/L, the heart rate decreased to below 80 beats per minute, but never dropped below 57 beats per minute. A similar picture was seen at pH 9. The first concentration that lowered the heart rate to below 100 beats per minute was 24 mg/L. With the exception of the concentration of 45 mg/L, at which no heartbeat could be detected in living individuals, the heart rate varied between 24 mg/L and the highest concentration of 50 mg/L between 76 and 96 beats per minute. At pH 5 and 6, the influence of lidocaine on the heartbeat was relatively low, which was certainly due to the low toxic concentrations in these ranges.
In general, the test series of lidocaine showed a clear delay of the hatching, which, however, partly also affected the control. First, mostly few hatching events were usually observed only at 72 hpf. The majority of individuals hatched at 96 hpf. Here, too, the difference between pH 5 and 6 on the one hand and pH 8 and 9 on the other was clearly reflected. While at pH 5 hatching success was consistently between 30 and 100 % and at pH 6 was almost absent only at the higher concentrations ≥ 1000 mg/L, at pH 8 and 9 hatching occurred exclusively at the lowest two to three concentrations. One factor that may have favoured the low hatching success with lidocaine is its effect as an anaesthetic, which already led to reduced to absent spontaneous movements in the chorion in the embryos and thus inhibited a key element of the hatching process.

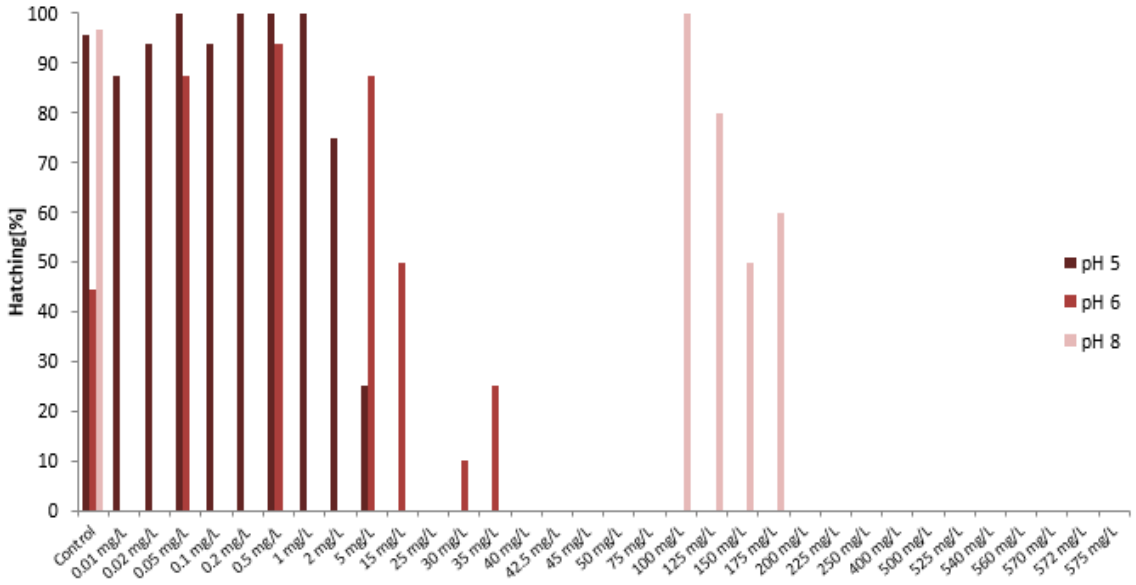
Figure 5.5a: Regression curves of mortality upon exposure of embryos to lidocaine



Regression curves of mortality upon exposure of embryos to lidocaine showing LC50 values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

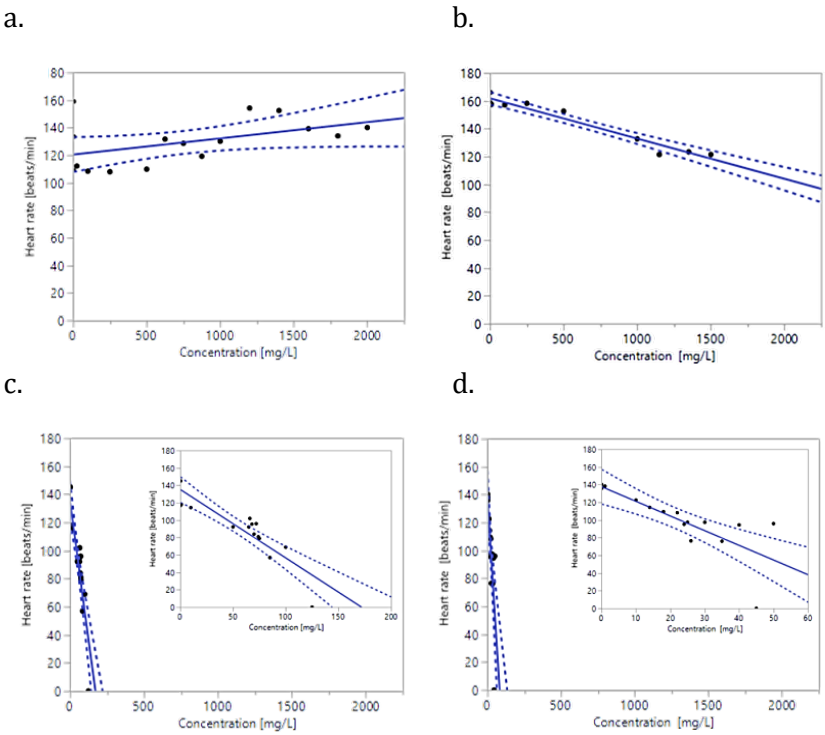
In summary, the base lidocaine showed, as expected, a clear increase in toxicity with increasing pH. Due to the missing or only extrapolated LC_{50} values for pH 5 and 6, the log D prediction can only be compared with the observed toxicity shift to a limited extent. For the difference between pH 8 and 9, however, there was relatively good agreement between the observed toxicity shift and the log D shift.

Figure 5.5b: Mortality of embryos depending on lidocaine concentration



Mortality of embryos at time 96 hpf in percent depending on lidocaine concentration and pH.
Source: Own depiction

Figure 5.5c: Linear regression of heart rate as a function of lidocaine concentration



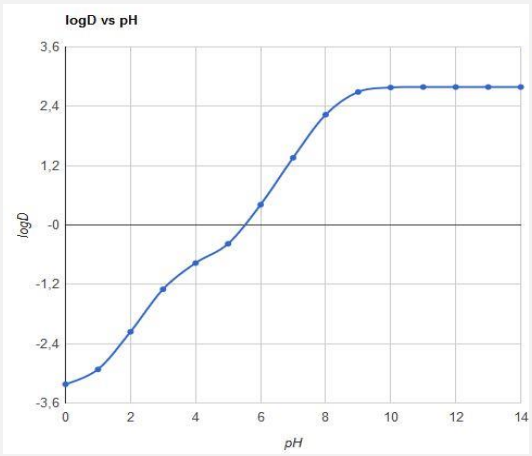
Linear regression of heart rate [beats/min] at time 48 hpf as a function of lidocaine concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

b. Tetracaine

Based on the log D curve, tetracaine was tested as a base at pH 5, 6, 8 and 9. The tests included two range findings as well as the main test. The log D curve for tetracaine as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.6. The corresponding regressions are shown in Figure 5.6a.

Table 5.6: Overview of the prepared tetracaine concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for tetracaine as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.10	(788.50)	170.13	6	0.10	179.56	59.235
	1.00				1.00		
	10.0				10.0		
	100.0				20.0		
	120.0				30.0		
	140.0				40.0		
	160.0				50.0		
	180.0				60.0		
	200.0				70.0		
	250.0				80.0		
	300.0				100.0		
	400.0				150.0		
	500.0				200.0		
8	0.05	6.109	3.938	9	0.0001	-	3.912
	0.10				0.001		
	0.20				0.10		
	0.30				0.50		
	0.40				1.00		
	0.50				2.00		
	0.75				3.00		
	1.00				3.50		
	2.00				3.75		
	4.00				4.00		
	5.00				5.00		
	7.50				10.0		
	50.0				0.0001		



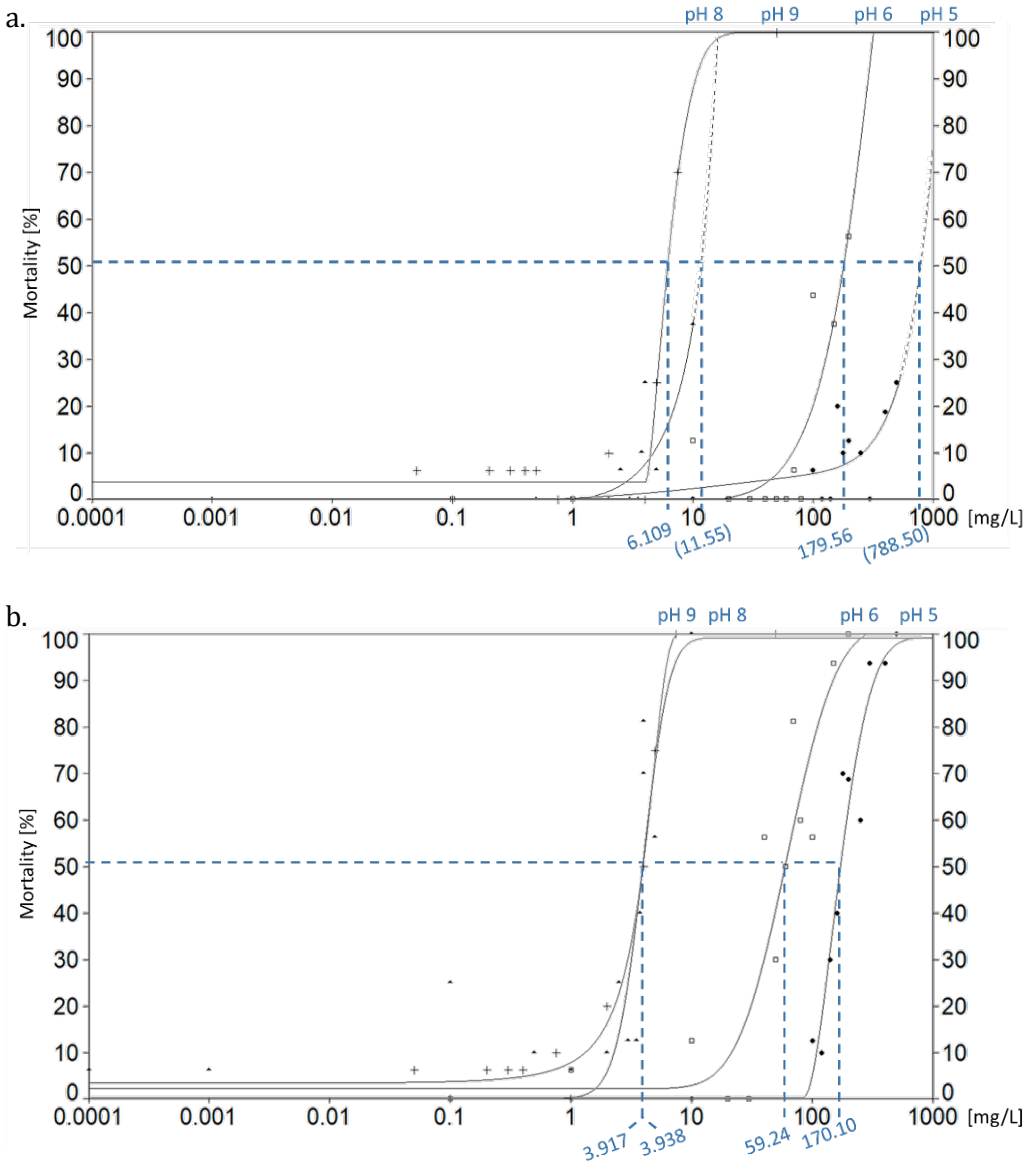
Source: Own depiction

The base tetracaine showed a marked pH-dependent toxicity. The LC_{50} values at time 96 hpf were 170.13 mg/L for pH 5, 59.24 mg/L for pH 6, 3.94 mg/L for pH 8 and 3.91 mg/L for pH 9. The increase in mortality was successive for pH 5 to pH 8, while hardly any difference in toxicity was seen between pH 8 and 9. For pH 5, mortality increased particularly in the last 24 h of the test, which meant that even at the highest concentration of 500 mg/L tetracaine, only 25% mortality could be induced at the 72 hpf time point. Therefore, it was not possible to define a reliable LC_{50} for pH 5 at 72 hpf. Based on the regression curve, an LC_{50} of 788.50 mg/L was finally derived. At pH 8, a mortality of 100 % was observed for the concentration 0.5 mg/L at the time 96 hpf. Since mortalities at concentrations below, or especially above, were still very low and within the control range, only slowly increasing to 20 % at concentrations of 2 mg/L tetracaine and above, the 0.5 mg/L concentration was excluded from further data analysis. Similarly to pH 5, tetracaine at pH 9 did not induce sufficient mortality at 72 hpf to determine the LC_{50} reliably but based on the available data, the LC_{50} was at least extrapolated to 11.553 mg/L. The 72 hpf LC_{50} for pH 9 was higher than for pH 8, which was driven by the late induction of mortality even in the highest concentration at pH 9 and the fact that the difference between pH 8 and 9 was not expected (log D) and not observed to be large, anyways.
In terms of heart rate, at pH 5, there was already a marked slowing to approximately 130 beats per minute at 100 mg/L compared to the control average of 151 beats per minute (Figure 5.6b). The decrease in heart rate continued steadily to 52.5 beats per minute at the highest concentration of 500 mg/L tetracaine. A similar picture emerged at the other pH values. At pH 6, the heart rate dropped significantly below the control level for the first time at a concentration of 20 mg/L with 132 beats per minute. At the highest concentration of pH 6 (200 mg/L), 32.5 beats per minute were finally still counted. At pH 8, the first clear drop in heart rate was seen at 0.30 mg/L to about 136 beats per minute. Here, too, the heart rate decreased successively for the most part. However, at the highest tetracaine concentration (50 mg/L), no more heartbeat could be measured. At pH 9, heart rate was within the control range for concentration up to 4 mg/L. At 4 and 5 mg/L, the heart rate decreased slightly to 140-145 beats per minute before it dropped drastically to 46 beats per minute at a concentration of 10 mg/L.
Comparing the hatching rates in general, it was observed that the embryos at pH 8 showed a significantly higher probability of hatching already at 60 hpf than was the case at pH 5, 6 or 9. Furthermore, hatching success at the 96 hpf time point decreased significantly or failed completely at the high concentrations of all pH values. At pH 6 and 8, the lack of hatching success occurred abruptly: At pH 6, 60 % of the embryos hatched at 30 mg/L, at 40 mg/L only 12.5 %. At all subsequent concentrations combined, only a single individual hatched. At pH 8, the critical

point was between 2 and 4 mg/L tetracaine. While at 2 mg/L hatching success was still 80 %, it dropped to 10 % at 2 mg/L and was completely absent at concentrations above this. At pH 5, the hatching rate fluctuated between 10 and 50 % at concentrations of 140 to 180 mg/L, before hatching finally failed almost completely from 200 mg/L onwards. At pH 9, the hatching success was generally lower compared to the other pH levels across all concentrations including the control. Furthermore, no hatching occurred before 72 hpf and above 4 mg/L tetracaine.

Overall, the toxicity of tetracaine showed a clear dependence on the pH value, although the difference was less pronounced than the log D curve would have suggested. For both mortality and heart rates, the effect increased successively with increasing concentration at all pH values. Only hatching success, especially at pH 6 and 8, showed a sudden decrease, indicating a critical threshold concentration.

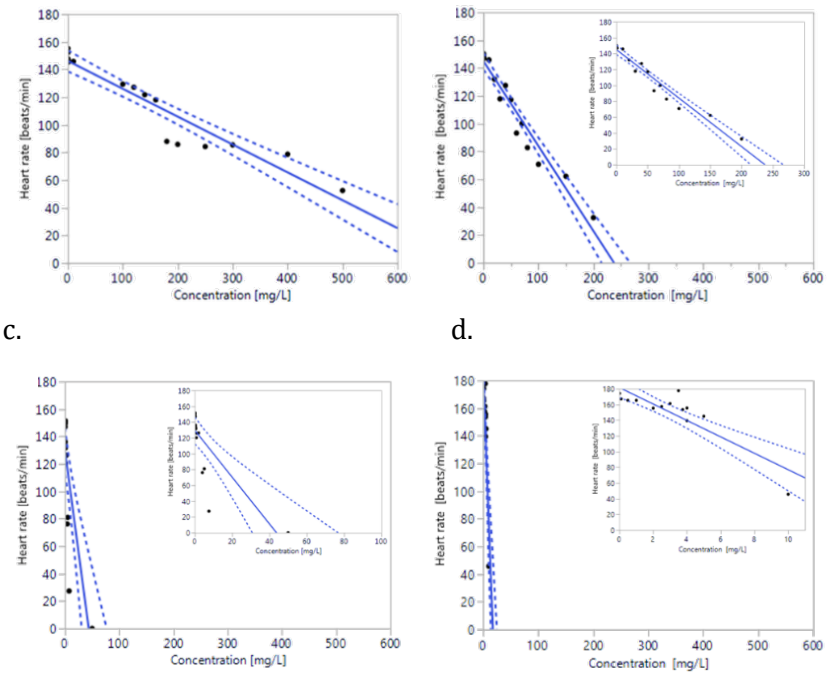
Figure 5.6a: Regression curves of mortality upon exposure of embryos to tetracaine



Regression curves of mortality upon exposure of embryos to tetracaine showing LC_{50} values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

Figure 5.6b: Linear regression of heart rate as a function of tetracaine concentration

a. b.



c. d.

Linear regression of heart rate [beats/min] at time 48 hpf as a function of tetracaine concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

5.2.1.3 Opioids

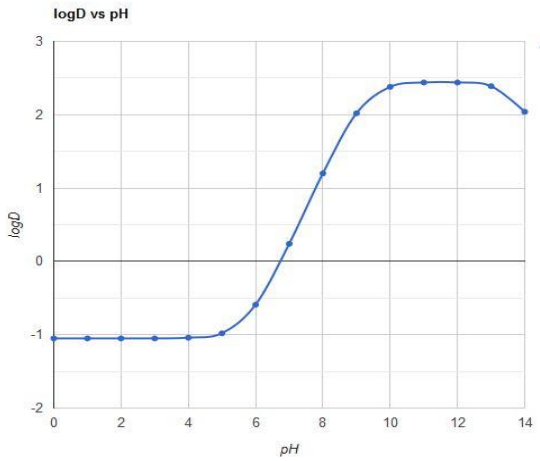
a. Tramadol

The opioid tramadol was tested at pH 6, 8 and 9. The tests included two range findings as well as the main experiment. The attempt to also test tramadol at pH 5 could not be implemented due to the high concentrations required and the associated solubility problems. The log D curve for tramadol as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.7. The corresponding regressions are shown in Figure 5.7a.

Table 5.7: Overview of the applied tramadol concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for tramadol as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
6	1000.0	4992.9	3110.5	8	1.00	90.466	46.280
	1250.0				5.00		
	1500.0				7.50		
	2000.0				10.00		
	2100.0				25.00		
	2200.0				30.00		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	2250.0				35.00		
	2300.0				40.00		
	2500.0				45.00		
	2750.0				50.00		
	3000.0				60.00		
	5000.0				100.0		
	7500.0				250.0		
9	0.01	25.0	18.445				
	0.10						
	1.00						
	10.00						
	12.00						
	14.00						
	16.00						
	18.00						
	20.00						
	25.00						
	50.00						
	75.00						
	100.0						



Source: Own depiction

For tramadol, a clear increase in toxicity with increasing pH could be seen. The LC_{50} values at 96 hpf were 3110.5 mg/L for pH 6, 46.28 mg/L for pH 8 and 18.45 mg/L for pH 9. While for pH 8 and 9 concentrations in the low and mid two-digit mg range were already sufficient to induce mortalities, concentrations above 1 g/L were needed at pH 6 to cause first lethal effects.

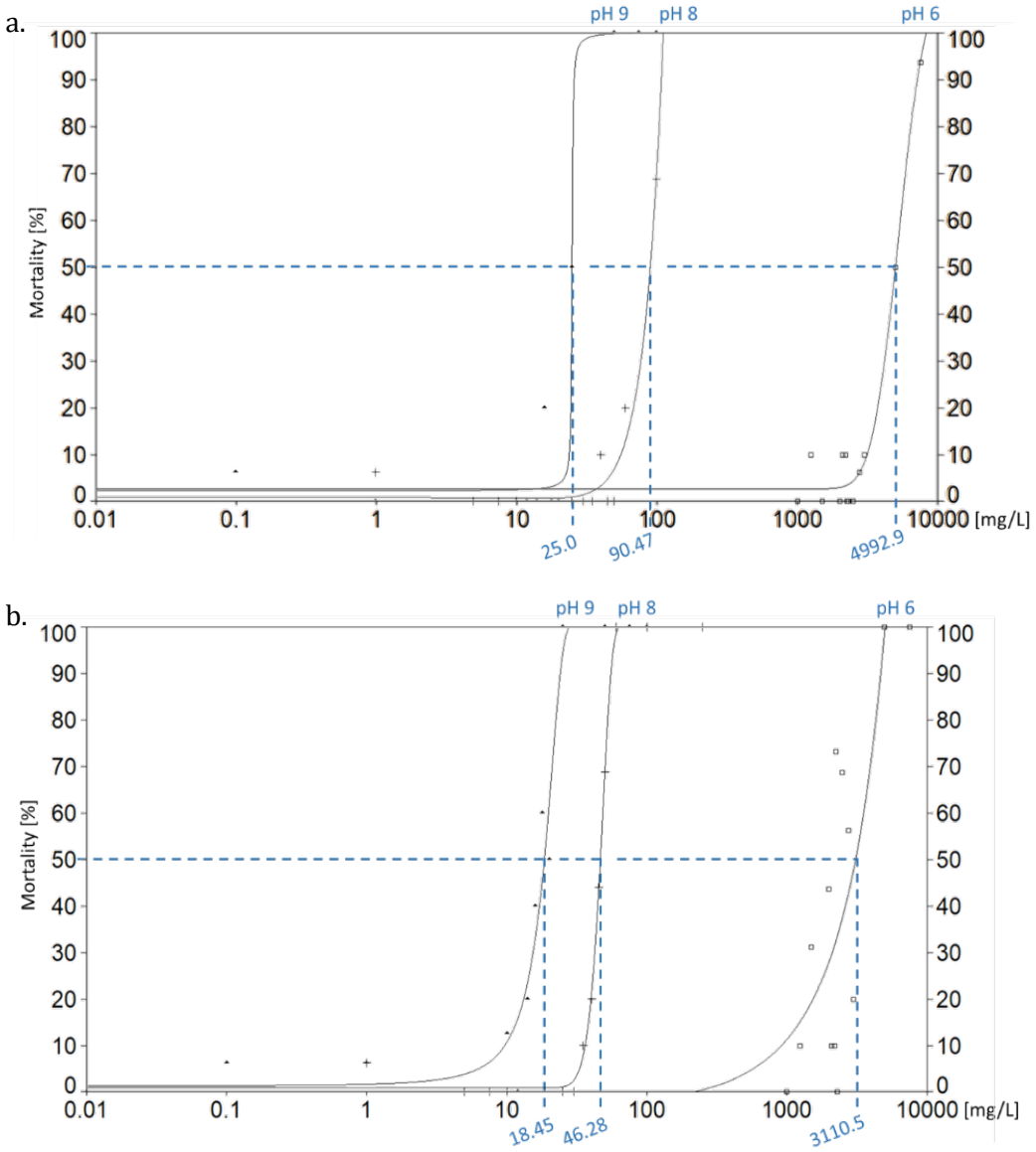
Effects on heart rate were rather moderate. In the range of LC_{50} concentrations, heart beat rates of 145 to 149 beats per minute were counted for pH 6 and 8, which represented a decrease of about 10 to 15 beats compared to the respective controls (average 160 to 162 beats per minute) (Figure 5.7b). Even at very high concentrations (e.g. 5000 mg/L [pH 6] or 100 mg/L [pH 8]), which already produced clear lethal effects at 48 hpf, the heart rate was still well above (pH 6) or just below (pH 8) 100 beats per minute. Only at pH 9 was the reduction in heart rate in the LC_{50} range more pronounced: from an average of 171 beats per minute in the control to 121.5 beats per minute at a tramadol concentration of 18 mg/L.

With regard to the hatching rate, a clear correlation between increasing tramadol concentrations and decreasing hatching success was particularly evident. While at 45 mg/L almost 90 % of the individuals hatched, the hatching rate at 50 mg/L was already only 50 % and fell to 0 % by 100

mg/L. At pH 9, the hatching rate also decreased with increasing concentrations, whereby the control values were only between 50 and 70 % and thus reflected the fundamentally reduced hatching success at pH 9. At pH 6, the hatching rate was relatively variable, due in particular to the second range finding, where hatching success was already significantly reduced in the control (25 %). At the corresponding tramadol concentrations, the hatching success then varied between 0 and 25 %, regardless of the level of the concentrations.

In principle, tramadol shows a clear pH-dependent toxicity, which, as expected for a weak base, increases with increasing pH. The increase in toxicity was particularly marked between pH 6 and pH 8. The observed toxicity differences, especially in the comparison of pH 6 to 8, were close to those shifts derived from log D.

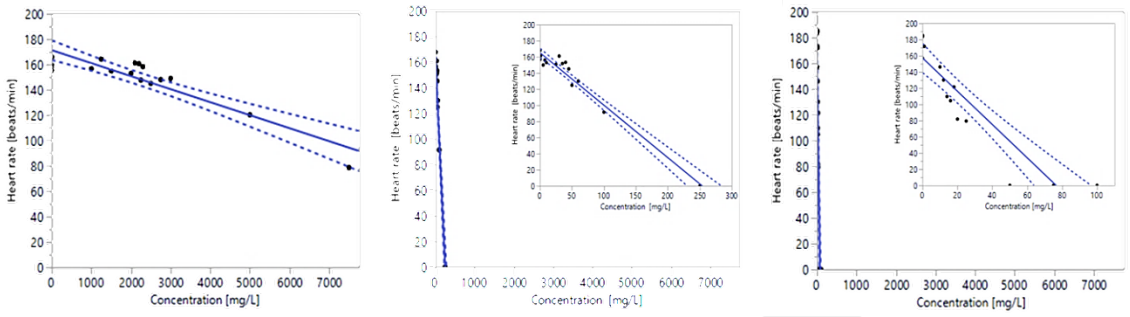
Figure 5.7a: Regression curves of mortality upon exposure of embryos to tramadol



Regression curves of mortality upon exposure of embryos to tramadol showing LC_{50} values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

Figure 5.7b: Linear regression of heart rate as a function of tramadol concentration

a. b. c.



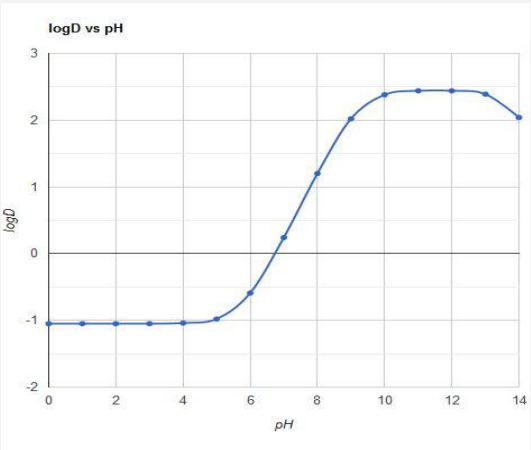
Linear regression of heart rate [beats/min] at time 48 hpf as a function of tramadol concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 6; b. pH 8; c. pH 9.

5.2.1.4 Antihistamines

a. Ketotifen

Ketotifen was tested at pH 9. The tests comprised three runs, whereby the first run was excluded due to too high control mortality. Instead, runs two and three were performed with six and seven concentrations, respectively. The log D curve for ketotifen as a function of pH and the concentrations applied as well as the LC_{50} values determined are noted in Table 5.8. The corresponding regressions are shown in Figure 5.8a. Since solubility problems already occurred at pH 9, no further investigations are planned for ketotifen at lower pH values.

Table 5.8: Overview of the applied ketotifen concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for ketotifen as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		
		72 hpf	96 hpf	
9	0.01	4.653	1.9206	
	0.05			
	0.10			
	0.50			
	1.00			
	2.00			
	2.50			
	3.00			
	4.00			
	4.50			
	5.00			
	10.00			

Source: Own depiction

The base ketotifen was tested exclusively at pH 9 and induced LC_{50} values of 4.65 mg/L (72 hpf) and 1.92 mg/L (96 hpf). Although ketotifen is expected to dissolve better at lower pH values, at the same time the log D suggests that toxicity would decrease significantly and thus the amount of substance used would have to be increased considerably. To make matters worse, ketotifen tended to dissolve promisingly at first, only to precipitate again after a few days, making the substance problematic for the test.

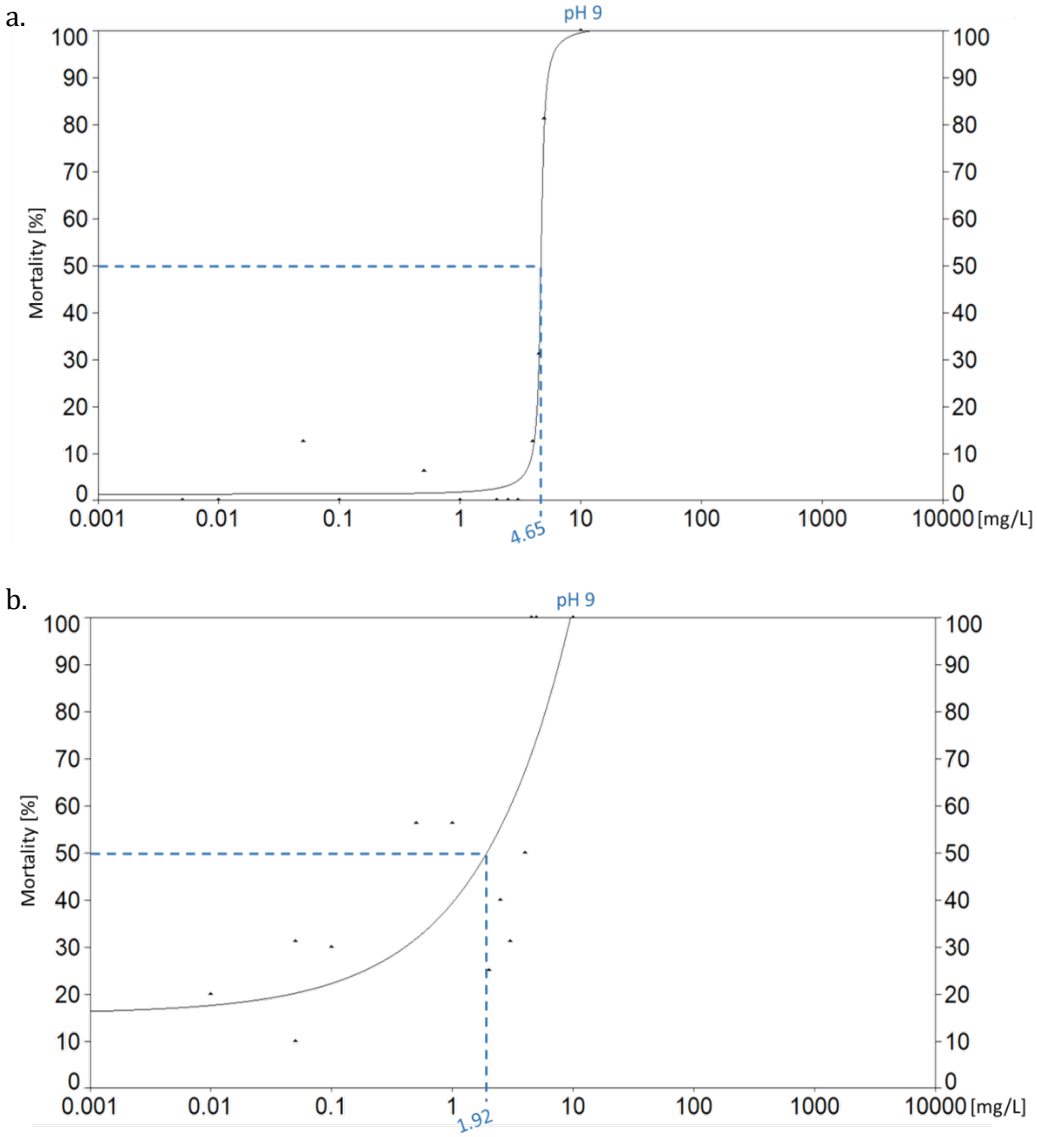
Apart from the highest concentrations (5 and 10 mg/L), lethal effects only occurred at 96 hpf.

In terms of heart rate, the lower concentrations remained at control levels (157 to 167 beats per minute) or were even above control, as in the case of 0.05 and 0.1 mg/L with 169 and 168 beats per minute compared to 157 beats per minute in the control (Figure 5.8b). Subsequently, while the heart rate drops moderately to 135 beats per minute up to 3 mg/L, it already drops to 110 beats per minute at 4 mg/L and then drops drastically to 53 at 4.5 mg/L and to just 36 beats per minute at 5 mg/L.

As in most pH 9 trials, the hatching rate is already significantly reduced in the control with an average of 37.5 %. The highest hatching rate of 50 % was observed at 0.05 mg/L ketotifen. In the remaining concentrations ≤ 2 mg/L, hatching success was between 10 and 31 %. At concentrations > 2 mg/L, hatching failed completely.

Due to only one pH value tested, no statement can be made for ketotifen about the agreement of the observed toxicity shifts with those expectations derived from the log D curve.

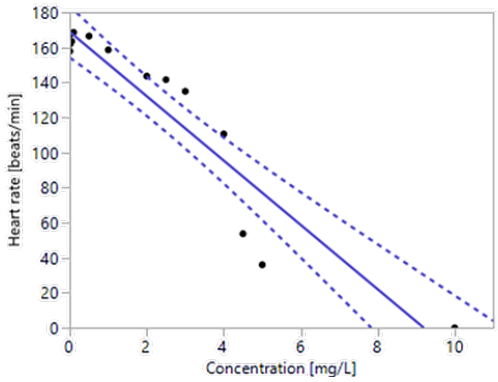
Figure 5.8a: Regression curves of mortality upon exposure of embryos to ketotifen



Regression curves of mortality upon exposure of embryos to ketotifen showing LC_{50} values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

Figure 5.8b: Linear regression of heart rate as a function of ketotifen concentration

a.



Linear regression of heart rate [beats/min] at time 48 hpf as a function of ketotifen concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 9.

5.2.1.5 Beta blockers

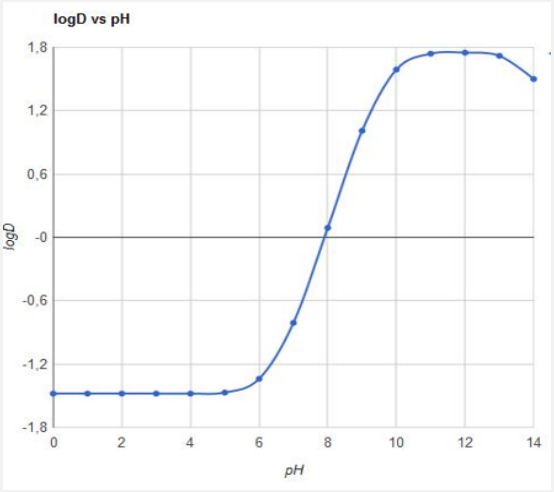
a. Metoprolol

Based on the log D curve, metoprolol was tested as a base at pH 5, 6, 8 and 9. The tests included two range-findings as well as the main test. The log D curve for metoprolol as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.9. The corresponding regressions are shown in Figure 5.9a.

Table 5.9: Overview of the applied metoprolol concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for metoprolol as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	400.0	-	(6739.0)	6	320.0	(7008.3)	3183.4
	1000.0				800.0		
	2500.0				2000.0		
	6250.0				3000.0		
	6400.0				3200.0		
	6600.0				3400.0		
	6800.0				3500.0		
	7000.0				3750.0		
					4000.0		
					4150.0		
					4300.0		
					4500.0		
					5000.0		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
8	1.00	-	77.34	9	0.10	48.39	11.32
	10.00				0.50		
	40.00				1.00		
	50,00				2.50		
	75.00				3.50		
	80.00				4.00		
	85.00				4.50		
	90.00				5.00		
	95.00				10.00		
	100.0				12.50		
	105.0				15.00		
	125.0				25.00		
	150.0				50.00		



Source: Own depiction

Since metoprolol in increasingly ionised form turned out to be relatively atoxic for *Danio rerio* embryos, very high concentrations had to be used for pH 5 and 6. Concentrations up to 7000 mg/L were used at pH 5. Concentrations above this were no longer soluble. Already at 6800 and 7000 mg/L, the metoprolol crystallised out again during the test week, which was reflected in a further decrease in the mortality rate. Because of this, firstly only two range findings were carried out and secondly the two highest concentrations were excluded from the analyses. Since the mortality rate even at the highest dissolving concentration of 6600 mg/L only increased to 31.25 %, the LC_{50} value of 6739 mg/L could not be determined directly, but had to be extrapolated. Furthermore, metoprolol induced mortalities that occurred almost exclusively in the last 24 hours of the test, which is why no LC_{50} values for 72 hpf could be determined directly, except for pH 9. At pH 6, the highest mortality rate at the 72 hpf time point was 25 % for a concentration of 4500 mg/L. In this case, an LC_{50} of 7008.3 mg/L was extrapolated. For pH 9, an

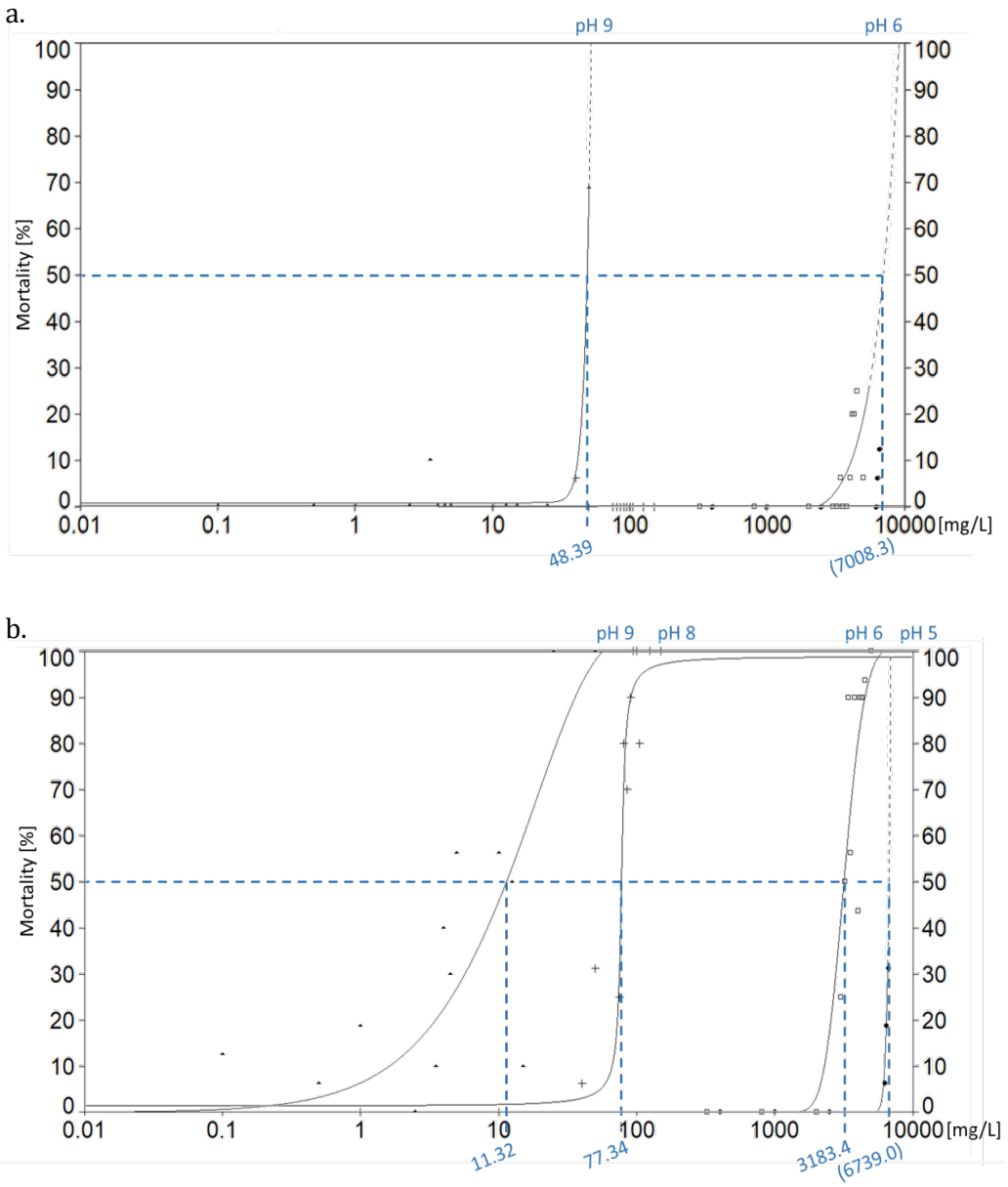
LC_{50} for 72 hpf of 48.39 mg/L could be determined. The LC_{50} values at 96 hpf were 3183.4 mg/L for pH 6, 77.34 mg/L for pH 8 and 11.32 mg/L for pH 9.

At all four pH values, metoprolol decreased the heart rate only to a relatively limited extent, even at high concentrations (Figure 5.9a). Although the heart rate fell rapidly from ranges around 160 beats per minute to 120 to 130 beats compared to controls even at lower concentrations, it then fell only slowly with increasing concentrations. Only in the highest concentrations of pH 8 (150 mg/L) and pH 9 (50 mg/L) was a heart rate < 100 measured, with 99 and 51 beats per minute, respectively.

With regard to the hatching rate, a clear decrease was already evident in the range of slight mortality increases and was therefore clearly reduced. While in the low pH 5 concentrations up to 2500 mg/L the larvae even hatched significantly faster than the control individuals, the hatching rate from 6250 mg/L onwards was clearly reduced to below 20 %. This effect could not be observed at pH 6 and 8. However, it was also evident here that the larvae either hatched early at time 60 hpf or not at all. At pH 9, hatching in the low concentrations ≤ 2.5 mg/L even began to a small extent at 48 hpf and then slowly increased further up to 96 hpf. However, the final hatching success ranged from 38 to 73 % and was thus mostly below the control. At concentrations ≥ 3.5 mg/L, hatching then almost completely failed to occur.

Metoprolol showed pH-dependent toxicity, with particularly large differences between pH 6 and 8. However, both the toxicity differences between pH 5 and 6 and between pH 6 and 8 as well as pH 8 and 9 were quite close to those predicted by log D.

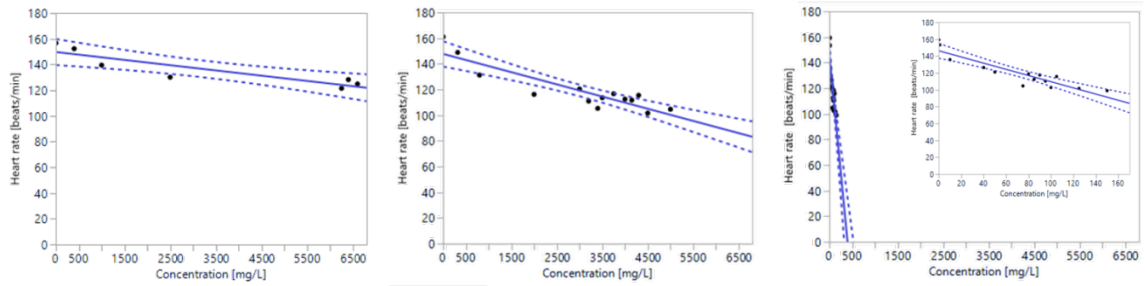
Figure 5.9a: Regression curves of mortality upon exposure of embryos to metoprolol



Regression curves of mortality upon exposure of embryos to metoprolol showing LC_{50} values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

Figure 5.9b: Linear regression of heart rate as a function metoprolol concentration

a. b. c.



Linear regression of heart rate [beats/min] at time 48 hpf as a function of metoprolol concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8.
Source: Own depiction

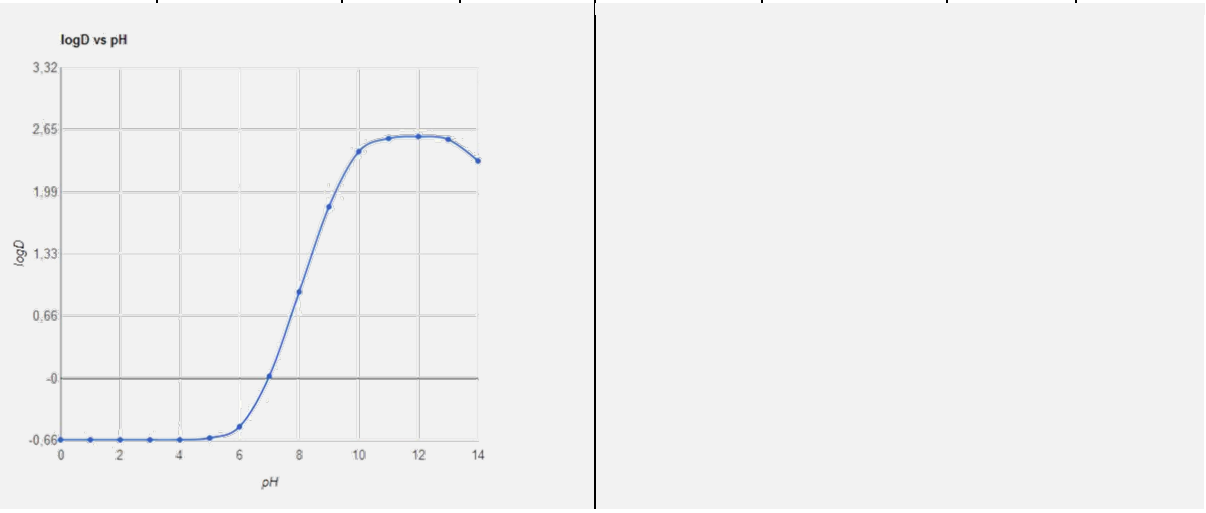
b. Propranolol

The base propranolol was tested at pH 5, 6, 8 and 9. The tests included two range-findings as well as the main test. The log D curve for propranolol as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.10. The corresponding regressions are shown in Figure 5.10a.

Table 5.10: Overview of the applied propranolol concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for propranolol as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	100.0	1619.5	1599.4	6	8.80	514.8	436.5
	250.0				88.0		
	500.0				220.0		
	750.0				440.0		
	1000.0				462.0		
	1200.0				475.2		
	1400.0				488.4		
	1600.0				501.6		
	1650.0				514.8		
	1700.0				528.0		
	1750.0				704.0		
	1800.0				880.0		
	2000.0				1560.0		
8	4.40	14.67	9.06	9	0.009	4.13	0.671
	6.60				0.044		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	8.80				0.088		
	13.20				0.880		
	17.66				1.760		
	22.00				3.520		
	26.40				4.400		
	35.20				5.280		
	52.80				7.040		
	70.40				8.800		
	88.00				88.00		
	220.0				220.0		
	440.0				440.0		



Source: Own depiction

The base propranolol shows a very marked difference in toxicity between pH values. Although the LC_{50} value at pH 5 of 1599 mg/L at 96 hpf was only about three times as high as at pH 6 with 437 mg/L, the toxicity difference between pH 6 and pH 8 with an LC_{50} of 9.06 mg/L was already 40-fold. The LC_{50} of 0.671 mg/L at pH 9 was still 16 times lower than at pH 8. If one looked at the mortalities more closely, it was noticeable that, similar to citalopram, the course of mortality at pH 5 and 6 (96 hpf) in particular was extremely erratic: At pH 5, up to 1400 mg/L only single individuals died, at the next higher concentration already almost 40 % of the embryos died and from 1650 mg/L the mortality was 100 %. At pH 6, mortality remained constant at 6.25 % from 8.80 to 440 mg/L propranolol, until it rose directly to 100 % at 462 mg/L. Similar tendencies were also observed in the case of the pH 5. Similar trends were also seen at pH 8, albeit to a somewhat lesser extent. Concentrations between 4.40 and 8.80 mg/L induced mortalities of 20 - 31 %, while at the next higher concentrations from 13.20 mg/L there was already 100 % mortality. At pH 9, on the other hand, mortality increased gradually from 25 - 80 % between 0.044 mg/L and 1.76 mg/L, before reaching 100 % from 3.52 mg/L onwards.

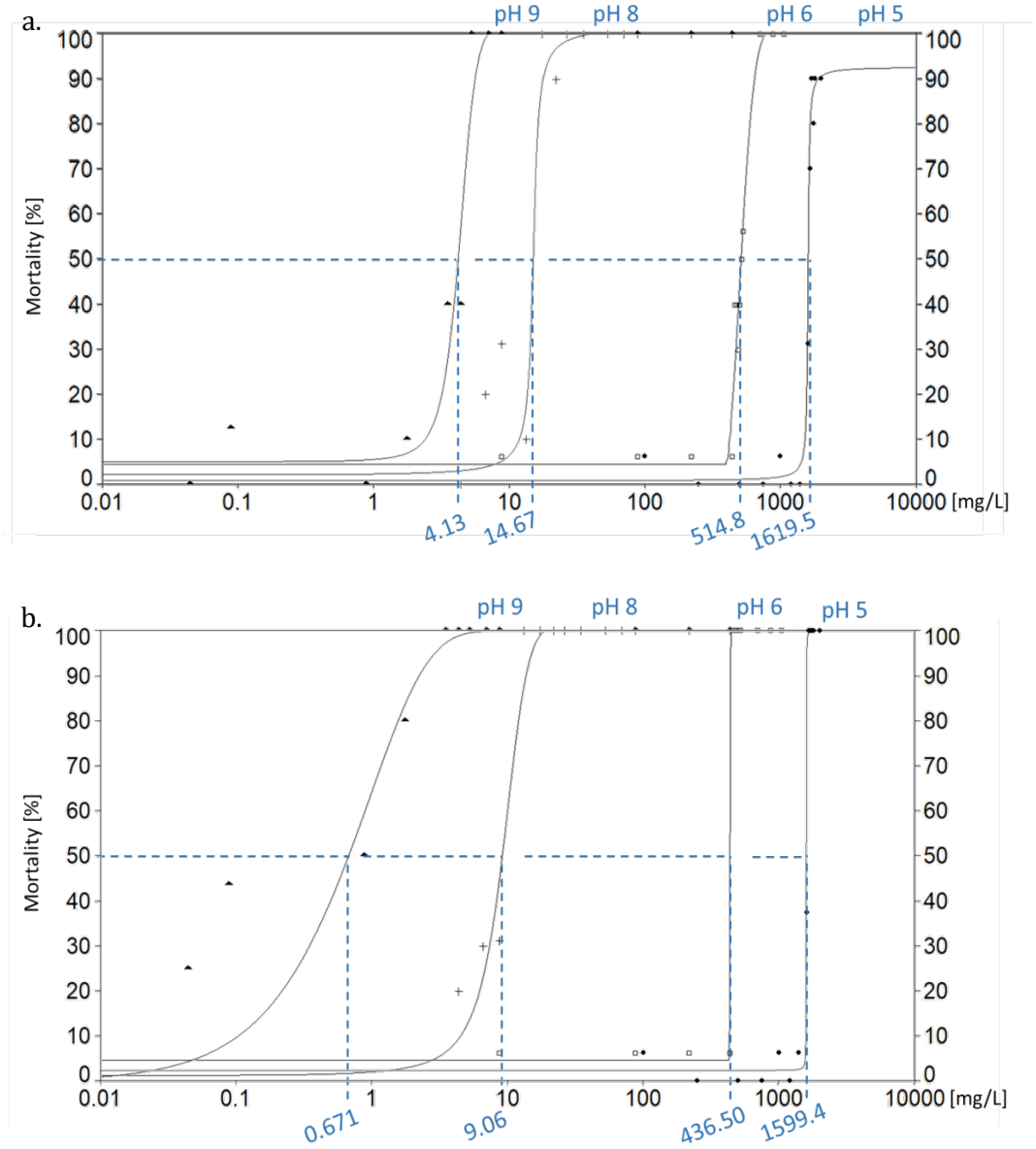
A similar picture was seen for the heart rates at time 48 hpf (Figure 5.10b). At pHs 5, 6 and 8, heartbeats per minute decreased steadily with increasing propranolol concentrations,

fluctuating at a low level of 50 to 70 beats per minute at pH 6 from 462 mg/L and not decreasing further. At pH 9, the picture was different. In embryos exposed to propranolol concentrations between 0.0088 and 1.760 mg/L, the heartbeat fluctuated continuously between 115 and 134 beats per minute before collapsing to 47 beats at 3.520 mg/L.

Finally, if we look at the hatching rate, the results from pH 5, 6 and 8 are again similar. At the 96 hpf time point, at the concentrations where the embryos reached the hatching stage, approximately 10 - 100 % hatched at pH 5 and 8 and 12 - 81 % hatched at pH 6. The hatching rate at pH 9 was limited to only a few individuals in both the test concentrations and the control.

Overall, propranolol showed a clear pH-dependent toxicity. The differences between the individual pH values were even more pronounced than with citalopram. Furthermore, similar trends supporting the mortality results could also be seen in the sublethal endpoints, especially the heart rate.

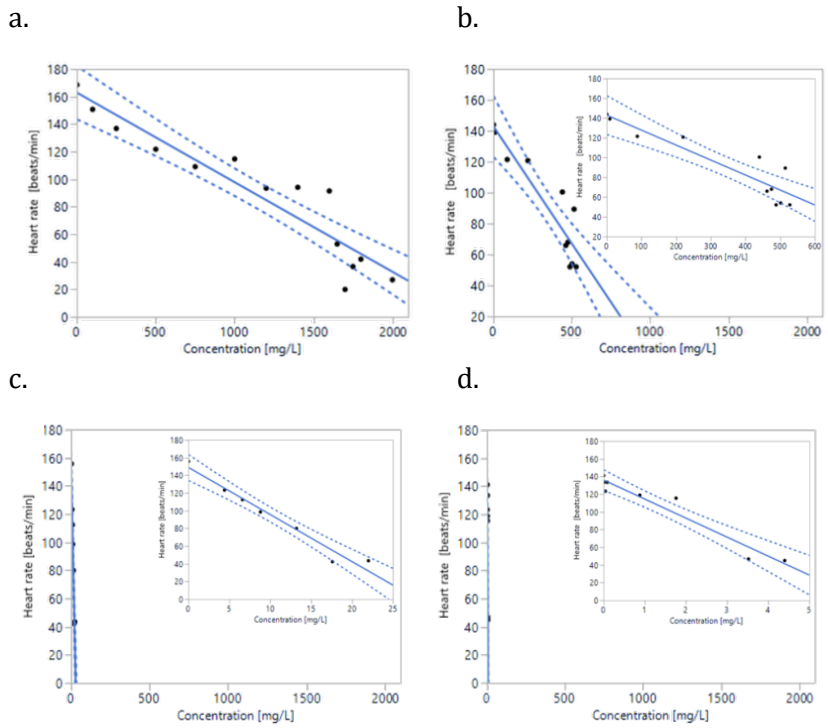
Figure 5.10a: Regression curves of mortality upon exposure of embryos to propranolol



Regression curves of mortality upon exposure of embryos to propranolol showing LC_{50} values; a. after 72 hpf; b. after 96 hpf

Source: Own depiction

Figure 5.10b: Linear regression of heart rate as a function propranolol concentration



Linear regression of heart rate [beats/min] at time 48 hpf as a function of propranolol concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

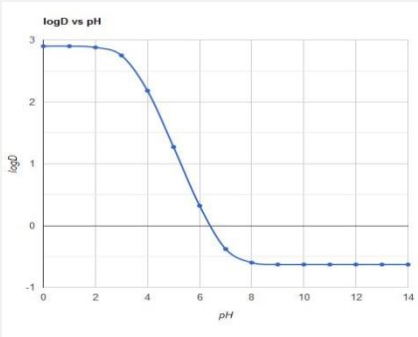
5.2.1.6 Cholesterol-lowering agents

a. Clofibric acid

Clofibric acid was tested at pH 5, 6 and 9. The tests were stopped after two range-findings due to low mortality rates and at the same time low solubility of the substance. The log D curve for clofibric acid as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.11. The corresponding log D curves and mortality rates are shown in Figure 5.11a and Figure 5.11b.

Table 5.11: Overview of the prepared clofibric acid concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for clofibric acid as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.01	67.778	66.832	8	0.10	-	-
	0.10				1.00		
	1.00				10.00		
	10.00				50.00		
	25.00				100.0		
	50.00				150.0		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	75.00				200.0		
	100.0				250.0		
9	0.10	-	(469.01)				
	1.00						
	10.00						
	50.00						
	100.0						
	150.0						
	200.0						
	250.0						

Source: Own depiction

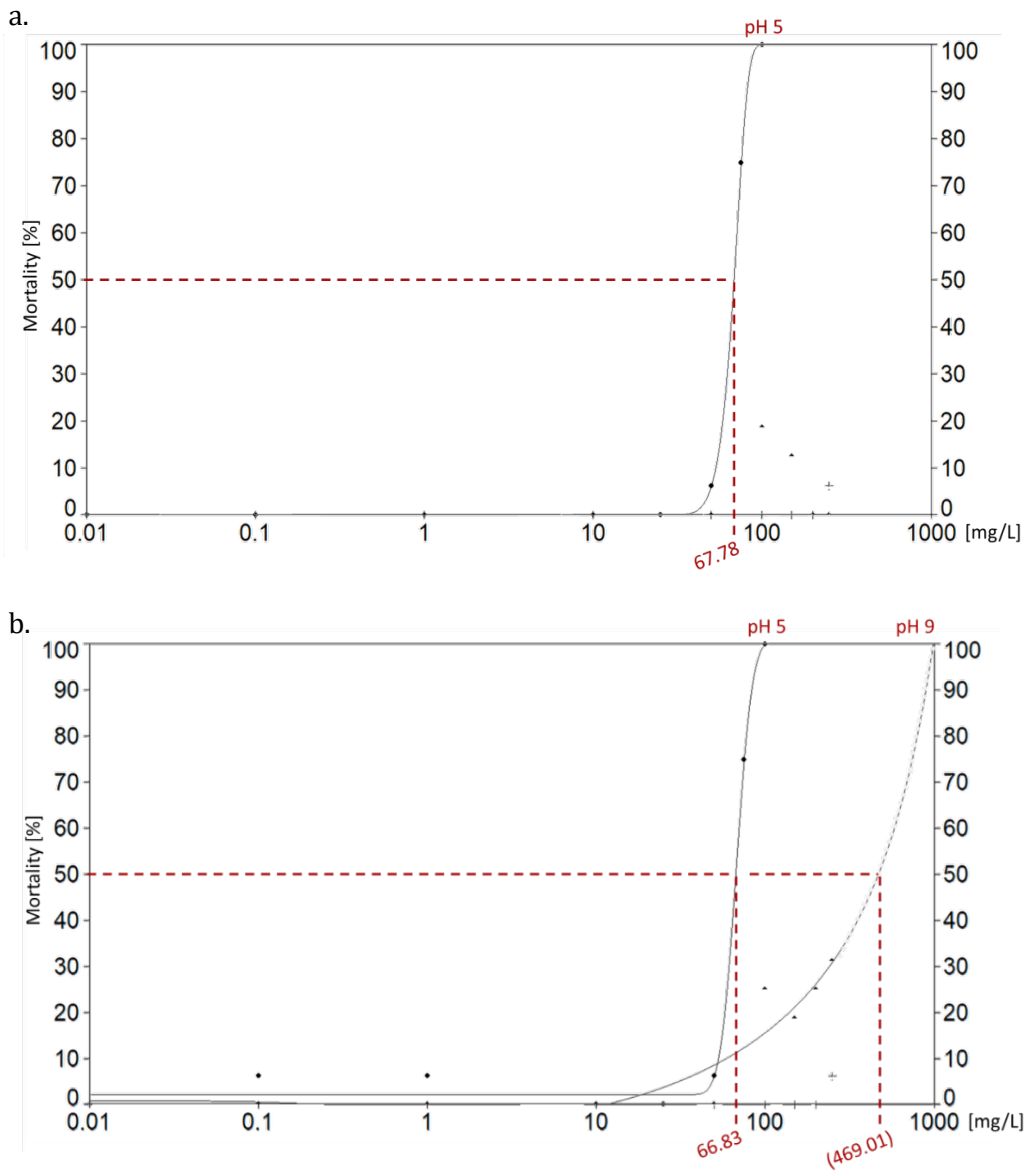
Due to low mortalities at pH 8 and 9 with simultaneous solubility problems, only two runs were carried out with clofibric acid, which allowed the determination of an LC_{50} only at pH 5, or extrapolated also at pH 9 (96 hpf). The LC_{50} values at pH 5 differed only very slightly between 72 hpf with 67.78 mg/L and 96 hpf with 66.83 mg/L, whereby the concentration resolution was rather coarse-meshed due to the two runs carried out. While only a few individuals died up to and including 50 mg/L, the mortality rate at the next higher concentration was already 75 %. At pH 9, an LC_{50} of 469 mg/L could be extrapolated for 96 hpf. The first lethal effects occurred from 100 mg/L and increased to a maximum of 31 % up to 250 mg/L. At pH 8, only one individual died at the highest concentration of 250 mg/L tested.

An effect on the heart rate could only be observed at pH 5 (Figure 5.11c). Up to and including 25 mg/L, the heart rate of the embryos was in the extended control range (between 150 and 175 beats per minute), whereas it then dropped to just under 100 beats per minute at 50 mg/L and to just 56 beats per minute at 75 mg/L. At 100 mg/L, no heartbeat could be detected even in still-living individuals at time 48 hpf. Heart rates at pH 8 and 9 were consistently between 170 and 190 beats per minute and showed no differences between controls and clofibric acid concentrations.

In terms of hatching rate, variability was a little higher. Hatching success at pH 5 was over 90 % up to and including 10 mg/L, but then almost completely failed at concentrations ≥ 25 mg/L. At pH 8, up to and including 100 mg/L, all individuals hatched without exception. Only from a clofibric acid concentration of 150 mg/L did the hatching rate drop minimally to 93.75 % and then only decreased to 81.25 % up to 250 mg/L. At pH 9, hatching success was generally unusually high and varied between 62.5 and 100 %. It was noticeable at all three pH values that the hatching rate at 60 hpf was already relatively high, mostly between 40 and 75 %. At pH 8 and 9, single individuals hatched already at 48 hpf. Clofibric acid therefore seems to tend to promote hatching. Furthermore, it was shown that in the case of clofibric acid, the endpoint hatching rate reacted more sensitively than the endpoint heartbeat. While at pH 5 the hatching was almost absent already at 25 mg/L, a clear reduction of the heartbeat could only be observed at 50 mg/L. The endpoint of the heartbeat was also reduced at pH 8 and 9. A decrease in hatching success

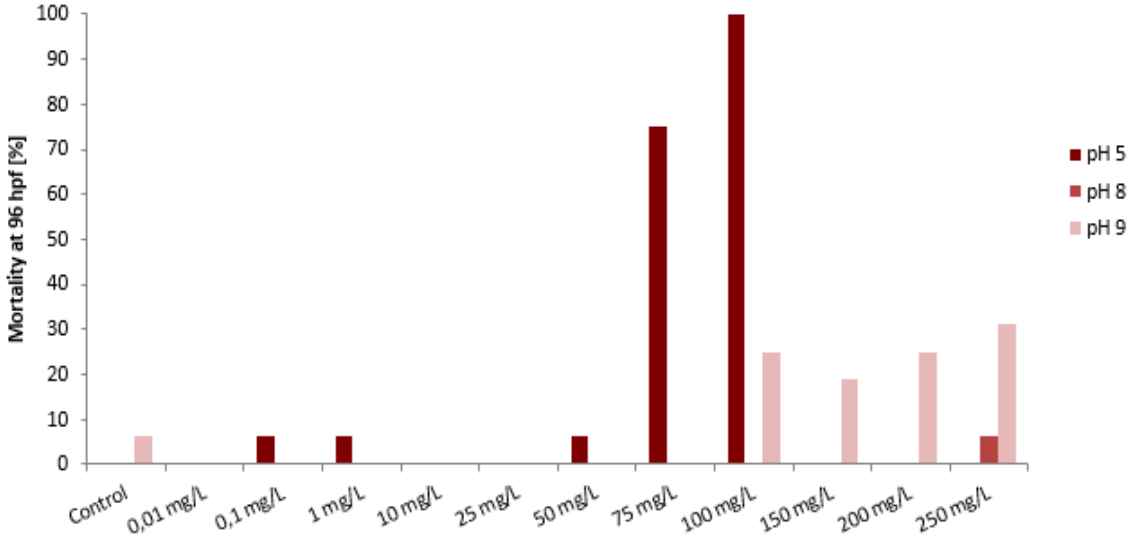
could also be seen at pH 8 and 9, albeit to a rather small extent, while the heart rate remained constant at both pH values.
For clofibric acid, a pH-dependent toxicity can be identified, which, according to an acid, decreases with increasing pH. Due to the reduced test range and low mortalities combined with solubility problems at pH 8 and 9, no LC50-based comparison can be made between the observed toxicity shifts and the expectations derived from the log D curve and the interpretation of the data can be done with appropriate caution.

Figure 5.11a: Regression curves of mortality upon exposure of embryos to clofibric acid



Regression curves of mortality upon exposure of embryos to clofibric acid, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.
Source: Own depiction

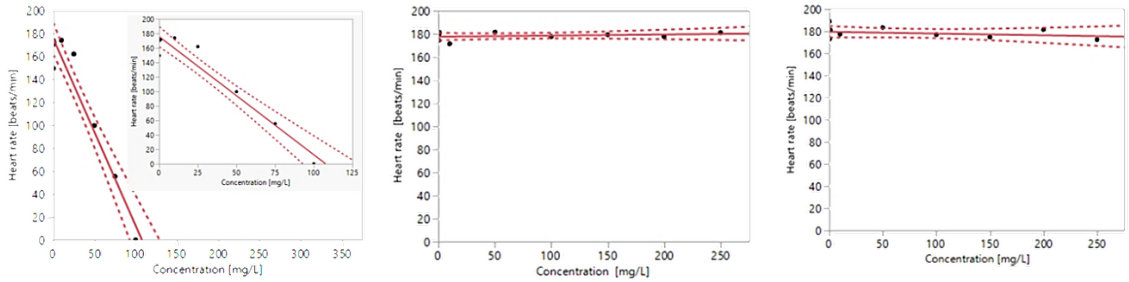
Figure 5.11b: Mortality of embryos depending on clofibric acid concentration



Mortality of embryos at time 96 hpf in percent depending on clofibric acid concentration and pH.
Source: Own depiction

Figure 5.11c: Linear regression of heart rate as a function of clofibric acid concentration

a. b. c.



Linear regression of heart rate [beats/min] at time 48 hpf as a function of clofibric acid concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

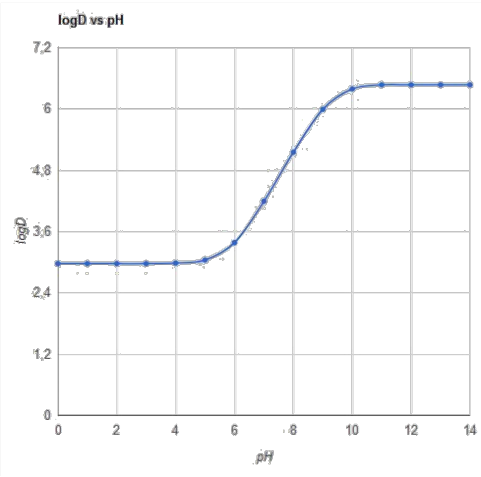
5.2.1.7 Estrogen receptor modulators

a. Enclomiphene

The base enclomiphene was tested at pH 8 and 9. Due to the small amount of substance and the assumption that toxicity decreases with decreasing pH and therefore the amount of enclomiphene used would have to be increased accordingly, the investigation of pH 6 was dispensed with. The tests included two range-findings as well as a reduced-scope main experiment. The log D curve for enclomiphene as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.12. The corresponding regressions are shown in Figure 5.12a.

Table 5.12: Overview of the prepared enclomiphene concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for enclomiphene as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
8	0.005	5.579	3.127	9	0.001	-	-
	0.020				0.005		
	0.100				0.020		
	0.500				0.100		
	1.000				0.250		
	2.000				0.500		
	3.000				1.000		
	5.000				1.500		
	10.00				2.000		
					2.500		
					5.000		



Source: Own depiction

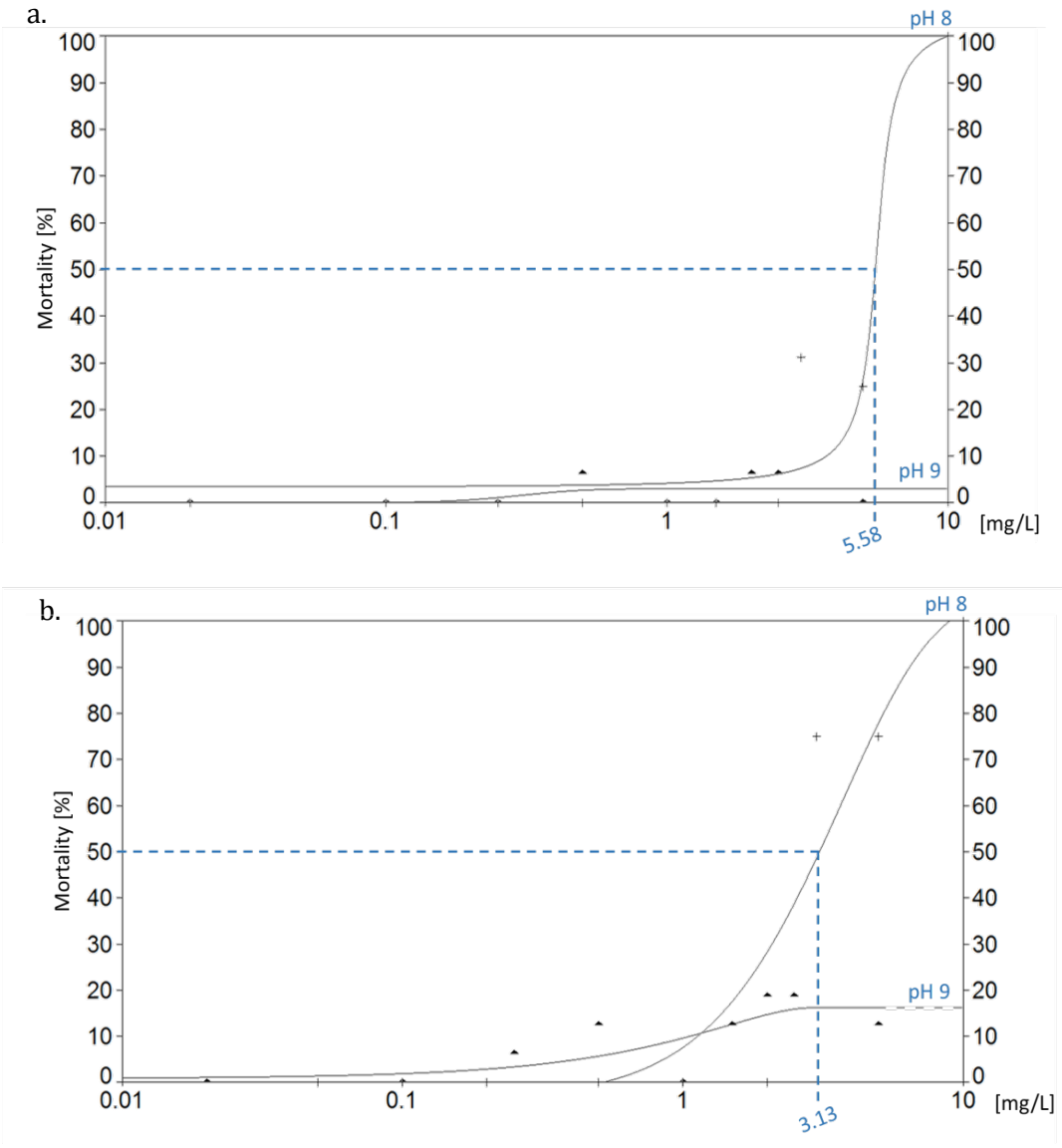
The mortality of the embryos exposed to enclomiphene at 96 hpf was at a consistently low level between 0 and 25 % at pH 9, independent of concentration (Figure 5.12b). Therefore, no non-linear regression could be performed for pH 9 to determine the LC_{50} value. Although it would have been expected that a higher mortality would be induced at pH 8, since more neutral species are present at this pH than is the case at pH 8 and thus the toxicity potential increases, this was not the case. Although no mortality occurred at all at the lower concentrations up to 2.5 mg/L, it jumped to 75 % at 3 mg/L and then reached 100 % at 10 mg/L. The LC_{50} for 96 hpf was thus higher than the LC_{50} for the same concentration. The LC_{50} for 96 hpf was thus 3.127 mg/L enclomiphene.

With regard to the heart rate, a similar picture emerged (Figure 5.12c). While the heart rate of the embryos at pH 9 was constant between 131 and 158 beats per minute independent of the concentration and thus on average slightly below the control level of 155 beats per minute, a

concentration-dependent slowing of the heart rate from 151 beats per minute at 0.005 mg/L to 16 beats per minute at 10 mg/L enclomiphene could be measured at pH 8.
The hatching rate at pH 8 was consistently high (94 - 100 %) up to 2 mg/L. The hatching success then dropped significantly to just under 50 % at 3 mg/L and further reduced to 0 % by 10 mg/L. At pH 9, hatching was somewhat higher in the lower enclomiphene concentrations up to 0.1 mg/L than in the medium concentrations and then failed completely in the high concentrations from 2.5 mg/L onwards. However, similar to citalopram and propranolol, the control also showed a low hatching rate of 21 % on average. It therefore seems likely that pH 9 may have a fundamentally unfavourable effect on the normal hatching process, or prevent or slow down hatching. Therefore, the investigation of the hatching rate at pH 9 does not seem to be very useful in general.

Overall, the toxicity of enclomiphene as a function of pH did not behave as previously assumed. Although more neutral species should be present at pH 9 than at pH 8 and thus enclomiphene should be more toxic to embryos at pH 9, the opposite effect was observed, which was reflected not only in mortality but also in results of a sublethal endpoint (heart rate).
The suitability of enclomiphene for inclusion in the final overall project model is therefore limited.

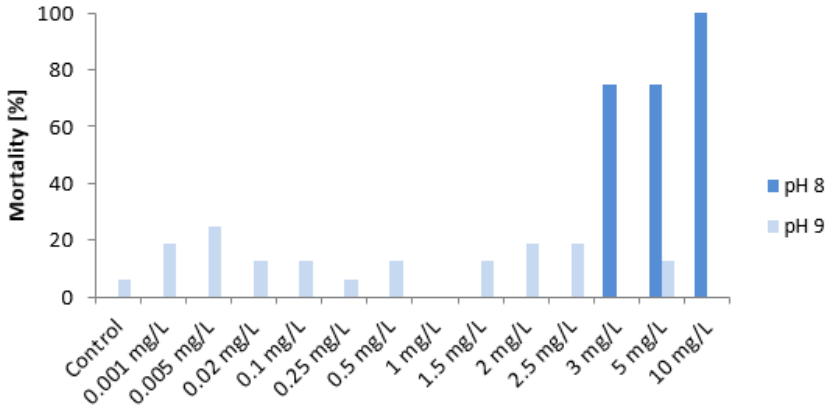
Figure 5.12a: Regression curves of mortality upon exposure of embryos to enclomiphene



Regression curves of mortality upon exposure of embryos to enclomiphene, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.

Source: Own depiction

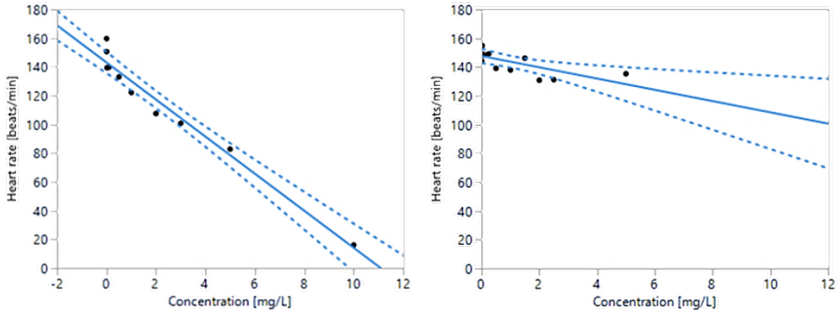
Figure 5.12b: Mortality of embryos depending on enclomiphene concentration



Mortality of embryos at time 96 hpf in percent depending on enclomiphene concentration and pH.
Source: Own depiction

Figure 5.12c: Linear regression of heart rate as a function of enclomiphene concentration

a. b.



Linear regression of heart rate [beats/min] at time 48 hpf as a function of clofibric acid concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 8; b. pH 9.
Source: Own depiction

5.2.1.8 Anti-depressants

a. Amitriptyline

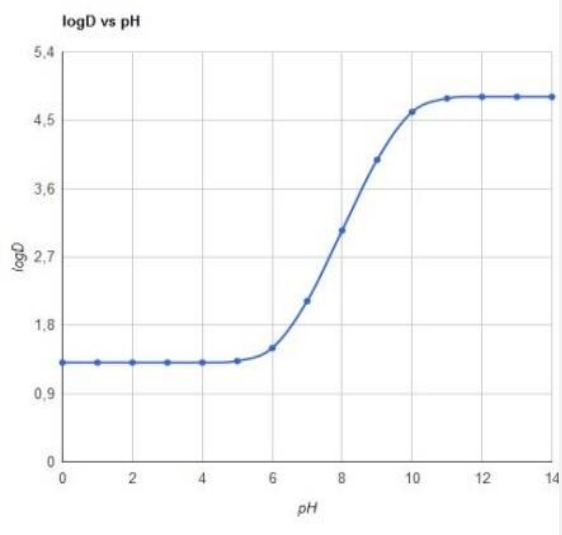
Amitriptyline was tested as a base at pH 5, 6, 8 and 9. The tests included two range-findings as well as the main test. The log D curve for amitriptyline as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.13a. The corresponding regressions are shown in Figure 5.13a.

Table 5.13a: Overview of the prepared amitriptyline concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for amitriptyline as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	10.00	303.08	245.03	6	0.01	95.178	89.159
	50.00				0.10		
	100.0				1.00		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	150.0				10.0		
	175.0				50.0		
	185.0				60.0		
	195.0				70.0		
	200.0				75.0		
	225.0				80.0		
	250.0				90.0		
	275.0				100.0		
	300.0				250.0		
	500.0				500.0		
8	0.01	3.1139	2.0967	9	0.01	1.6366	1.5996
	0.10				0.10		
	1.00				0.50		
	1.50				0.75		
	2.00				1.00		
	2.25				1.50		
	2.50				1.65		
	2.75				1.85		
	3.00				2.00		
	4.00				2.25		
	6.00				2.50		
	8.00				5.00		
	10.00				10.00		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf

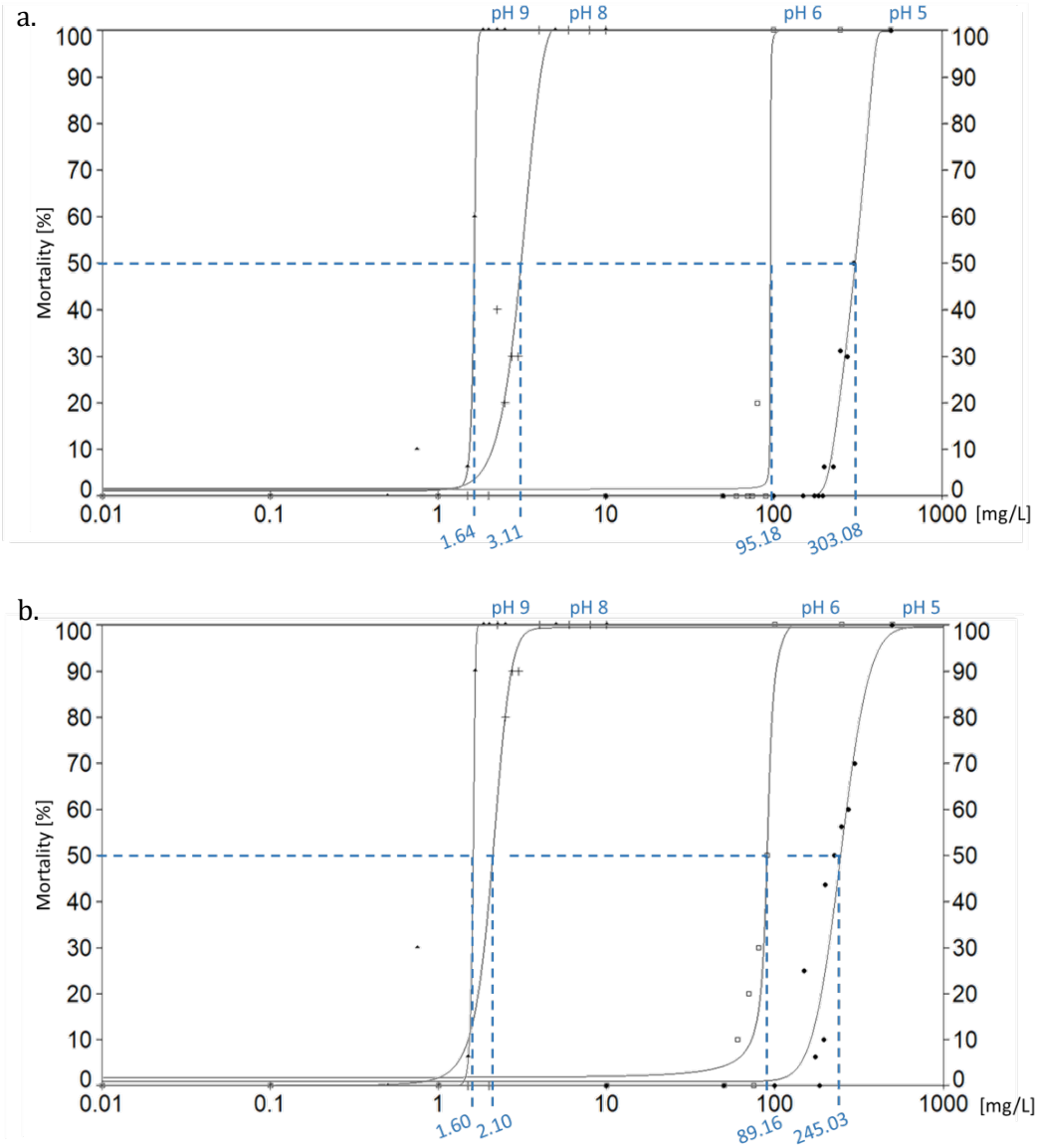


Source: Own depiction

For amitriptyline, there was a clear increase in mortality with increasing pH. While the LC_{50} value for pH 5 at time 96 hpf was still 245.03 mg/L, it decreased significantly for pH 6 to 89.16 mg/L, for pH 8 to 2.10 mg/L and for pH 9 to 1.60 mg/L. Furthermore, with the exception of pH 5, only slight differences in the LC_{50} values at the 72 and 96 hpf time points were observed. It was noticeable that for pH 5 and pH 6 a rather slow increase in mortality was recorded, whereas for pH 8 and 9 mortality increased abruptly.
Heartbeats were not counted for amitriptyline exposure, but for hatching rates it was shown that there tended to be threshold concentrations for pH 6 and 8 above which hatching no longer occurred, while at pH 9 hatching success successively decreased (Figure 5.13.b). For pH 6, this threshold concentration was ≥ 70 mg/L, for pH 8 ≥ 2.0 mg/L. At pH 9, hatching success was already somewhat lower in the control (75 % on average) than in the pH 6 and 8 controls (97-100 %), then decreased to 6.25 % up to 1.5 mg/L and stopped completely from 1.65 mg/L onwards. Although no threshold concentration could be defined at pH 5, the hatching success decreased very rapidly between 225 and 300 mg/L. While at 225 mg/L still 87.5 % of the individuals hatched, which was only slightly below the control level of ≥ 90 %, the hatching rate already dropped to 37.5 % at 250 mg/L and dropped 10 % for 275 mg/L. In contrast, from concentrations of 300 mg/L onwards, hatching was completely absent.
The principle of threshold concentrations was also shown for amitriptyline for all pH values for other abnormalities. Thus, spontaneous movements were absent at the 24 hpf time point and extremely increased and pronounced oedema formation was observed. In addition, amitriptyline led to malformations of the spine. On the one hand, kinks appeared in the tail tip, on the other hand, the tails were too short or completely crippled. In addition, necrosis could be observed in the area of the tail tip. The corresponding threshold concentrations are listed in Table 5.13b.

Overall, amitriptyline showed a clear pH-dependence of toxicity, whereby the difference between pH 8 and 9 was significantly smaller than could be expected from the log D curve. In addition, amitriptyline induced striking effects at the sublethal level. Oedema and, in particular, malformations of the spinal column were observed.

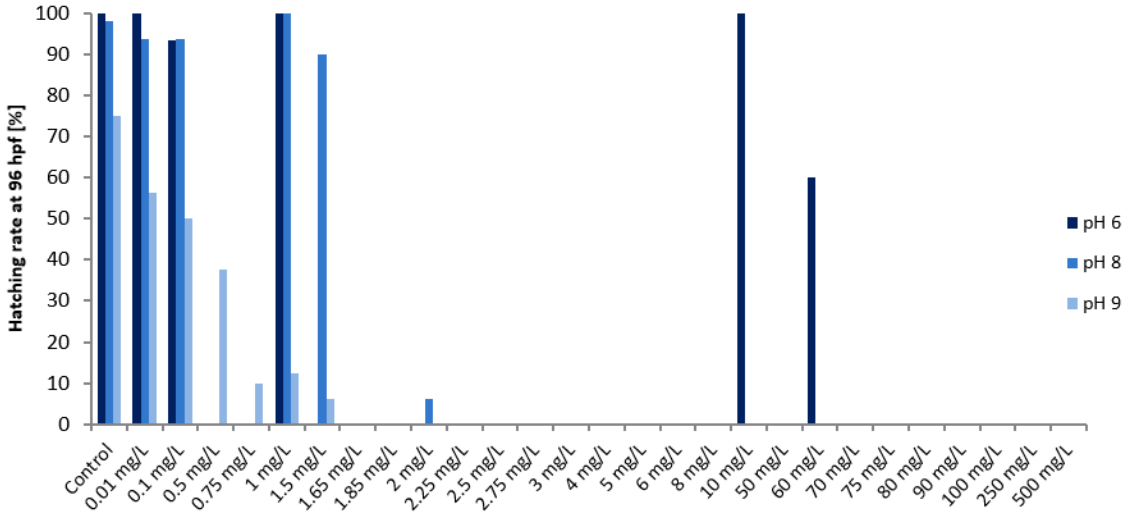
Figure 5.13a: Regression curves of mortality upon exposure of embryos to amitriptyline



Regression curves of mortality upon exposure of embryos to amitriptyline, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.

Source: Own depiction

Figure 5.13b: Mortality of embryos depending on amitriptyline concentration



Mortality of embryos at time 96 hpf in percent depending on amitriptyline concentration and pH.
Source: Own depiction

Table 5.13b: Threshold concentrations at which the listed effects occurred in the majority of individuals

	Movement (24 hpf)	Tail (96 hpf)	Oedema (96 hpf)	Hatching (96 hpf)
pH 5	150 mg/L	≥ 300 mg/L	≥ 300 mg/L	≥ 300 mg/L
pH 6	≥ 10 mg/L	≥ 50 mg/L	≥ 50 mg/L	≥ 70 mg/L
pH 8	≥ 0.1 mg/L	≥ 2 mg/L	≥ 2 mg/L	≥ 2 mg/L
pH 9	≥ 0.01 mg/L	≥ 1.5 mg/L	≥ 1 mg/L	≥ 0.75 mg/L

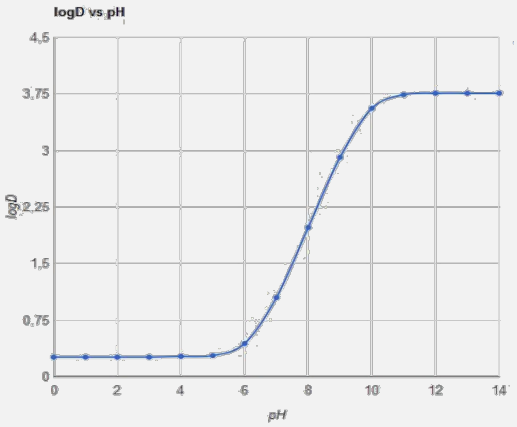
Threshold concentrations at which the listed effects (lack of spontaneous movement, spinal malformations in the tail region, oedema) occurred in the majority of individuals, or less than 10 % of the embryos hatched.
Source: Own depiction

b. Citalopram

Citalopram was tested as a base at pH 5, 6, 8 and 9. The tests included two range-findings as well as the main experiment. The pH 9 approach was repeated in its entirety. The concentrations and results discussed here refer exclusively to data that were collected again. The log D curve for citalopram as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.14. The corresponding regressions are shown in Figure 5.14a.

Table 5.14: Overview of the applied citalopram concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for citalopram as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	250.0	1231.1	1213.6	6	0.8	426.2	400.0
	500.0				8.0		
	750.0				40.0		
	1000.0				80.0		
	1100.0				120.0		
	1120.0				160.0		
	1140.0				200.0		
	1150.0				240.0		
	1160.0				320.0		
	1180.0				400.0		
	1200.0				480.0		
	1300.0				560.0		
	1500.0				720.0		
8	0.8	35.04	19.99	9	0.10	-	5.855
	8.0				0.50		
	12.0				1.00		
	20.0				2.50		
	22.0				4.00		
	24.0				5.00		
	28.0				5.50		
	32.0				6.00		
	36.0				6.50		
	40.0				7.00		
	48.0				7.50		
	64.0				10.00		
	80.0						

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
							

Source: Own depiction

In contrast to acids, for bases the neutral species are increasingly present at increasing pH values, i.e. the toxicity increases accordingly in the basic range. Therefore, the highest LC_{50} value of 1213 mg/L at 96 hpf was present for pH 5, followed by 400 mg/L for pH 6, 19.99 mg/L for pH 8 and 5.86 mg/L for pH 9. Thus, citalopram is about three and a half times more toxic at pH 9 than at pH 8, about twenty times more toxic at pH 8 than at pH 6 and about three times more toxic at pH 6 than at pH 5.

Looking at the mortality curve, at pH 5 and pH 6 a moderate increase in mortality from 0 % to 500 mg/L to 40 % at 1180 mg/L and to 100 % at 1500 mg/L at pH 5, and from 0 % at 320 mg/L to 50 % at 400 mg/L and to 100 % at 480 mg/L at pH 6 was observed. Furthermore, at the two low pH values, mortality already increased significantly in the first hours of the test and then generally remained relatively constant before increasing slightly again at 96 hpf. At pH 8, on the other hand, mortality was less than 20 % at concentrations ≤ 20 mg/L, but then jumped to 100 % at 22 mg/L when the concentration was increased by only 2 mg/L. A particular increase in mortality was seen at pH 8 in the last 48 hours of the test. In contrast to the previous pH values, no mortality was induced at pH 9 up to and including 72 hpf. Lethal effects occurred exclusively at the time 96 hpf, whereby these varied between 20 and 80 %, especially in the medium to high concentrations (4 to 7.5 mg/L).

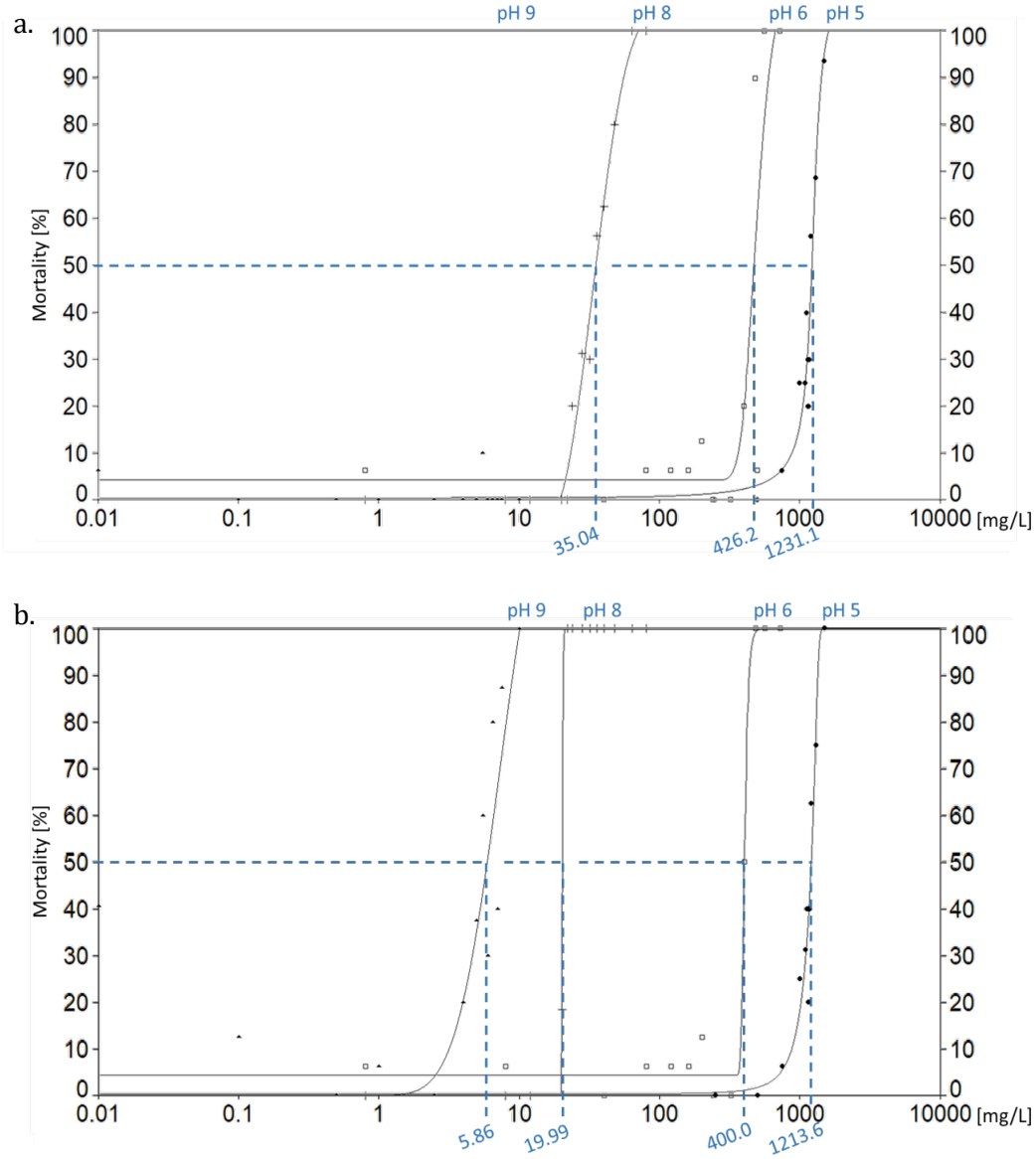
A comparable picture was shown for the heart rates (Figure 5.14b). While at pH 8 the heart rates decreased continuously with increasing citalopram concentrations to about 20 beats per minute at the highest concentration, the heart rate at pH 6 never dropped below 59 beats per minute. At pH 5, the heart rate varied relatively independently of concentration between 130 and 150 beats (control between 157 and 168 beats per minute) and never fell below 114 beats per minute. At pH 9, embryo heartbeats at concentrations ≤ 1 mg/L were in the control range between 156 and 184 beats per minute. In the medium to high concentrations between 4 and 7.5 mg/L, heart rates remained fairly constant at 69 to 74 beats per minute. Only at the highest concentration of 10 mg/L did they increase again significantly to 132 beats per minute.

The hatching success at pH 5 was only at the control level (> 90 %) in the low concentrations (≤ 750 mg). In the medium concentrations (1000 - 1180 mg/L), the hatching rate varied between about 40 and 80 %, before dropping to ≥ 25 % from 1200 mg/L. A similar pattern was seen at pH 6 and 8. Up to 400 mg/L (pH 6), and 40 mg/L (pH 8), the hatching rate at time 96 hpf

remained constant at over 80 %, before dropping significantly to 60 % (pH 6), and 44 % (pH 8), respectively, at the next higher concentrations (500 mg/L; 45 mg/L). In the exposures at pH 9, between 19 and 44 % of the embryos hatched in almost all concentrations, without a concentration gradient being discernible. However, it was striking that hardly any individuals hatched at 60 hpf compared to the other pH values. The hatching success in the control was also significantly reduced compared to pH 5 to 8 and was only between 50 and 68 %.

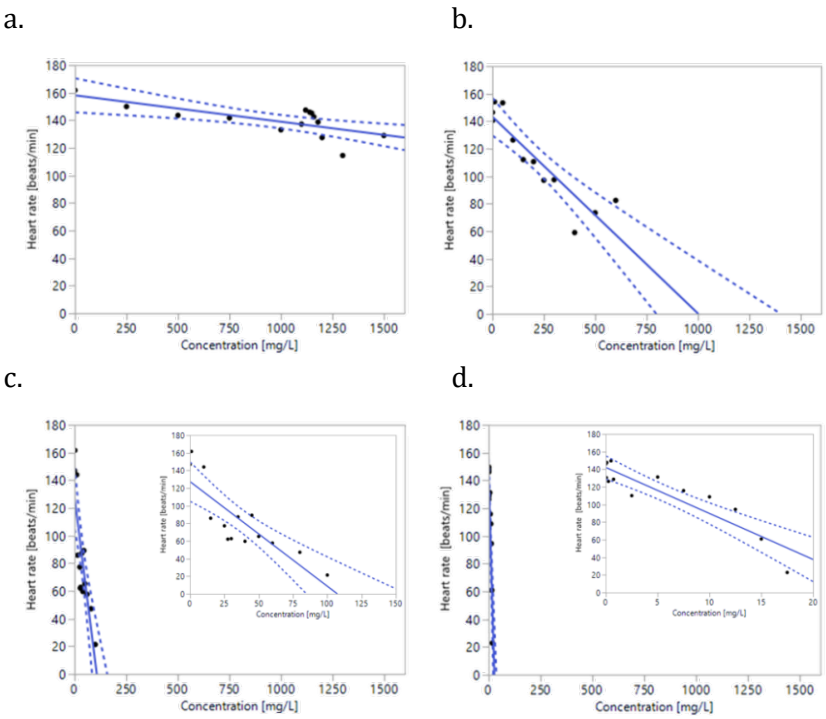
In summary, citalopram showed a clear increase in toxicity with a shift to higher pH values.

Figure 5.14a: Regression curves of mortality upon exposure of embryos to citalopram



Regression curves of mortality upon exposure of embryos to citalopram showing LC_{50} values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

Figure 5.14b: Linear regression of heart rate as a function citalopram concentration



Linear regression of heart rate [beats/min] at time 48 hpf as a function of citalopram concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

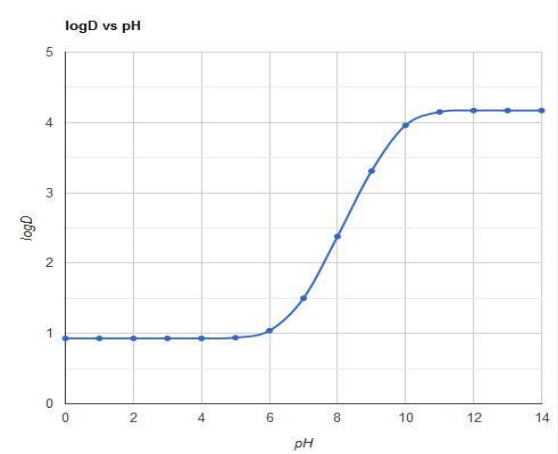
c. Fluoxetine

The base fluoxetine was tested at pH 5, 6, 8 and 9. The tests included two range-findings as well as the main test. The log D curve for fluoxetine as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.15. The corresponding regressions are shown in Figure 5.15a.

Table 5.15: Overview of the applied fluoxetine concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for fluoxetine as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.10	(470.91)	229.90	6	0.10	99.38	58.33
	1.00				1.00		
	10.00				10.00		
	50.00				50.00		
	100.0				55.00		
	150.0				58.00		
	200.0				61.00		
	220.0				65.00		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	240.0				68.00		
	250.0				71.00		
	260.0				75.00		
	280.0				95.00		
	300.0				100.0		
8	0.05	16.075	2.205	9	0.0001	1.927	0.333
	0.50				0.001		
	1.00				0.005		
	2.00				0.01		
	2.20				0.05		
	2.40				0.10		
	2.50				0.25		
	2.60				0.50		
	2.80				1.00		
	3.00				1.25		
	4.00				1.50		
	5.00				1.75		
	50.00				2.00		
					2.25		
					2.50		



Source: Own depiction

Fluoxetine showed a clear correlation between pH and toxicity. As expected for a base, the toxicity increased with increasing alkalinity. While for pH 5 the LC_{50} was still 470.9 mg/L (72 hpf), respectively 229.9 mg/L (96 hpf), at pH 6 it already decreased to 99.38 mg/L (72 hpf),

respectively 58.33 (96 hpf), at pH 8 to 16.08 mg/L (72 hpf), respectively 2.21 mg/L (96 hpf) and finally at pH 9 to 1.93 mg/L (72 hpf), respectively 0.33 mg/L (96 hpf). Similar to metoprolol, fluoxetine acted primarily in the last 24 hours of the test. Except at the very high concentrations, only single individuals died up to 72 hpf.

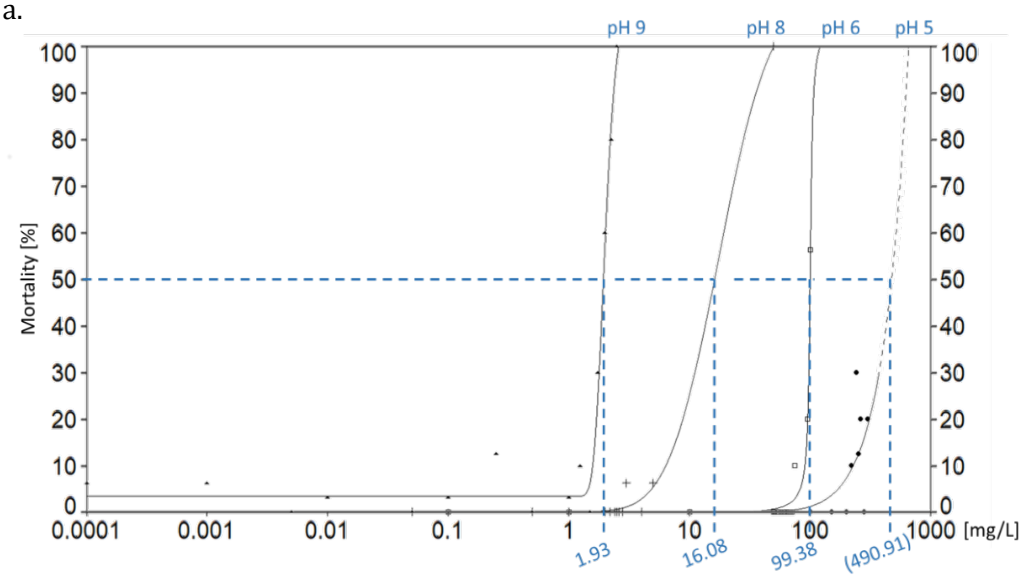
The later effect of fluoxetine was also reflected in the rather small effect on heart rate at time 48 hpf (Figure 5.15b). At pH 5, the heartbeats of the embryos from almost all concentrations were in the control range between 150 and 180 beats per minute. Only at the second highest concentration of 280 mg/L did the heart rate drop to 146 beats per minute. Even at the highest concentration of 300 mg/L, the heart rate was still well into the three-digit range at 136 beats per minute. Both concentrations triggered 100 % mortality after 96 hpf. Even at pH 6, only the embryos from the two highest concentrations (95 and 100 mg/L) had heart rates below 150 and 100 beats per minute, with 128 and 91 beats per minute, respectively. The fact that no heartbeat was measured at pH 8 in the highest concentration of 50 mg/L was due to complete mortality already after 12 hpf. Otherwise, all heart rates counted were above 150 beats per minute except for the second highest concentration of 5 mg/L with 137 beats per minute. Only at pH 9 was a decrease in heart rate observed in embryos from more than two concentrations. At exposures to 1.5 mg/L fluoxetine, the heart rate dropped to 130 beats per minute and reduced to 51 beats per minute by the highest concentration of 2.5 mg/L.

Hatching was also rather little affected by fluoxetine. In particular, in the pH 5 and pH 8 exposures, the hatching rate was above 80 % in almost all concentrations except the highest concentration, although even in the highest concentration, hatching success was still at least 50 %. Even at pH 9, 50 to 80 % of embryos hatched at concentrations ≤ 1 mg/L. Hatching also tended to start early at the three pH values mentioned, resulting in an already relatively high hatching rate at 60 hpf. Only at pH 6 was hatching success significantly reduced. However, even in the control only between 50 and 75 % of the embryos hatched. While in the range of 0.1 to 55 mg/L the hatching success was still between 31 and 93 %, in concentrations ≥ 58 mg/L only 0 to 30 % of the embryos hatched. In contrast to the other three pH values, hatching events at time 60 hpf were completely absent, apart from a single individual.

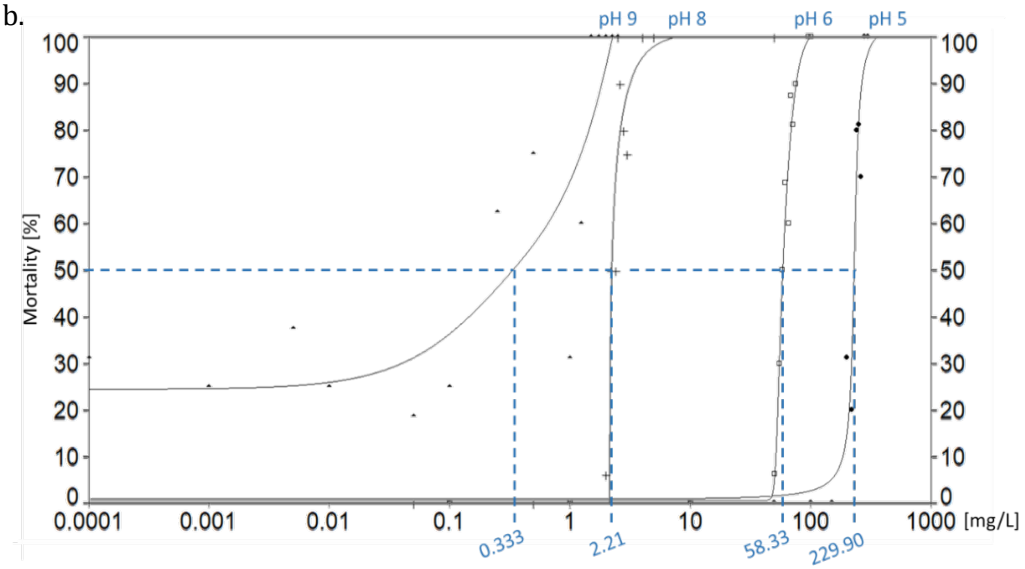
In conclusion, fluoxetine exhibits pH-dependent toxicity, with toxicity increasing with increasing pH. With regard to the endpoints investigated, this dependence was particularly evident in mortality, while the sublethal effects were less sensitive to fluoxetine. However, the prediction of the toxicity shift based on log D agreed well with the observed results, especially for the differences between pH 6 and 8 and pH 8 and 9.

Figure 5.15a: Regression curves of mortality upon exposure of embryos to fluoxetine

a.



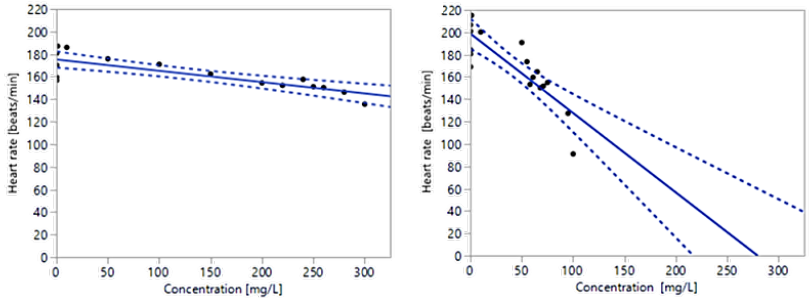
b.



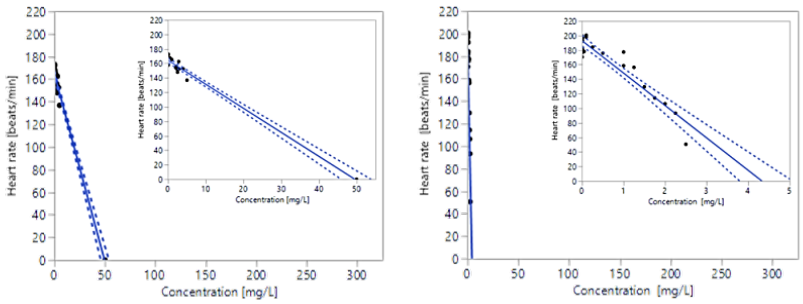
Regression curves of mortality upon exposure of embryos to fluoxetine showing LC_{50} values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

Figure 5.15b: Linear regression of heart rate as a function fluoxetine concentration

a. b.



c. d.



Linear regression of heart rate [beats/min] at time 48 hpf as a function of fluoxetine concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

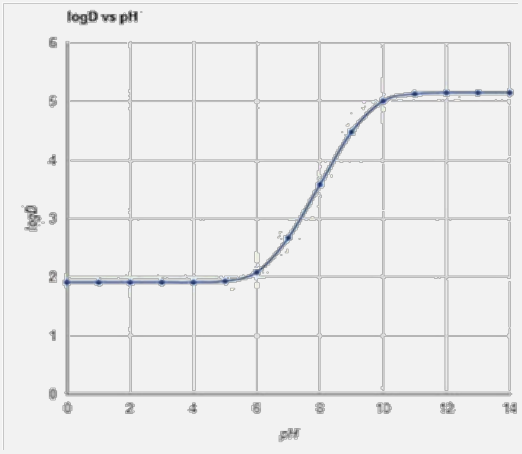
d. Sertraline

For sertraline, it was planned to test all four pH values, pH 5, 6, 8 and 9. The tests included two range findings as well as the main test. The log D curve for sertraline as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.16. The corresponding regressions are shown in Figure 5.16a. Since sertraline proved to be extremely poorly soluble in water even in small amounts, in this case it was necessary to resort to the solubiliser DMSO (dimethyl sulphoxide). The final DMSO concentration was 0.01 %, which is considered unproblematic in zebrafish embryos (Kais et al. 2013). In addition to the negative control, a DMSO control per pH was always performed in parallel. However, even 0.01 % DMSO could only dissolve sertraline to a limited extent, which is why the solubility limit was already reached at 30 mg/L at pH 5. Concentrations ≤ 30 mg/L, however, did not induce mortalities, whereupon pH 5 was not pursued further after one run.

Table 5.16: Overview of the prepared sertraline concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for sertraline as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	1.00	-	-	6	0.01	14.081	10.554
	10.00				0.10		
	20.00				1.00		
	30.00				5.00		
					7.50		
					10.0		
					11.0		
					12.0		
					13.0		
					14.0		
					15.0		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
					20.0		
					25.0		
8	0.01	1.200	1.194	9	0.01	0.5906	0.0961
	0.10				0.04		
	0.50				0.07		
	1.00				0.10		
	1.10				0.40		
	1.15				0.70		
	1.20				0.80		
	1.40				0.90		
	1.50				1.00		
	1.60				2.00		
	1.80				5.00		
	2.00				10.00		
	4.00						
	5.00						
	6.00						
	8.00						
	10.00						



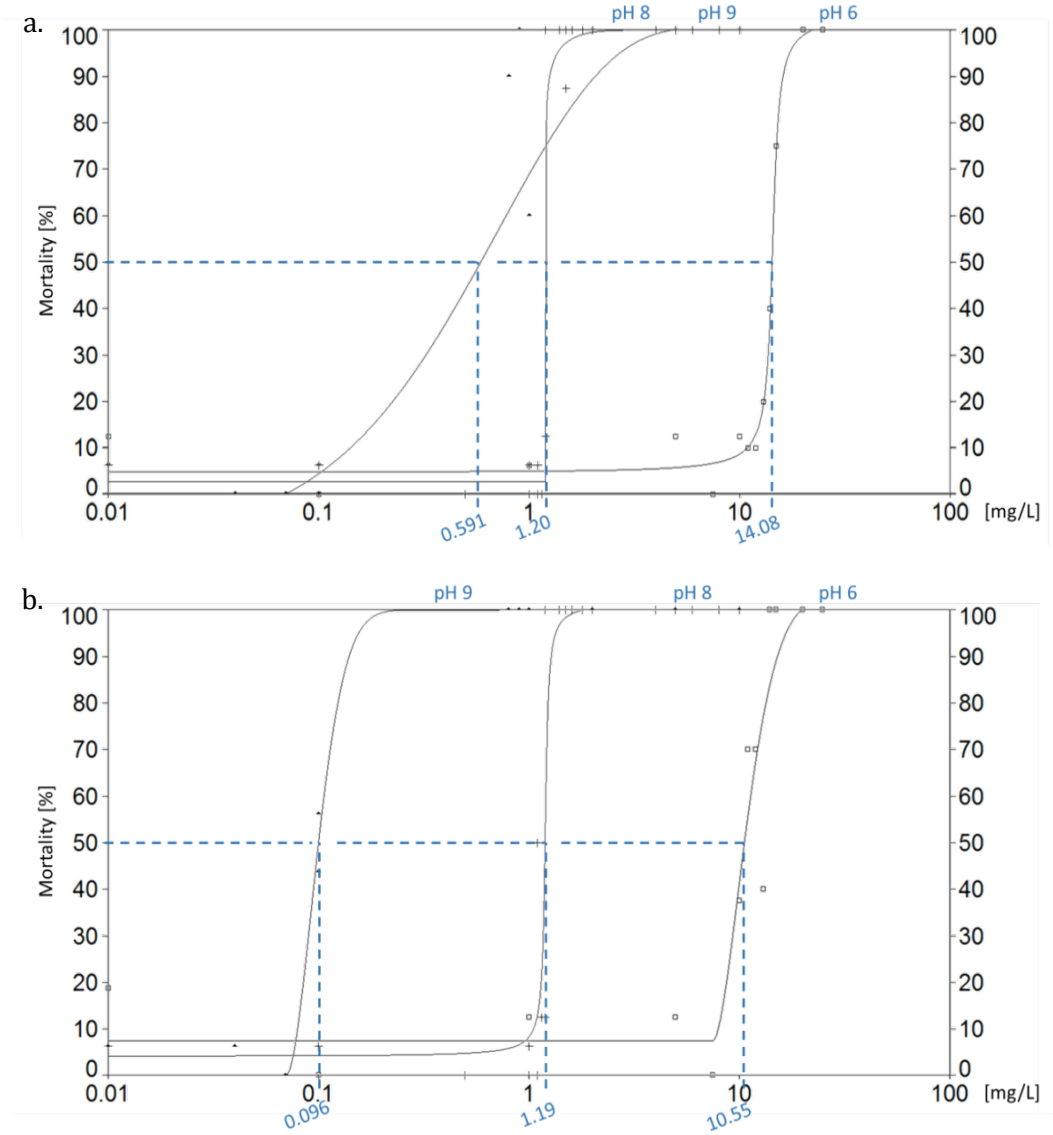
Source: Own depiction

Since it was difficult to generate mortalities at pH 8 that were not either around 10% or equal to 100 %, additional concentrations were tested and some concentrations were repeated to check the data. Concentrations around the LC_{50} were found to induce both no and complete mortality, indicating that the critical concentration for embryos was in a very narrow range. Based on the

data at pH 9, the concentration 0.1 mg/L was tested twice. Since the replicate gave very similar results (43.75 and 56.25 % mortality at 96 hpf), this data point was considered very robust and the two concentrations above it, 0.4 and 0.7 mg/L, at which mortalities of 6.25 and 12.5 % (96 hpf) occurred, were subsequently excluded from the regression.
The shifts in LC_{50} values as a function of pH were not as pronounced as for other substances, but were in line with expectations according to the log D curve, especially from pH 8 to pH 9 with a factor of about 10. The LC_{50} values at time 96 hpf were 10.554 mg/L for pH 6, 1.194 mg/L for pH 8 and 0.096 mg/L for pH 9. Compared to pH 8, the mortality curves of pH 6 and 9 were flatter.
The effect of sertraline on heart rate was less pronounced (Figure 5.16b). In the low and medium concentrations of all pH values, the heart rate was generally in the range of the control level between 150 and 170 beats per minute. Only in the high concentrations did the heart rate drop rapidly and markedly, or was no longer present.
Regarding the hatching rate, a clear hatching acceleration was observed for pH 5. Already at 48 hpf up to 31 % of the individuals hatched, at 60 hpf correspondingly 75 to 94 % and at 72 hpf the hatching process was almost completely finished. The pH values 6 and 8, on the other hand, were largely inconspicuous, with hatching success at 96 hpf exceeding 80 % (pH 6) and 90 % (pH 8) at concentrations up to 11 and 1 mg/L, respectively, with the exception of a few outliers. With further increasing concentrations, the hatching rate decreased slowly, never falling below 40 % (pH 6), or 60 % (pH 8), except in concentrations with 100 % mortality. At pH 9, there was no clear trend. The hatching log fluctuated relatively independently of the concentration and was on average significantly lower than at pH 6 and 8.

Overall, a pH-dependent toxicity could be observed for sertraline, which was less pronounced than for other substances, but which reflected well the expectations based on the log D curve. The effects on the sublethal endpoints were either weak (low to medium concentrations) or very pronounced (high concentrations) and were therefore only partially in agreement with the expression of the lethal effects.

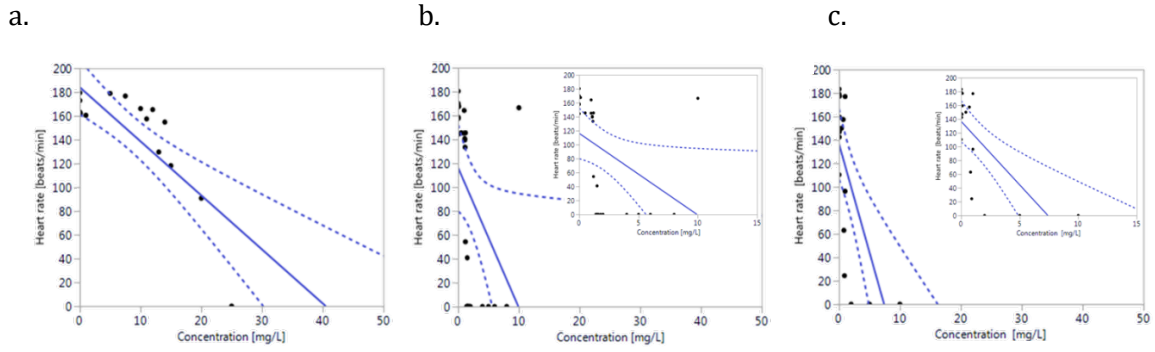
Figure 5.16a: Regression curves of mortality upon exposure of embryos to sertraline



Regression curves of mortality upon exposure of embryos to sertraline, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.

Source: Own depiction

Figure 5.16b: Linear regression of heart rate as a function of sertraline concentration



Linear regression of heart rate [beats/min] at time 48 hpf as a function of sertraline concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 6; b. pH 8; c. pH 9.

Source: Own depiction

5.2.2 Pesticides

5.2.2.1 Chlorophenols

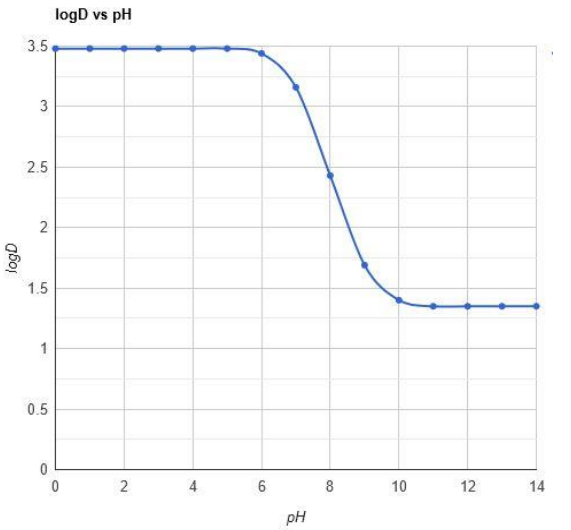
a. 2,3-DCP

The chlorophenol 2,3-DCP (dichlorophenol) was tested at pH 5, 6 and 8. The tests included two range findings as well as the main test. Due to the low solubility of DCP in water, isopropanol at a concentration of 0.01 % was used as solubiliser. The log D curve for DCP as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.17. The corresponding regressions are shown in Figure 5.17a.

Table 5.17: Overview of the prepared DCP concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for DCP as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.01	10.0	6.70	6	0.01	8.972	6.50
	0.1				0.1		
	1.0				1.0		
	2.5				5.0		
	3.5				5.5		
	5.0				6.0		
	5.5				6.25		
	6.0				6.5		
	6.5				7.0		
	7.0				7.5		
	7.5				8.5		
	10				9.5		
	15				10		
8	0.05	17.545	8.36				
	0.5						
	5.0						
	6.0						
	7.0						
	8.0						
	9.0						
	10.0						
	12.0						

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	20.0						
	25.0						
	30.0						
	50.0						

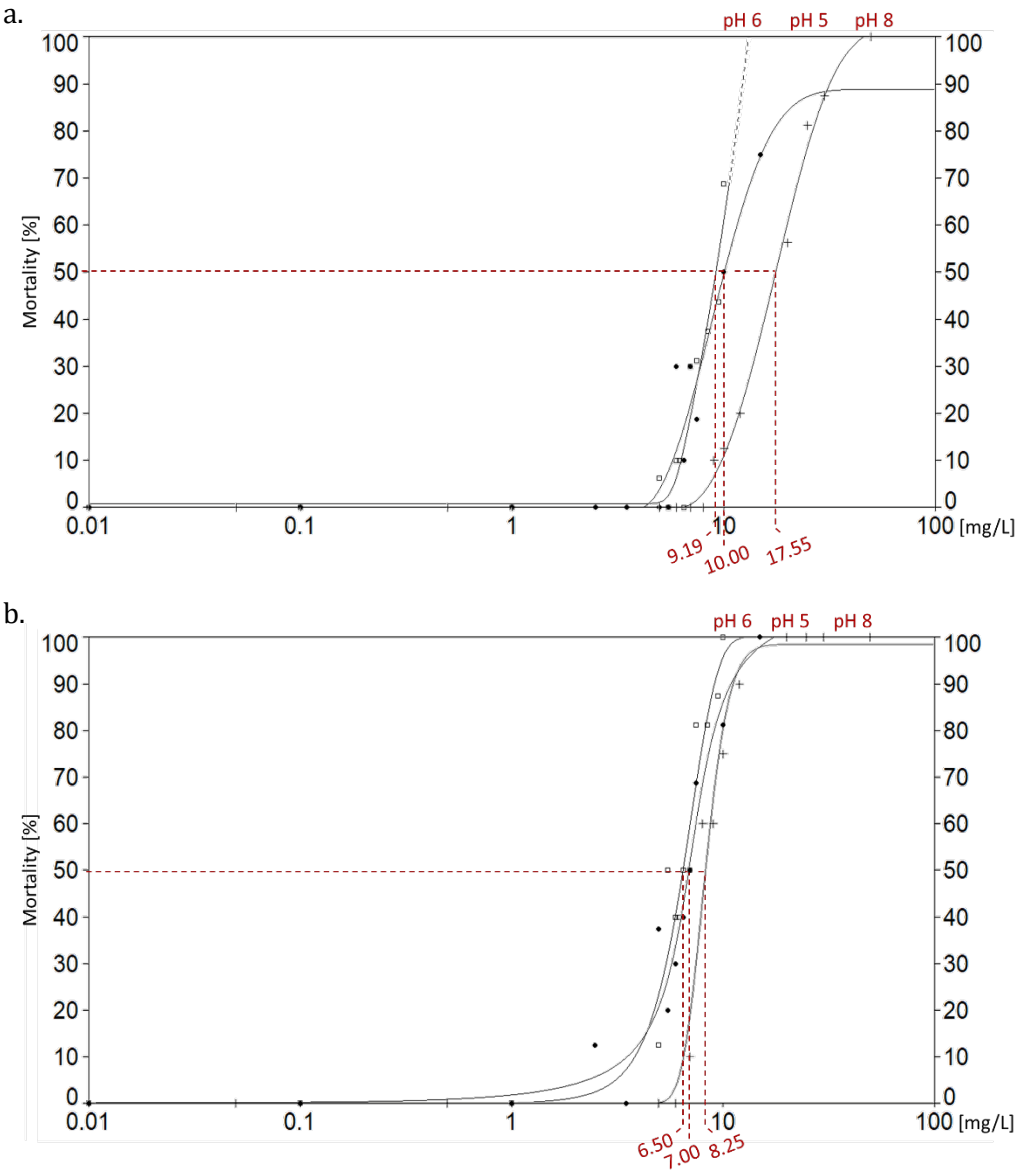


Source: Own depiction

As log D predicted, differences between the pH levels were rather small, particularly between pH 5 and 6. This was reflected in the LC_{50} values which were basically higher at pH 8 with 8.36 mg/L (96 hpf) than at pH 5 and 6 as usual for acids. Due to the little difference in log D between pH 5 and 6 and simultaneous biological variability, the LC_{50} values for pH 6 with 8.97 mg/L (72 hpf) and 6.5 mg/L (96 hpf) were even slightly lower than for pH 5 with 10 mg/L (72 hpf) and 6.7 mg/L (96 hpf). This was also reflected in the first concentration, which induced 100 % mortality at 96 hpf. For pH 8, this concentration was 20 mg/L, for pH 6 it dropped to 10 mg/L and increased to 15 mg/L at pH 5, again. As 10 mg/L was a concentration tested for all three pH levels, the comparison is particularly straightforward: the mortality amounted to 81.25 % (pH 5), 100 % (pH 6) and 75 % (pH 8).
In terms of heart rate, embryos exposed to lower concentrations were mostly within the control range between 140 and 160 beats per minute and decreased drastically at a certain concentration across all three pH levels. At pH 5, the threshold concentration was 3.5 mg/L inducing a heart rate of 144 beats per minute and then dropping to only 75 beats per minute at 5 mg/L. In subsequent concentrations heart rates varied between around 40 and 80 beats per minute before lacking completely at 15 mg/L. At pH 6, 1 mg/L induced a heart rate of 170 beats per minute while at 5 mg/L only 53 beats per minute were measured. At pH 8, the decrease occurred between 8 and 9 mg/L from 138 to 90 beats per minute.
A similar picture was seen in hatching rate with hatching success significantly decreasing at about the same threshold concentrations as observed in heart rate. In case of pH 5, the hatching rate dropped from control range to 25 %. An even more drastic decrease occurred at pH 6 and 8 with hatching successes below 20 %. While a complete lack of hatching at pH 5 and 8 was clearly coinciding with a total mortality at 96 hpf, at pH 6 only three individuals in total hatched above the threshold concentration of 1 mg/L.

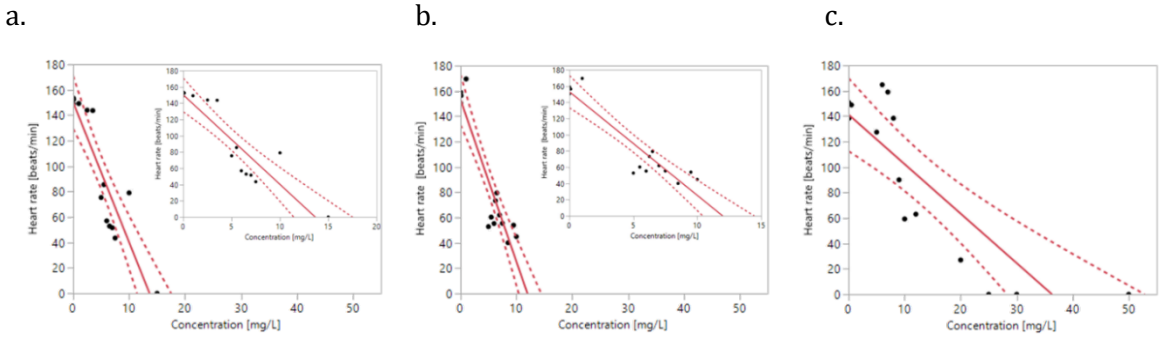
In total, the toxicity induced by DCP reflected the pH-dependent predictions of log D quite well, particularly concerning the very small mortality differences between pH 5 and 6 underlining the “plateau” effect.

Figure 5.17a: Regression curves of mortality upon exposure of embryos to DCP



Regression curves of mortality upon exposure of embryos to DCP, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.
Source: Own depiction

Figure 5.17b: Linear regression of heart rate as a function of DCP concentration



Linear regression of heart rate [beats/min] at time 48 hpf as a function of DCP concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8.
Source: Own depiction

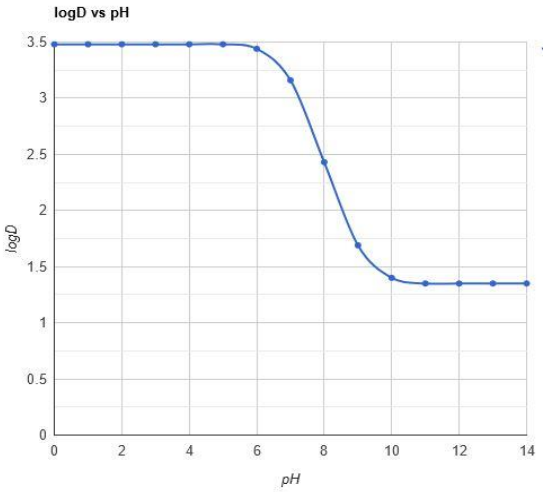
b. 2,4,5-TCP

2,4,5-TCP (trichlorophenol), used as herbicide or fungicide, was tested at pH 5, 6 and 8. The tests included two range findings as well as the main test. In addition, one (pH 6) or two (pH 5/8) further runs were carried out in the range-finding mode to narrow down the LC_{50} range more closely. Due to the low solubility of TCP in water, isopropanol at a concentration of 0.01 % was used as solubiliser. The log D curve for TCP as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.18. The corresponding regressions are shown in Figure 5.18a.

Table 5.18: Overview of the prepared TCP concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for TCP as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.050	0.600	0.595	6	0.05	0.860	0.830
	0.100				0.10		
	0.500				0.20		
	0.525				0.30		
	0.550				0.40		
	0.575				0.42		
	0.580				0.44		
	0.590				0.45		
	0.600				0.46		
	0.700				0.48		
	0.750				0.49		
	0.800				0.50		
	0.900				0.70		
	1.00				1.50		
	2.00				5.00		
8	0.05	1.481	1.425				
	0.50						
	1.00						
	2.00						
	2.05						
	2.10						
	2.15						
	2.20						

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	2.30						
	2.40						
	2.50						
	2.75						
	3.00						
	4.00						
	5.00						
	50.0						

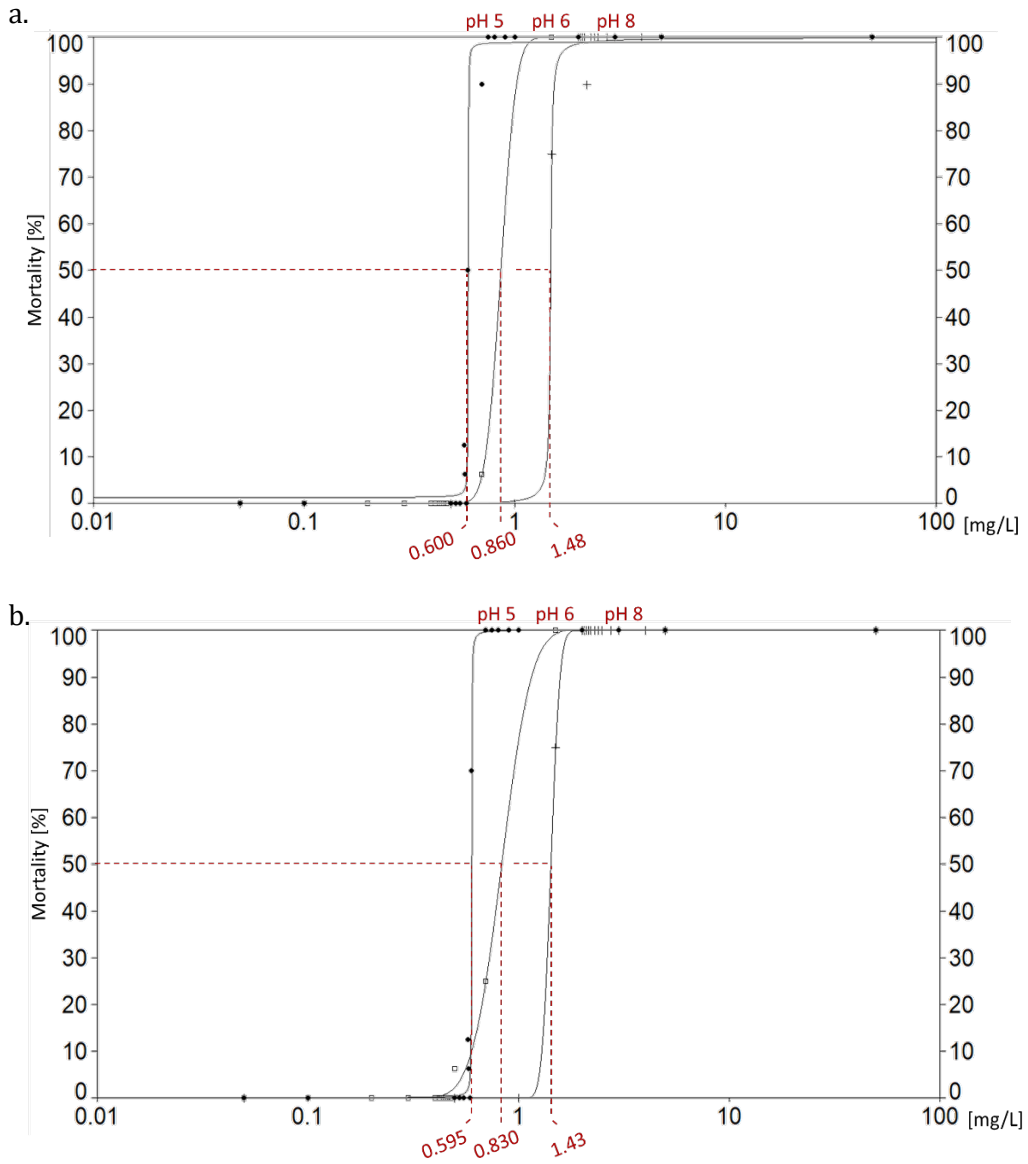


Source: Own depiction

As expected, the toxicity of TCP decreased with increasing pH. However, as predicted by log D, the differences were rather small. The LC_{50} values at the time point 96 hpf were 0.595 mg/L for pH 5, 0,83 mg/L for pH 6 and 1.425 mg/L for pH 8. However and in contrast to DCP, TCP showed a very steep increase in toxicity, which made it difficult to induce mortalities that were not extremely low, or absent, or not immediately total, despite additional test runs. This was also reflected in the sublethal endpoints of hatching and heart rate (Figure 5.18b), where the compound-exposed embryos were either at control levels (80 - 100 % hatching; 150 - 180 beats per minute) or near total failure (no hatching; no heartbeat). In most cases, the total failure was due to mortality present at these time points. Mortality usually occurred very early, mostly between 12 and 48 hpf. For all three pH values tested, there was only one relevant concentration in each case that induced a more or less average mortality rate. For pH 5 this concentration was 0.6 mg/L, for pH 6 0.7 mg/L and for pH 8 1.5 mg/L. The respective mortalities at 96 hpf were 70 % (pH 5), 25 % (pH 6) and 75 % (pH 8). In addition, a significantly reduced hatching rate was observed at these concentrations, ranging from 20 to 50 %. The heart rate also fell in these ranges, in some cases drastically, below the control level. For embryos at pH 5, only just under 41 beats per minute were measured, while for pH 6 and pH 8 embryos still about 148, and 100 beats per minute, respectively, were recorded.

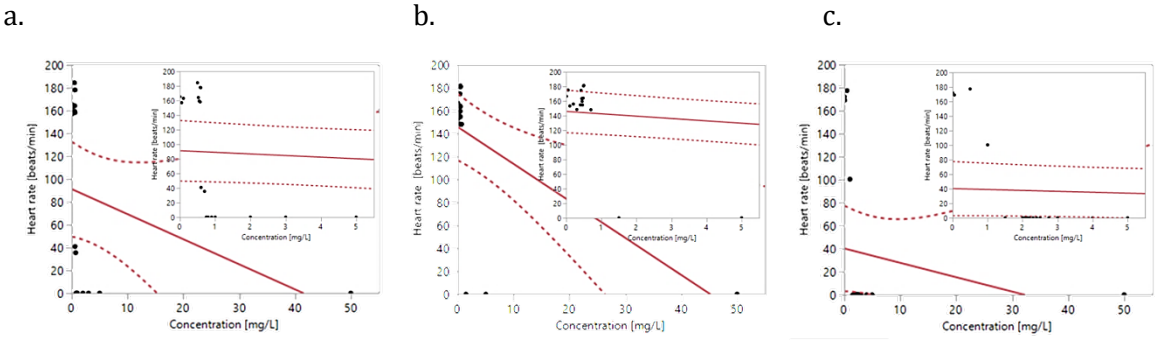
Overall, TCP showed a pH-dependent toxicity shift that was rather small, as predicted by log D. The observations for the difference between pH 5 and 6 matched the prediction quite well, whereas the difference between pH 6 and 8 deviated from the log D prediction by about a factor of seven.

Figure 5.18a: Regression curves of mortality upon exposure of embryos to TCP



Regression curves of mortality upon exposure of embryos to TCP, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.
Source: Own depiction

Figure 5.18b: Linear regression of heart rate as a function of TCP concentration

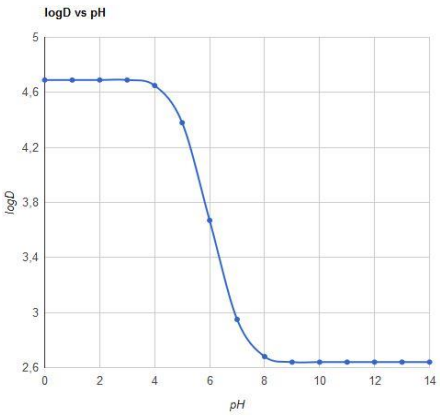


Linear regression of heart rate [beats/min] at time 48 hpf as a function of TCP concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8.
Source: Own depiction

c. PCP

The pesticide PCP (pentachlorophenol) was tested at pH 5, 6 and 8. The tests included two range findings as well as the main test. The log D curve for PCP as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.19. The corresponding regressions are shown in Figure 5.19.

Table 5.19: Overview of the prepared PCP concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for PCP as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.001	0.0712	0.0663	6	0.005	0.0649	0.0560
	0.010				0.050		
	0.020				0.052		
	0.040				0.054		
	0.045				0.056		
	0.060				0.058		
	0.065				0.060		
	0.070				0.070		
	0.075				0.075		
	0.080				0.080		
	0.085				0.090		
	0.100				0.100		
	1.00				0.200		
					0.300		
					0.400		
					0.500		
					5.00		
8	0.010	0.2650	0.2633				
	0.100						
	0.200						
	0.235						
	0.240						
	0.250						
	0.260						
	0.265						

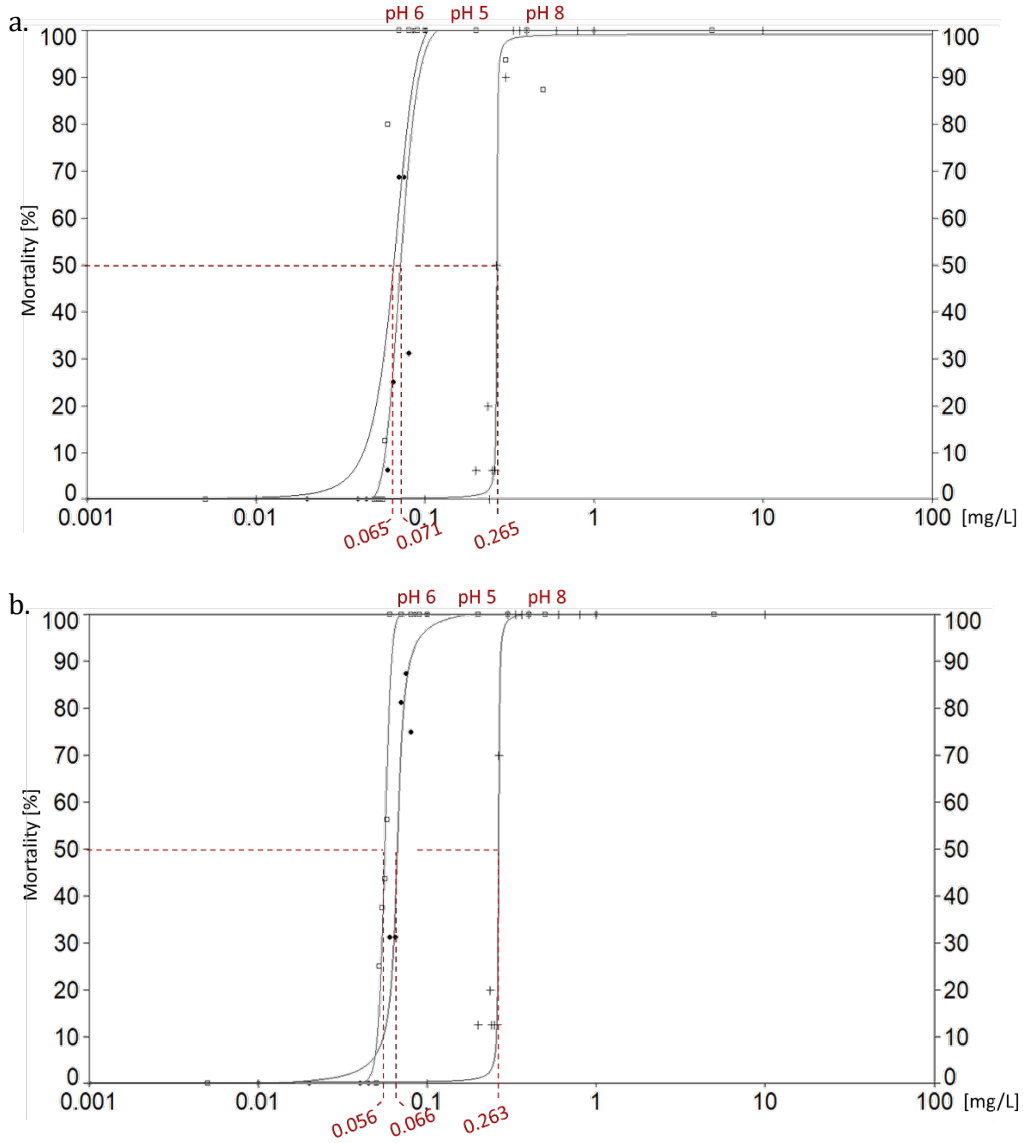
pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	0.300						
	0.335						
	0.400						
	0.600						
	0.800						
	1.00						
	10.0						

Source: Own depiction

The toxicity differences predicted by log D were rather small for the acid PCP and were approximately between a factor of 5 (pH 5/6) and 10 (pH 6/8), which on the one hand probably contributed to the fact that, contrary to expectation, PCP had a more toxic effect on the embryos at pH 6 than at pH 5. On the other hand, PCP already showed itself to be toxic at very low concentrations, which can lead to corresponding shifts in results in the case of potentially small concentration deviations. The LC_{50} values determined for PCP were 0.066 mg/L for pH 5, 0.056 mg/L for pH 6 and 0.263 mg/L for pH 8.
Particularly at pH 6, the substantial differences could be observed with little change in concentration. While concentrations up to 0.50 mg/L did not cause mortality, 0.60 mg/L was already sufficient to reach 100 % after 96 hpf. As already described, the effects on mortality were close to each other at pH 5 and 6, whereas pH 8 stood out clearly. At a concentration of 0.1 mg/L PCP, 100 % mortality was already achieved at pH 5 and 6 after 72 hpf, while at the same concentration at pH 8 no lethal effects occurred at all until the end of the experiment.
Heart rates were not counted for PCP.
Concerning hatching behaviour, PCP was found to cause hatching events before 60 hpf, occasionally, at pH levels 5 and 8 and at concentrations that induced less than 100 % mortality. For these two pH levels, the hatching rate at the low and medium concentrations was also basically in the control range (> 80 %). At pH 6, on the other hand, the hatching success already dropped significantly to 37.5 to 50 % in the medium concentrations.

Even though PCP was apparently more toxic at pH 6 than at pH 5, the deviation from the prediction from the log D model for the toxicity shift was < a factor of 5. There was better agreement between prediction and observed toxicity shift for the difference between pH 6 and 8. Model and observation differed only by a factor of 2. Thus, PCP shows a pH-dependent toxicity that can be predicted relatively well with the log D model, even if the statement about a consistently decreasing toxicity with increasing pH cannot be made unambiguously on the basis of the data collected here.

Figure 5.19: Regression curves of mortality upon exposure of embryos to PCP



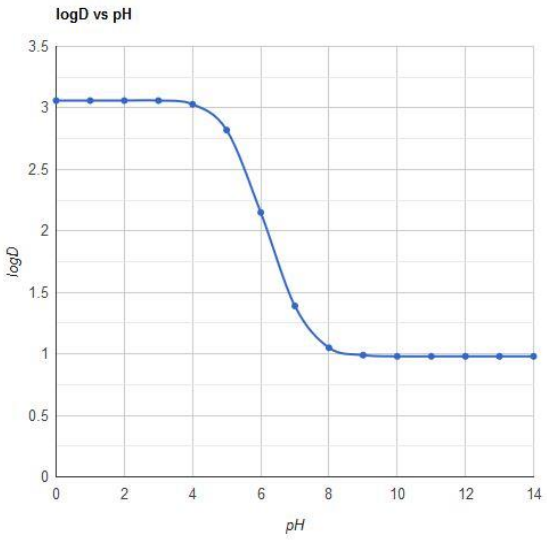
Regression curves of mortality upon exposure of embryos to PCP, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.
Source: Own depiction

5.2.2.2 Herbicides

a. Bromoxynil

The herbicide bromoxynil was tested at pH 5, 6 and 8. The tests included two range findings as well as the main experiment. Due to the poor solubility of bromoxynil in water, isopropanol at a concentration of 0.01 % was used as solubiliser. The log D curve for bromoxynil as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.20. The corresponding regressions are shown in Figure 5.20a.

Table 5.20: Overview of the prepared bromoxynil concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for bromoxynil as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.05	0.1880	0.1612	6	0.02	0.4612	0.3591
	0.10				0.20		
	0.12				0.30		
	0.14				0.40		
	0.15				0.45		
	0.16				0.50		
	0.18				0.55		
	0.20				0.60		
	0.30				0.65		
	0.40				0.80		
	0.50				1.00		
	5.00				2.00		
	50.0				20.0		
8	0.10	26.993	20.266				
	1.00						
	10.0						
	15.0						
	20.0						
	22.5						
	25.0						
	27.5						
	30.0						
	40.0						
	60.0						
	80.0						
	100.0						

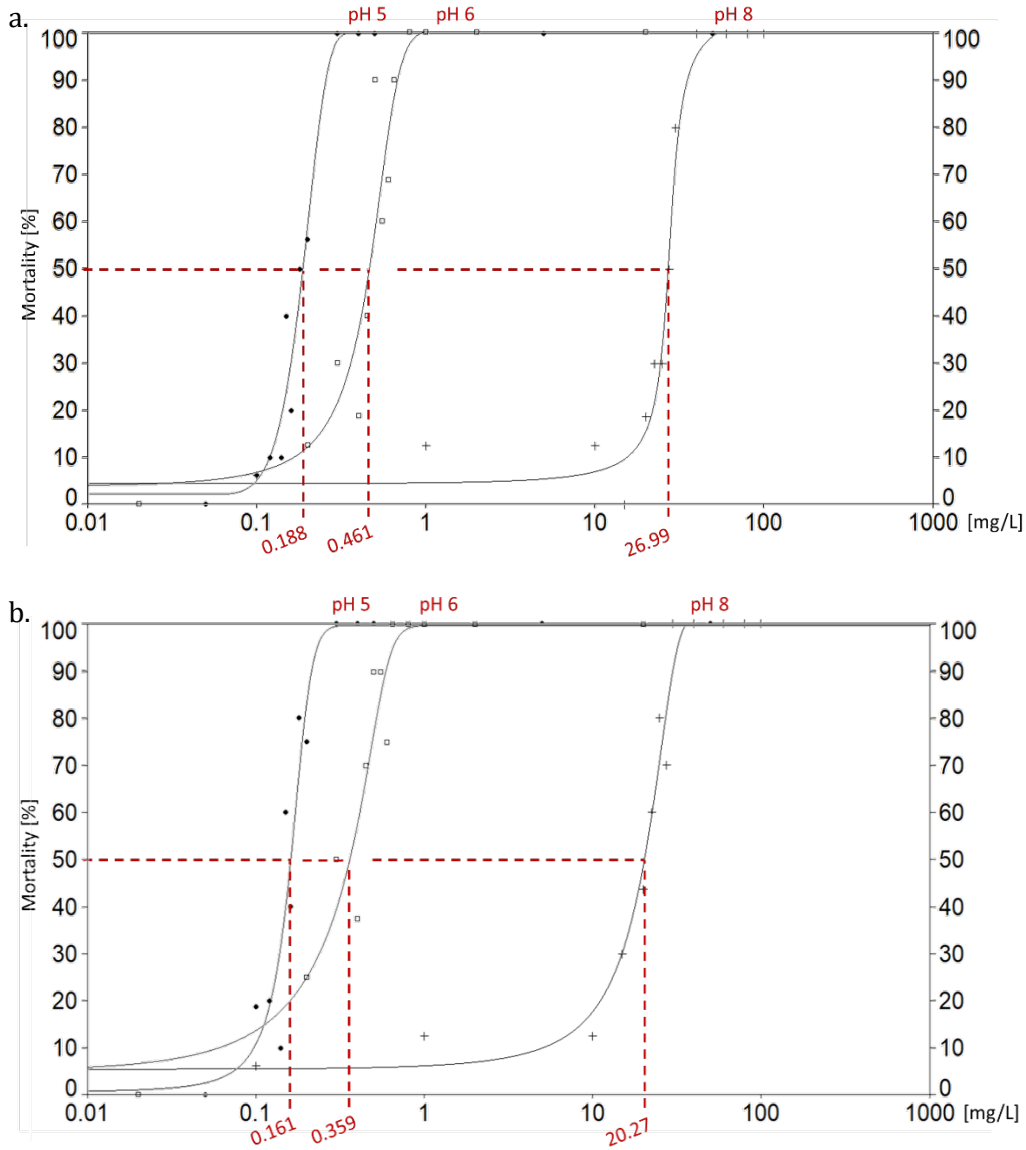
Source: Own depiction

In the range of pH 5 and 6, the acid bromoxynil was toxic at very low concentrations already, which was reflected in LC_{50} values of 0.16 mg/L (pH 5) and 0.36 mg/L (pH 6) at the 96 hpf time

point, whereas an LC50 of 20.27 mg/L was calculated for pH 8. Thus, the LC_{50} values showed increasing toxicity with decreasing pH, as expected for an acid. Basically, first mortalities started early, partly already at 12 hpf, and increased successively over time.
The heart rate at pH 5 decreased rapidly and considerably over the course of the concentration (Figure 5.20b). Only the lowest concentration of 0.1 mg/L was still within the control range of about 149 beats per minute on average. Already at a concentration of 0.12 mg/L, the heart rate dropped to about 131 beats per minute. If the overall mortality in the concentrations at time 48 hpf increased to over 30 %, (0.18 mg/L, ≥ 0.3 mg/L), hardly any heartbeat could be detected in the remaining individuals still alive. At pH 6 and 8, heartbeat decreased only at intermediate concentrations (pH 6 ≥ 0.5 mg/L; pH 8 ≥ 15 mg/L), with complete absence of heartbeat correlating exclusively with complete mortality at time 48 hpf.
At pH 5 and 8, bromoxynil did not yet trigger hatching activity at 48 hpf, however, a majority of hatching events were observed at 60 hpf. Subsequently (72/96 hpf), only single individuals hatched. Low or absent hatching correlated mostly with high mortalities, which occurred up to and including 60 hpf. At pH 6, hatching success was significantly lower, reaching 75% only in the lowest bromoxynil concentration of 0.02 mg/L, while in all other concentrations tested, a maximum of 50% of embryos hatched, but with hatching rates exceeding 90% in controls relevant to these concentrations.

Overall, bromoxynil showed the expected decrease in toxicity with increasing pH. The small difference between pH 5 and 6 corresponded relatively well to the prediction with log D, while the toxicity shift between pH 6 and 8 was more pronounced than assumed by about 4.5-fold.

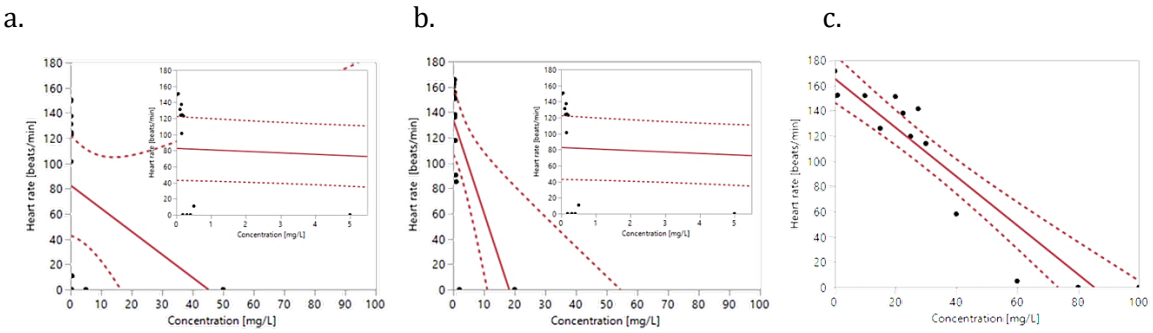
Figure 5.20a: Regression curves of mortality upon exposure of embryos to bromoxynil



Regression curves of mortality upon exposure of embryos to bromoxynil, indicating LC_{50} values; a. after 72 hpf; b. after 96 hpf.

Source: Own depiction

Figure 5.20b: Linear regression of heart rate as a function of bromoxynil concentration



Linear regression of heart rate [beats/min] at time 48 hpf as a function of bromoxynil concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8.

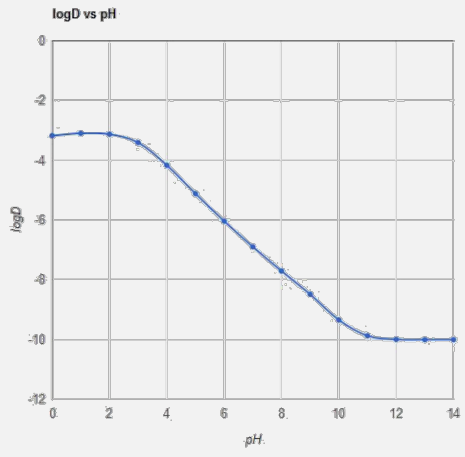
Source: Own depiction

b. Glyphosate

The acid glyphosate was only tested at pH 5 and 6, since it was already known from a published study (Schweizer et al. 2019) that no mortalities occur up to at least 1700 mg/L at pH 7 and the solubility limit of glyphosate in water is approx. 2000 mg/L. Based on the log D curve as a function of pH, it can be assumed that at pH 8 substance amounts above the solubility limit are needed to induce mortalities. Therefore, the investigation at pH 8 was dispensed with. Furthermore, the problem already became apparent at pH 6, so that the scope of the test series was limited to two range findings. Due to the low mortalities, only one LC_{50} value could be determined for pH 5 at the time 96 hpf. The log D curve for glyphosate as a function of pH and the concentrations applied in each case as well as the LC_{50} value determined for pH 5 are noted in Table 5.21. The corresponding regression is shown in Figure 5.21a.

Table 5.21: Overview of the prepared glyphosate concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for glyphosate as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	10.0	-	239.59	6	250.0	-	-
	50.0				500.0		
	100.0				750.0		
	500.0				1000		
	750.0				1250		
	1000				1500		
	1500				1750		
	2000				2000		

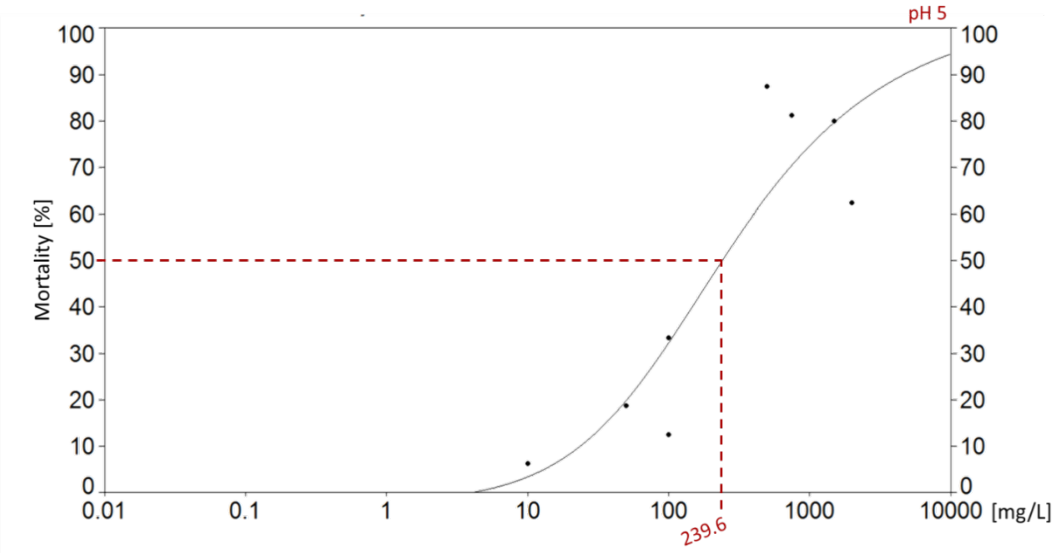


Source: Own depiction

The LC_{50} value for glyphosate at pH 5 at the 96 hpf time point was The LC_{50} value for glyphosate at pH 5 at the 96 hpf time point was 239.59 mg/L, although it should be noted that this value probably overestimates the toxicity of glyphosate somewhat, as it was not possible to test concentrations that caused 100 % mortality even at pH 5. In the tests carried out at pH 5, mortality at 100 mg/L was 33 %; at 500 mg/L it dropped again by more than half to 13 % in the

meantime. According to the regression derived from the results, the real LC_{50} should lie between these two concentrations. However, based on the values collected here, it was between 500 and 750 mg/L glyphosate. At 750 mg/L glyphosate, the highest mortality of 88 % was also reached in the experiment. It then slowly decreased again to 63 % at 2000 mg/L. At 72 hpf, mortalities occurred at pH 5 only at the three highest concentrations (1000, 1500, 2000 mg/L). However, these were consistently below 20 %, which is why it was not possible to determine the LC_{50} value for 72 hpf. Since this experimental set-up was still carried out with the undiluted buffer system, control mortalities of 0 % were observed at 72 hpf, but 50 % at 96 hpf. I.e. the control mortality was even higher than the mortality effects of glyphosate up to a concentration of 500 mg/L inclusive. However, since glyphosate proved to be fundamentally a rather unsuitable substance for the pHION model, the runs were not repeated again.
At pH 6, no lethal effects occurred up to the highest concentration of 2000 mg/L, neither up to 72 hpf nor up to 96 hpf.
The measured heart rates decreased almost continuously at pH 5 compared to the control, only at 2000 mg/L there was a slight increase again to the level of 1000 mg/L (Figure 5.21b and 5.21c). The deviation of the heart rate compared to the control reached almost 20 %. At pH 6, a pattern already described by Schweizer et al. (2019) for the effect of glyphosate on *Danio rerio* embryos at pH 7 was observed: first, a slowing of the heartbeat was observed up to a certain concentration, a kind of "turning point", followed by an acceleration of the heartbeat, which was even above the control level at particularly high concentrations. This "turning point" occurred in this series of experiments at pH 6 in the range of 1000 mg/L. At concentrations of 1500 mg/L glyphosate, the heart rate was already about 7 % above the average control rate measured.
Furthermore, the hatching rate also showed parallels to the results of Schweizer et al. (2019), who demonstrated a clear hatching acceleration under the influence of glyphosate, which was particularly pronounced at the lower concentrations. While in this set of experiments between 12 and 13 % of the embryos hatched at 60 hpf in the control at both pH 5 and pH 6, this percentage increased to 69 % (pH 5, 10 mg/L) and 75 % (pH 6, 250 mg/L), respectively, at the lowest glyphosate concentration tested (Figure 5.21d). With an increase in concentration, the hatching rate decreased significantly at pH 5 and remained at or slightly above the control level for all other concentrations. At pH 6, however, the hatching rate decreased continuously, but remained at a clearly increased level of > 30 % compared to the control.

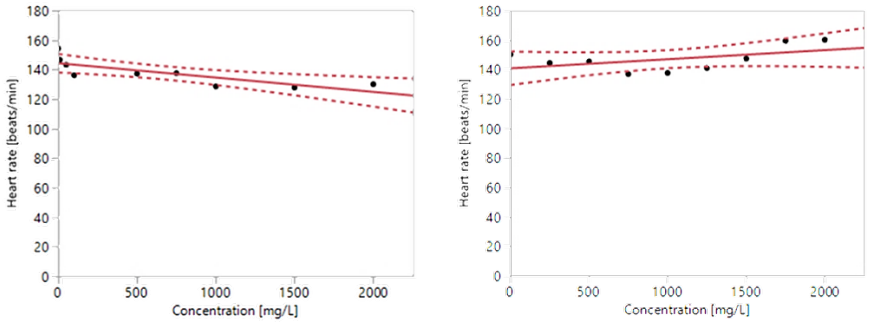
Figure 5.21a: Regression curves of mortality upon exposure of embryos to glyphosate



Regression curves of mortality upon exposure of embryos to glyphosate at pH 5 after 96 hpf, indicating the LC50 value.
Source: Own depiction

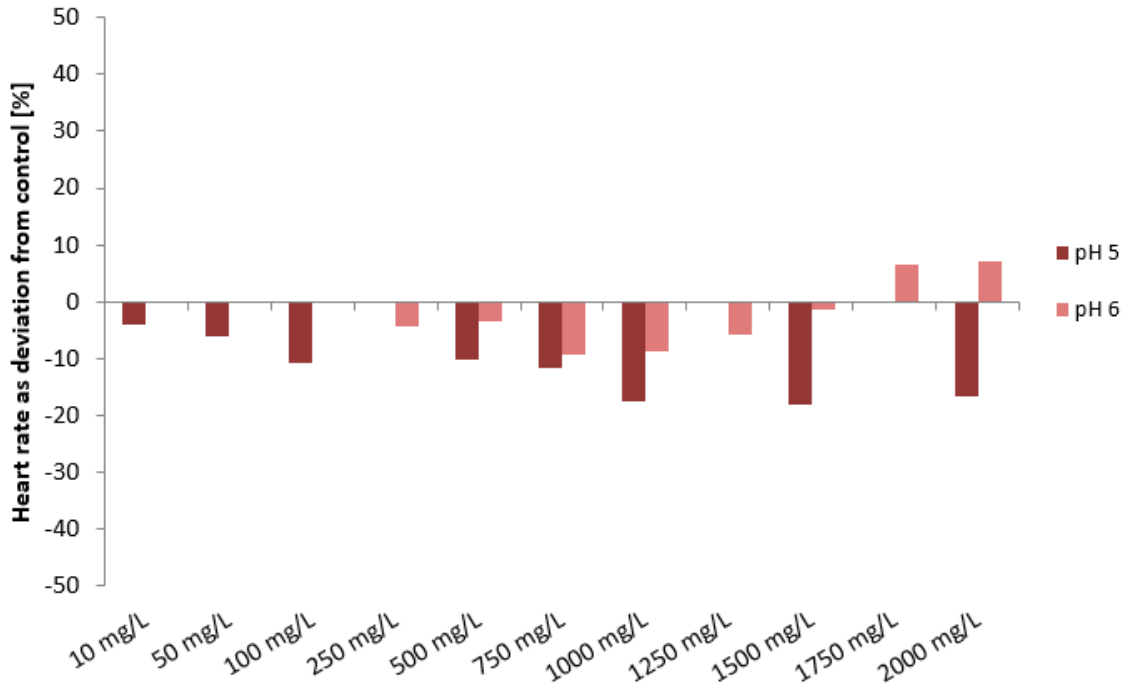
Figure 5.21b: Linear regression of heart rate as a function of glyphosate concentration

a. b.



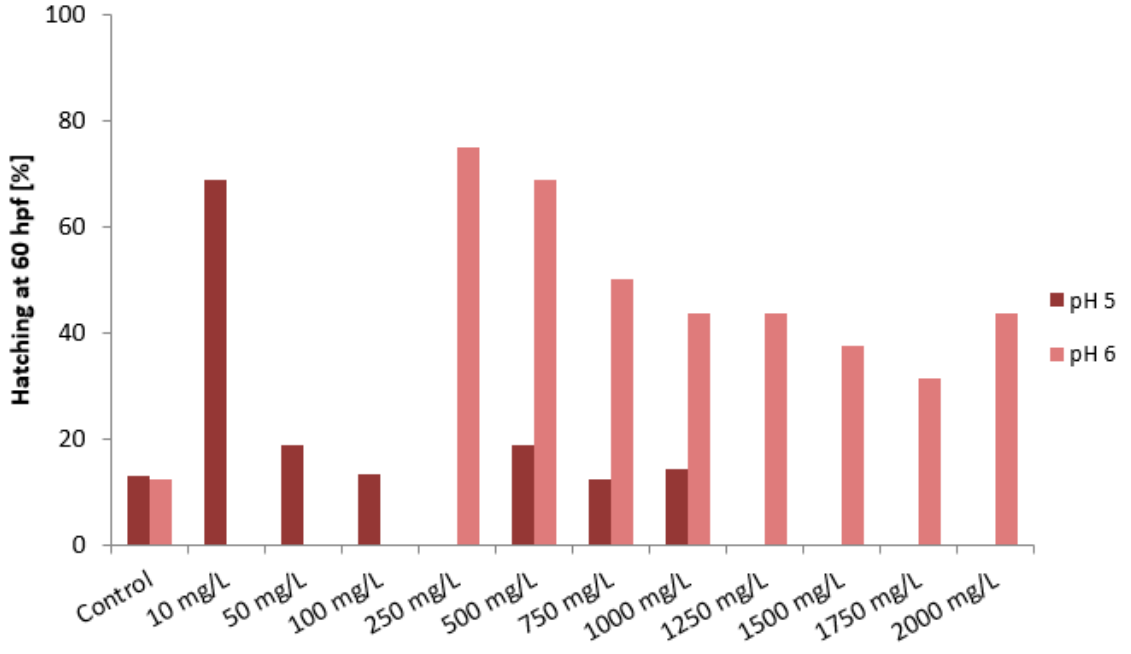
Linear regression of heart rate [beats/min] at time 48 hpf as a function of glyphosate concentration [mg/L]; a. pH 5; b. pH 6.
Source: Own depiction

Figure 5.21c: Mean deviation of heart rate as a function of glyphosate concentration and pH



Mean deviation of heart rate from control in percent at time point 48 hpf as a function of glyphosate concentration and pH.
Source: Own depiction

Figure 5.21d: Hatching rate of embryos at time 60 hpf in percent as a function of glyphosate concentration and pH



Source: Own depiction

Overall, glyphosate showed the expected increase in toxicity towards the acidic range. However, since glyphosate has only a very limited acute toxic effect on the embryos and the quantities needed to achieve the desired effects clearly exceed the solubility limit of glyphosate in water, further investigation of glyphosate within the PHION project is not carried out. The additionally included sublethal endpoints heartbeat and hatching rate showed clear parallels to effect

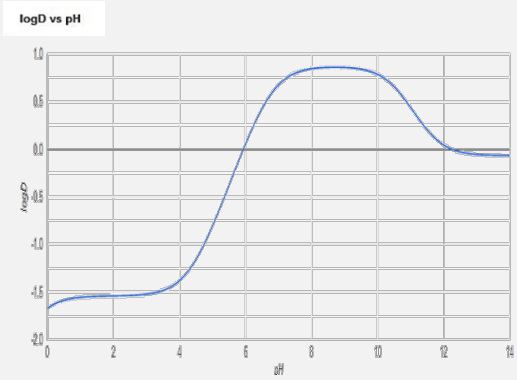
patterns already described by Schweizer et al. (2019), especially at pH 6, which further support the assumption of subacute toxicity of glyphosate.

5.2.2.3 Insecricides

a. Imidacloprid

Although imidacloprid is a base, its toxicity was tested at pH 5, 6 and 8 in order to find the range of greatest toxicity change predicted by the log D curve. However, the tests only included a range finding, since on the one hand hardly any effects were observed and on the other hand solubility problems already occurred at high concentrations of 750 mg/L and above. The log D curve for imidacloprid as a function of pH and the concentrations applied in each case are noted in Table 22. Due to the very low mortality, neither regressions nor LC_{50} values could be determined in the case of imidacloprid.

Table 5.22: Overview of the prepared imidacloprid concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for imidacloprid as a function of pH

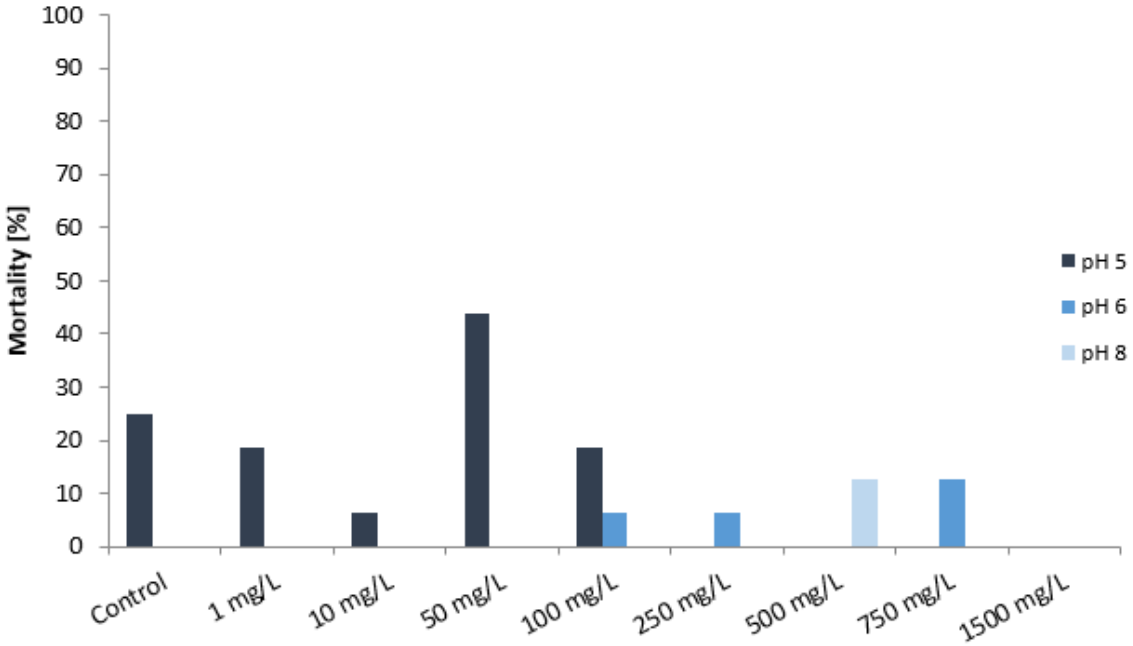
pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	1.0	-	-	6	10.0	-	-
	10.0				50.0		
	50.0				100.0		
	100.0				250.0		
					750.0		
					1500		
8	50.0	-	-				
	100.0						
	250.0						
	500.0						

Source: Own depiction

Although it would have been expected that imidacloprid would have a more toxic effect on embryos in the increasingly basic pH ranges and, in addition, higher concentrations were applied at pH 6 and 8 than at pH 5, only isolated mortalities occurred at these pH values (a maximum of two individuals per concentration) (Figure 5.22a). At pH 5, on the other hand, no mortality at all was observed until 72 hpf. At 96 hpf, mortality increased significantly and varied between 6 and 44 %, independent of concentration. However, the control also showed a

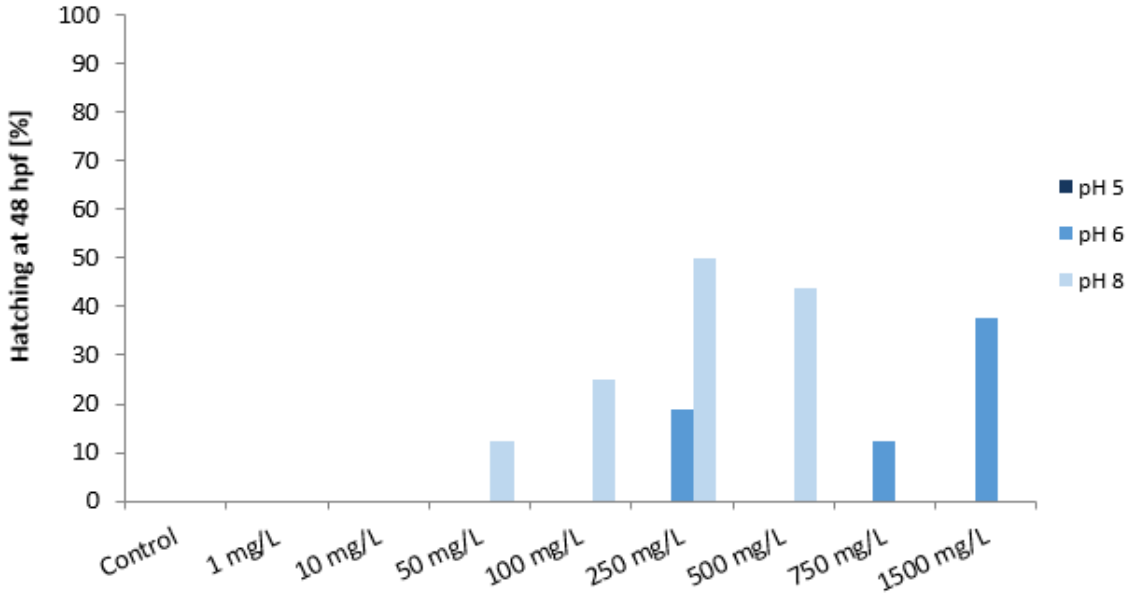
mortality of 25 %, which suggests that the lethal effects observed are artefacts that are negligible.
Heartbeats were not counted for imidacloprid, but hatch rates were recorded. These showed clear abnormalities. While usually the first hatching events can be recorded at time 60 hpf, imidacloprid induced clear hatching accelerations at pH 6 and 8, so that already at 48 hpf up to 50 % of the embryos had hatched (Figure 5.22b). This effect occurred particularly at pH 8, whereas at pH 5 and all controls there was no hatching at 48 hpf. In addition, a tendency for the effect to increase was observed at both pH values. I.e. in contrast to the mortality, both a most extensive concentration dependence and the effect amplification at higher pH values were shown here, as was to be expected with a base.
Overall, imidacloprid proved unsuitable for the pHION project due to its limited acute toxicity in combination with low solubility at high concentrations > 500 mg/L (pH 6). However, the sublethal endpoint hatching rate, which showed an effect enhancement with increasing pH, suggests that a pH dependence of mortality would probably have been observed at significantly higher concentrations, as expected.

Figure 5.22a: Mortality of embryos at time 96 hpf in percent depending on imidacloprid concentration and pH



Source: Own depiction

Figure 5.22b: Hatching rate of embryos at time 48 hpf in percent as a function of imidacloprid concentration and pH



Source: Own depiction

5.2.2.4 Anti-microbial agents

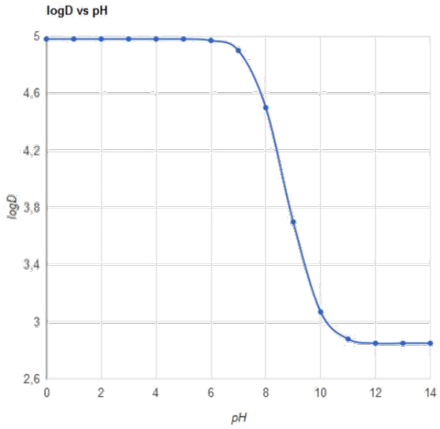
a. Triclosan

The acid triclosan was tested at pH 5, 6 and 8 and to a limited extent at pH 9. The tests included two range-findings as well as the main test. The log D curve for triclosan as a function of pH and the concentrations applied in each case as well as the LC_{50} values determined are noted in Table 5.23. The corresponding regressions are shown in Figure 5.23a.

Table 5.23: Overview of the applied triclosan concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for triclosan as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.05	0.43	0.31	9	0.450	0.67	0.66
	0.10				0.475		
	0.20				0.500		
	0.30				0.650		
6	0.40	0.43	0.39		0.850		
	0.42				1.000		
	0.44				1.200		
	0.45				1.500		
8	0.46	0.47	0.41		1.800		
	0.48				2.000		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	0.50						
	0.60						
	1.00						



Source: Own depiction

Differences in toxicity at different pH values could only be shown to a very limited extent for triclosan. Looking at the log D curve as a function of pH, an extremely slight, or slight decrease in the proportion of neutral species, and thus toxicity, can be expected from pH 5 to pH 6 and from pH 6 to pH 8. This was reflected accordingly in the results for mortality. The LC_{50} values for 96 hpf were 0.31 mg/L, 0.39 mg/L and 0.41 mg/L for pH 5, 6 and 8, respectively. For all three pH values, 0.42 mg/L was the critical concentration at which mortalities first occurred before 96 hpf. While survival rates at the time of 72 hpf were similarly low at pH 5 and 6 from 0.42 mg/L (10 - 40 % compared to 90 % at pH 8), the collapse at pH 8 only occurred from a triclosan concentration of 0.46 mg/L onwards.

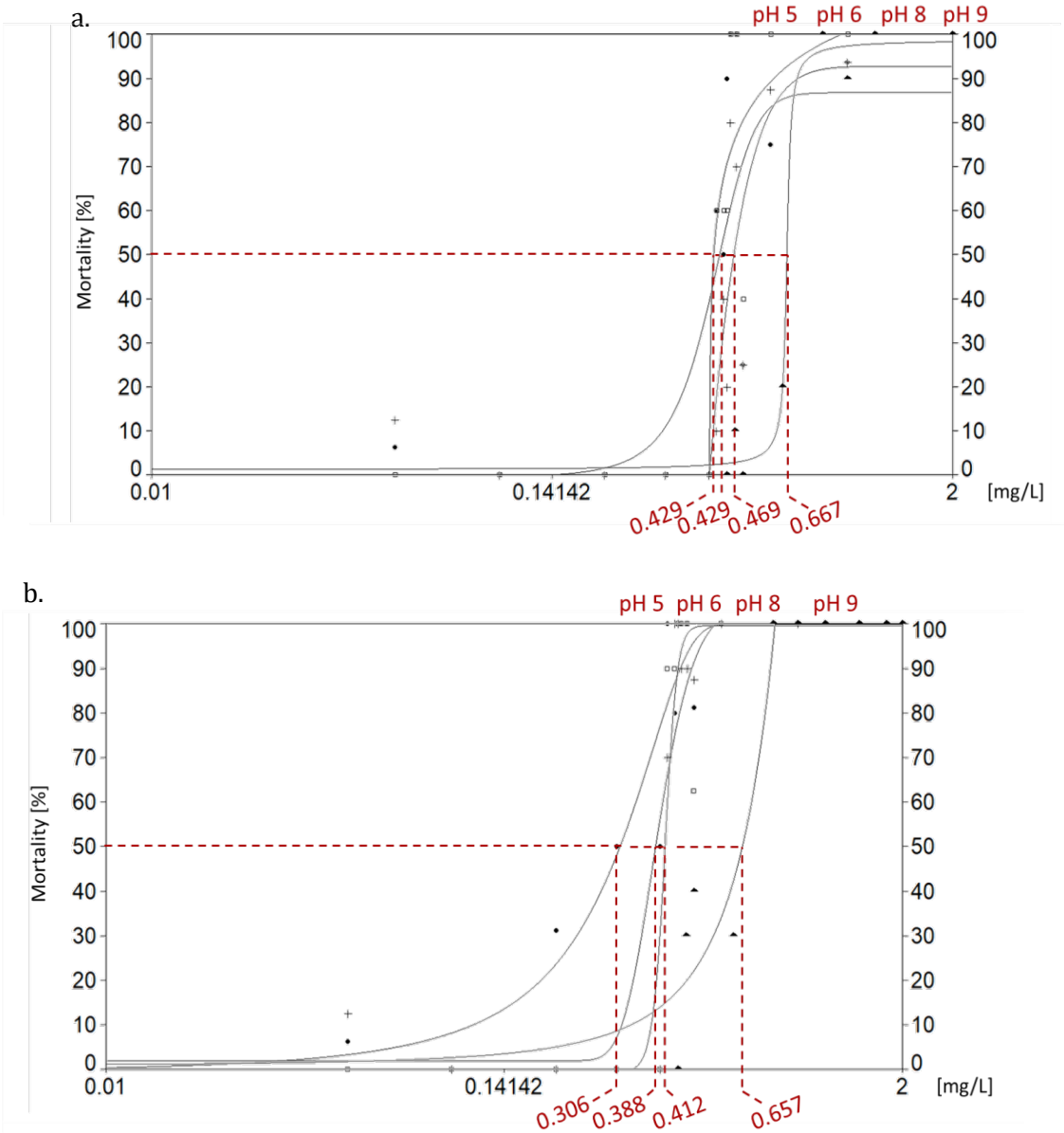
At the 96 hpf time point, the results of pH 8 approached those of pH 5 and 6 again, which can also be seen in the corresponding LC_{50} values for 72 and 96 hpf (Table 5.23, Figure 5.23a). Due to the slight differences in the results of these three pH values and the prediction of a significant drop in toxicity when the test environment shifted in the basic direction, additional concentrations were applied at pH 9. Mortalities occurred in these exposures only from 0.475 mg/L triclosan and 100 % mortality was reached only from concentrations of 0.85 mg/L. Compared to pH 5, 6 and 8, where complete mortality was already observed at 0.42 mg/L, respectively 0.46 mg/L and 0.6 mg/L, the results at pH 9 showed a further decrease in toxicity, which, however, remained below the predictions based on the log D curve. The LC_{50} value for 96 hpf was 0.66 mg/L for pH 9.

Regarding the sublethal endpoints, the heart rate was almost congruent with mortality (Figure 5.23b). At pH 5 and pH 6, heart beat rates were in the control range (between 150 and 160 beats per minute) up to a concentration of 0.40 mg/L and then decreased markedly from 0.42 mg/L (pH 5: 15 beats per minute; pH 6: 97 beats per minute). At pH 8, the decrease only occurs at 0.44 mg/L and remains in a range of 100 to 120 beats per minute up to and including a concentration of 0.50 mg/L triclosan. Only at 0.60 mg/L does the heart rate drop to 66 beats per minute. At pH 9, this effect is only observed at 0.85 mg/L (70 beats per minute).

With regard to the hatching rate, the exposures at pH 5 differed significantly from those at the higher pH values with hatching rates of a maximum of 69 %. At pH 6 and 8, the incision was again between concentrations of 0.40 and 0.42 mg/L triclosan. While at 0.40 mg/L 75-100 % of the embryos still hatched, from 0.42 mg/L onwards no hatching at all was recorded. At pH 9, the hatching of embryos was completely absent at any concentration.

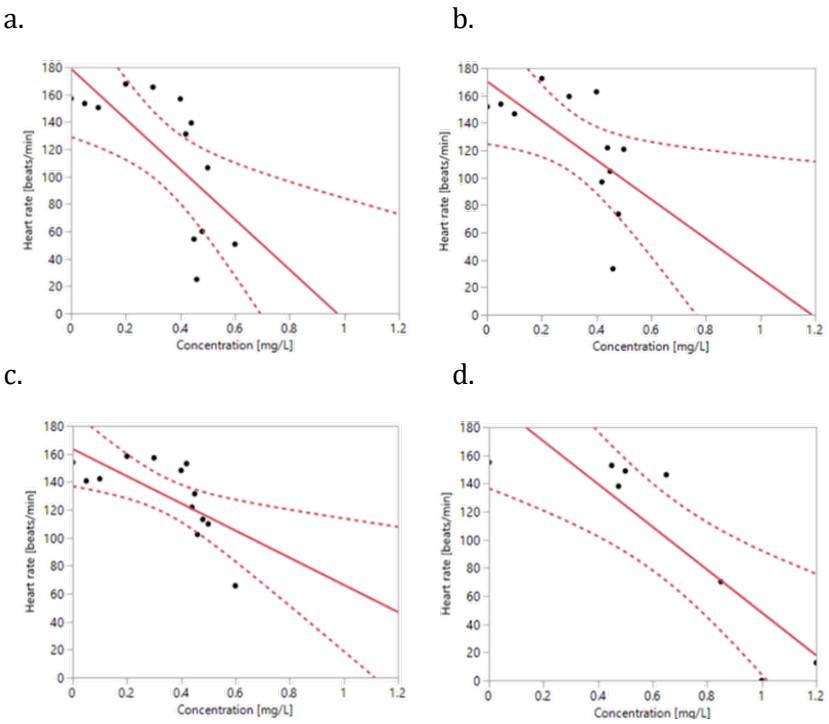
Overall, the decreasing toxicity with increasing pH was confirmed for triclosan at pH 9, with little variation in toxicity as predicted from the log D as a function of pH. However, the differences in toxicity were even smaller than expected.

Figure 5.23a: Regression curves of mortality upon exposure of embryos to triclosan



Regression curves of mortality upon exposure of embryos to triclosan showing LC_{50} values; a. after 72 hpf; b. after 96 hpf
Source: Own depiction

Figure 5.23b: Linear regression of heart rate as a function triclosan concentration



Linear regression of heart rate [beats/min] at time 48 hpf as a function of triclosan concentration [mg/L]; embedded small graphs with fitted y-axis; a. pH 5; b. pH 6; c. pH 8; d. pH 9.
Source: Own depiction

5.2.3 Other substances

5.2.3.1 Tricarboxylic acids

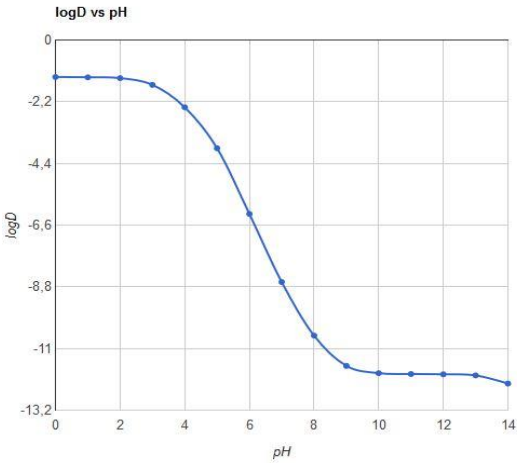
a. Citric acid

Citric acid was tested at pH 5, 6 and 8. The tests included two range findings as well as the main test. The log D curve for citric acid as a function of pH and the concentrations applied in each case, as well as the LC_{50} values obtained, are noted in Table 5.24. The associated regressions are shown in Figure 5.24a.

Table 5.24: Overview of the applied citric acid concentrations per pH and the corresponding LC_{50} values for 72 and 96 hpf as well as the log D curve for citric acid as a function of pH

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
5	0.5	-	-	6	5.0	4926.0	3250.0
	5.0				50.0		
	50.0				500.0		
	500.0				1000		
	750.0				2000		
	1000				3000		
	1500				3200		

pH	concentration [mg/L]	LC_{50} [mg/L]		pH	concentration [mg/L]	LC_{50} [mg/L]	
		72 hpf	96 hpf			72 hpf	96 hpf
	2500				3400		
	3000				3600		
	3500				3800		
	4000				4000		
	4500				4500		
	5000				5000		
8	50.0	4559.0	3102.0				
	500.0						
	2000						
	2500						
	3000						
	3250						
	3500						
	3750						
	4000						
	6000						
	8000						
	10000						
	12500						



Source: Own depiction

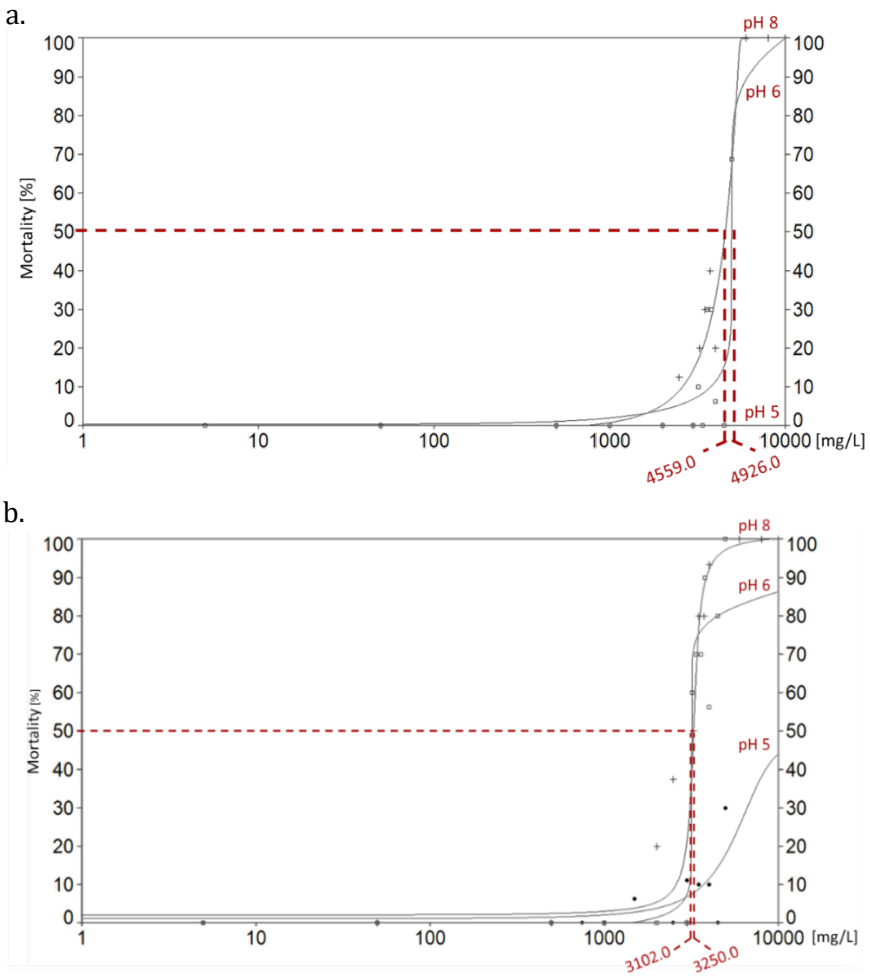
The results for citric acid behaved contrary to expectations. Ultimately, the most pronounced effects occurred at pH 8, i.e., in the more ionised range, with pH 6 and 8 differing only marginally, while there was little effect at pH 5. Therefore, LC_{50} values at time 96 hpf could only be determined for pH 6 (3250 mg/L) and pH 8 (3102 mg/L). The maximum induced mortality at pH 5 was 30% at 5000 mg/L citric acid. For comparison, at the same concentration and pH 6, mortality was 100 % and at pH 8, 93.33 % mortality was already achieved at 4000 mg/L citric acid (Figure 5.24b). At pH 6 and 8, a clear concentration-dependent increase in mortality could be seen. Even though the LC_{50} values of pH 6 and 8 were not far apart, a difference in hatching rates was evident (Figure 5.24c). While the hatching rates at pH 6 were comparable to the corresponding ones at pH 5 despite higher mortalities, the hatching success at pH 8 was much lower. That is, at pH 6 most individuals died only after hatching, while at pH 8 many embryos also coagulated. Heart beats were not counted.

The results, which are contrary to expectations, can be easily explained physiologically. As an important building block in the metabolism of many organisms, citric acid has its own transporters (e.g. Sun et al. 2010), i.e. its uptake into the cell is actively regulated. Thus, the dependence of the uptake of citric acid into the cells is largely independent of the degree of

ionisation. The fact that in this case the effects are significantly lower at pH 5 than at pH 6 and 8 could be due to the transporters, which may no longer function at too low a pH, i.e. active transport can no longer be guaranteed.

In conclusion, citric acid turned out to be an unsuitable substance for the pHION project due to its active transport mechanisms. However, the fact that citric acid has shown in an exemplary way that substances that enter the cells through active transport are not suitable for our modelling project can be seen as an important gain in knowledge and must be considered in the future selection of substances.

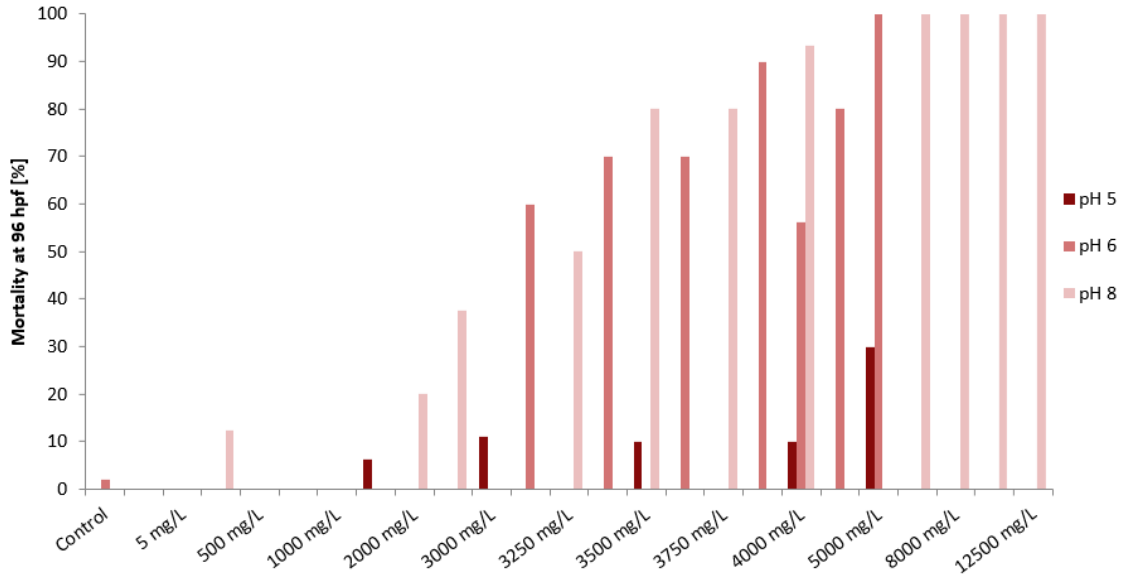
Figure 5.24a: Regression curves of mortality upon exposure of embryos to citric acid



Regression curves of mortality upon exposure of embryos to citric acid, indicating LC50 values; a. after 72 hpf; b. after 96 hpf.

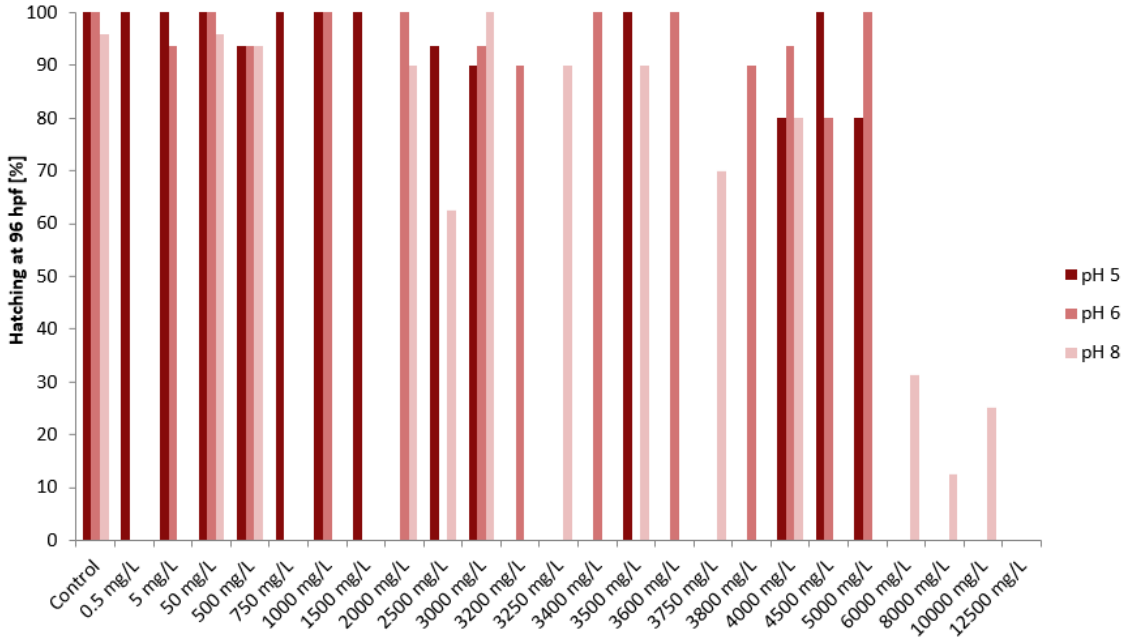
Source: Own depiction

Figure 5.24b: Mortality of embryos at time 96 hpf in percent as a function of citric acid concentration and pH



Source: Own depiction

Figure 5.24c: Hatching rate of embryos at time 96 hpf in percent as a function of citric acid concentration and pH



Source: Own depiction

5.3 Embedding toxicity results into log D prediction

To assess the relationship between log D and toxicity in a first step, the Δ LC50 (mortality) at 72 and 96 hpf (for an overview of log D values and determined LC_{50} levels see Appendix, Table A1 and Table A2) were calculated for all possible binary combinations of pH for a given substance according to *equations 1* and compared to the respective delogarithmised Δ D to determine the deviation from observed (mortality) to predicted (log D) toxicity shift ['shift ratios' Δ $TS_{D/LC}$ from

one pH to another] as depicted in *equation 2*. For $\Delta TS_{D/LC}$, the ideal outcome is 1, indicating no deviation from observed to log D predicted toxicity shifts.

$$\Delta LC_{50} = LC_{50\ (higher\ pH)} - LC_{50\ (lower\ pH)} \quad (1)$$

$$\Delta TS_{D/LC} = \Delta LC_{50} / \Delta D\ [for\ \Delta LC_{50} > \Delta D] \text{ and } TS_{D/LC} = \Delta D / \Delta LC_{50}\ [for\ \Delta D > \Delta LC_{50}] \quad (2)$$

Results showed that the log D model worked for most of the substances (Table 5.25). Leaving aside low toxicity coupled with solubility problems, the model has only failed for enclomiphene and citric acid. Enclomiphene as estrogen receptor modulator is a very specific hormone active substance which might target receptors that may not be expressed at such an early developmental stage, thus might not be very much effective in zebrafish embryos, yet. Cell transport mechanisms for citric acid, on the other hand, are well known and the failure to predict pH-dependent toxicity by log D is simply due to active transport of citric acid in and out of the cell. Thus, citric acid concentrations are kept rather constant within the cell independent of pH. Results for citric acid emphasised the fact that the applicability of the log D model is restricted to substances that diffuse through cell membranes along a gradient and in which the charge of the species is key to their transmission through biomembranes.

For all other substances $\Delta TS_{D/LC}$ was mainly between 1 and 4, at least for the neighbouring Δ pH (marked in bold in Table 5.25), which is very close to the prediction. The highest deviation from predicted to observed toxicity shift was seen in ibuprofen pH 6 vs. 8, other than that, the deviation was below a factor of 10. Particularly when considering biological variability, the reliability of results is to be rated highly making log D a convincing parameter for modelling pH-dependent toxicity.

Table 5.25: Factorial predicted and observed toxicity shifts

					Δ LC_{50}		Δ $TS_{DS/LC}$	
			Δ pH	**Δ D**	**72 hpf**	**96 hpf**	**72 hpf**	**96 hpf**
PHARMA-CEUTICALS	**NSAIDs**	**ASA**	5 vs. 6	8.91	8.05	4.95	1.11	1.80
			5 vs. 8	79.43	125.25	150.40	1.58	1.89
			6 vs. 8	8.91	15.56	30.37	1.75	3.41
		Diclofenac	5 vs. 6	8.91	5.48	9.61	1.63	1.08
			5 vs. 8	229.09	266.99	292.63	1.17	1.28
			5 vs. 9	295.12	801.72	1104.52	3.74	3.74
			6 vs. 8	25.70	48.69	30.44	1.89	1.18
			6 vs. 9	33.11	146.20	114.91	4.42	3.47
			8 vs. 9	1.29	3.00	3.77	2.33	2.93
		Ibuprofen	5 vs. 6	6.17	4.35	4.88	1.42	1.26
			5 vs. 8	407.38	18.01	21.48	22.62	18.96

					Δ LC_{50}		Δ $TS_{DS/LC}$	
			5 vs. 9	1122.02	-	40.44	-	27.75
			6 vs. 8	66.07	4.14	4.40	15.97	15.01
			6 vs. 9	181.97	-	8.29	-	21.96
			8 vs. 9	2.75	-	1.88	-	1.46
		Naproxen	6 vs. 8	34.67	38.29	39.55	1.10	1.14
			6 vs. 9	50.12	170.34	152.49	3.40	3.04
			8 vs. 9	1.45	4.45	3.86	3.08	2.67
	Anaesthetics	**Lidocaine**	5 vs. 6	8.51	-	-	-	-
			5 vs. 8	309.03	-	50.92	-	6.07
			5 vs. 9	457.09	-	127.30	-	3.59
			6 vs. 8	36.31	-	-	-	-
			6 vs. 9	53.70	-	-	-	-
			8 vs. 9	1.48	1.66	2.50	1.12	1.69
		Tetracaine	5 vs. 6	13.49	4.39	2.87	3.07	4.70
			5 vs. 8	891.25	129.08	43.20	6.90	20.63
			5 vs. 9	2570.40	68.25	43.44	37.66	59.17
			6 vs. 8	66.07	29.39	15.04	2.25	4.39
			6 vs. 9	190.55	15.54	15.12	12.26	12.60
			8 vs. 9	2.88	1.89	1.01	1.52	2.87
	Opioids	**Tramadol**	6 vs. 8	61.66	55.19	67.21	1.12	1.09
			6 vs. 9	407.38	199.716	168.64	2.04	2.42
			8 vs. 9	6.61	3.62	2.51	1.83	2.63
	Beta blockers	**Metoprolol**	5 vs. 6	1.35	-	2.12	-	1.57
			5 vs. 8	36.31	-	87.13	-	2.40
			5 vs. 9	302.00	-	595.11	-	1.97
			6 vs. 8	26.92	-	41.16	-	1.53
			6 vs. 9	223.87	144.82	281.12	1.55	1.26
			8 vs. 9	8.32	-	6.83	-	1.22
			5 vs. 6	1.32	3.15	3.66	2.39	2.78

					Δ LC_{50}		Δ $TS_{DS/LC}$	
			5 vs. 8	36.31	110.42	176.53	3.04	4.86
			5 vs. 9	295.12	392.13	2383.61	1.33	8.08
			6 vs. 8	27.54	35.10	48.18	1.27	1.75
			6 vs. 9	223.87	124.65	650.58	1.80	2.91
			8 vs. 9	8.13	3.55	13.50	2.29	1.66
	CLA	**Clofibric acid**	5 vs. 9	79.43	-	6.72	-	11.83
	Anti-depressants	**Amitriptyline**	5 vs. 6	1.48	3.18	2.75	2.15	1.86
			5 vs. 8	52.48	97.33	116.86	1.85	2.23
			5 vs. 9	446.68	185.19	153.18	2.41	2.92
			6 vs. 8	35.48	30.56	42.52	1.16	1.20
			6 vs. 9	302.00	58.16	55.74	5.19	5.42
			8 vs. 9	8.51	1.90	1.31	4.47	6.49
		Citalopram	5 vs. 6	1.45	2.89	3.03	2.00	2.10
			5 vs. 8	50.12	35.14	60.71	1.43	1.21
			5 vs. 9	426.58	-	207.26	-	2.06
			6 vs. 8	34.67	12.17	20.01	2.85	1.73
			6 vs. 9	295.12	-	68.31	-	4.32
			8 vs. 9	8.51	-	3.41	-	2.49
		Fluoxetine	5 vs. 6	1.26	4.74	3.94	3.76	3.13
			5 vs. 8	27.54	29.29	104.26	1.06	3.79
			5 vs. 9	234.42	244.35	689.42	1.04	2.94
			6 vs. 8	21.88	6.18	26.45	3.54	1.21
			6 vs. 9	186.21	51.57	174.92	3.61	1.06
			8 vs. 9	8.51	8.34	6.61	1.02	1.29
		Sertraline	6 vs. 8	31.62	11.74	8.84	2.69	3.58
			6 vs. 9	251.19	23.84	107.65	10.53	2.33
			8 vs. 9	7.94	2.03	12.18	3.91	1.53
PESTICIDES	**Chlorophenols**	**2,3-DCP**	5 vs. 6	1.05	1.11	1.03	1.06	1.02
			5 vs. 8	5.25	1.75	1.25	2.99	4.21

					Δ LC_{50}		Δ $TS_{DS/LC}$	
			6 vs. 8	5.01	1.96	1.29	2.56	3.90
		2,4,5-TCP	5 vs. 6	1.15	1.43	1.40	1.25	1.22
			5 vs. 8	14.13	2.47	2.40	5.72	5.90
			6 vs. 8	12.30	1.72	1.72	7.14	7.17
		PCP	5 vs. 6	5.13	1.10	1.18	4.68	4.33
			5 vs. 8	50.12	3.72	3.97	13.46	12.61
			6 vs. 8	9.77	4.08	4.70	2.39	2.08
	Herbicides	**Bromoxynil**	5 vs. 6	4.68	2.45	2.23	1.91	2.10
			5 vs. 8	58.88	143.55	125.72	2.44	2.14
			6 vs. 8	12.59	58.53	56.44	4.65	4.48
OTHER	**Tricarboxylic acids**	**Citric acid**	5 vs. 6	218.78	-	1.75	-	124.90
			5 vs. 8	4786300.92	-	1.84	-	2608141.35
			6 vs. 8	21877.62	1.08	1.05	20247.68	20881.34

Factorial predicted (Δ D) and observed (Δ LC50 [mortality at 72 and 96 hpf]) toxicity shifts, as well as the deviation of observed from predicted shift ratio: Δ TSD/LC [mortality]; values for Δ TSD/LC given in green/red indicate an overestimation (green) or underestimation (red) of toxicity by log D; table excludes ketotifen as it was only conducted at pH 9 and all substances with less than two LC50 values as no Δ TSD/LC can be calculated for any Δ pH; ASA – acetylsalicylic acid; CLA – cholesterol-lowering agent.
Source: Own depiction

5.4 Heart rate as early warning parameter for mortality

In terms of mortality, log D proved to be an excellent predictor, but for predicting toxicity in combination with following the principle of the '3 Rs' (refine, reduce, replace) according to Russell and Burch (1959), which underlie the 'European Directive on the protection of animals used for scientific purposes' (Directive 2010/63/EU), it is desirable to establish a sublethal alternative to the parameter 'mortality' at 96 hpf. To achieve that goal, the early warning potential of the parameter 'heart rate' at 48 hpf was evaluated for most of the substances but for ten exemplary compounds (the acids diclofenac, ibuprofen, naproxen, triclosan and the bases citalopram, fluoxetine, propranolol, metoprolol, tetracaine and tramadol) specifically.

To assess the early warning potential, heart rate EC_{20}s were determined and Δ EC_{20}s calculated similarly to the Δ LC_{50} calculation (equations 3 and 4). Finally, it was calculated to which extent [E] mortality and heart rate differed from each other (equation 5) to determine the fit of heart rate as early warning parameter. For Δ $TS_{D/LC}$, Δ $TS_{D/EC}$ and E, the ideal outcome is 1, indicating no deviation from observed to log D predicted toxicity shifts and no difference between the mortality and heart rate toxicity parameter.

$$\Delta EC_{20} = EC_{20\,(\text{higher pH})} - \log EC_{20\,(\text{lower pH})} \quad (3)$$

$$\Delta TS_{D/EC} = \Delta EC_{20} / \Delta D \; [for \; \Delta EC_{20} > \Delta D] \text{ and } TS_{D/EC} = \Delta D / \Delta EC_{20} \; [for \; \Delta D > \Delta EC_{20}] \quad (4)$$

$$E = \Delta TS_{D/LC} / \Delta TS_{D/EC} \; [for \; \Delta TS_{D/LC} > \Delta TS_{D/EC}] \text{ and } E = \Delta TS_{D/EC} / \Delta TS_{D/LC} \; [for \; \Delta TS_{D/EC} > \Delta TS_{D/LC}] \quad (5)$$

The results show that heart rate at 48 hpf (EC_{20}) can equally predicted by log D (for a contrasting juxtaposition of mortality and heart rate curves see Appendix, Figure A1) and that in some cases, heart rate is even a better predictor than mortality, at concerning the ten compounds investigated (Table 5.26). Generally, the difference between mortality and heart rate performed quite similar in relation to their predictability by log D with only two main exceptions: diclofenac pH 6 vs. 8 and citalopram pH 5 vs. 6. In both incidences, the heart rate was off while mortality reflected the log D prediction well. In average, mortality was slightly closer to the log D prediction than heart rate but the difference was minimal.

Table 5.26: Factorial predicted and observed toxicity shifts, $\Delta TS_{D/EC}$ and the extent to which the toxicity parameters deviate from each other

		Δ D	Δ LC_{50}	Δ EC_{20}	Δ $TS_{D/LC}$	Δ $TS_{D/EC}$	E	better predictor
Diclofenac	**pH 5 vs. 6**	8.91	9.61	6.68	1.08	1.34	1.24	mortality
	pH 6 vs. 8	25.70	30.44	189.85	1.18	7.39	6.24	mortality
	pH 8 vs. 9	1.29	3.77	1.02	2.93	1.26	2.32	heart rate
Ibuprofen	**pH 5 vs. 6**	6.17	4.88	5.93	1.26	1.04	1.22	heart rate
	pH 6 vs. 8	66.07	4.40	2.94	15.01	22.48	1.50	mortality
	pH 8 vs. 9	2.75	1.88	3.24	1.46	1.17	1.25	heart rate
Naproxen	**pH 6 vs. 8**	34.67	39.55	47.93	1.14	1.38	1.21	mortality
	pH 8 vs. 9	1.45	3.86	3.78	2.67	2.61	1.02	heart rate
Triclosan	**pH 5 vs. 6**	1.02	1.27	1.05	1.24	1.03	1.20	heart rate
	pH 6 vs. 8	2.95	1.06	1.10	2.78	2.68	1.04	heart rate
	pH 8 vs. 9	6.31	1.59	1.39	3.96	4.53	1.15	mortality
Citalopram	**pH 5 vs. 6**	1.45	3.03	16.95	2.10	11.72	5.59	mortality
	pH 6 vs. 8	34.67	20.01	13.61	1.73	2.55	1.47	mortality
	pH 8 vs. 9	8.51	3.41	4.81	2.49	1.77	1.41	heart rate
Fluoxetine	**pH 5 vs. 6**	1.26	3.94	3.60	3.13	2.86	1.10	heart rate
	pH 6 vs. 8	21.88	26.45	14.03	1.21	1.56	1.29	mortality
	pH 8 vs. 9	8.51	6.61	4.46	1.29	1.91	1.48	mortality
Propranolol	**pH 5 vs. 6**	1.32	3.66	1.78	2.78	1.35	2.06	heart rate
	pH 6 vs. 8	27.54	48.18	54.42	1.75	1.98	1.13	mortality

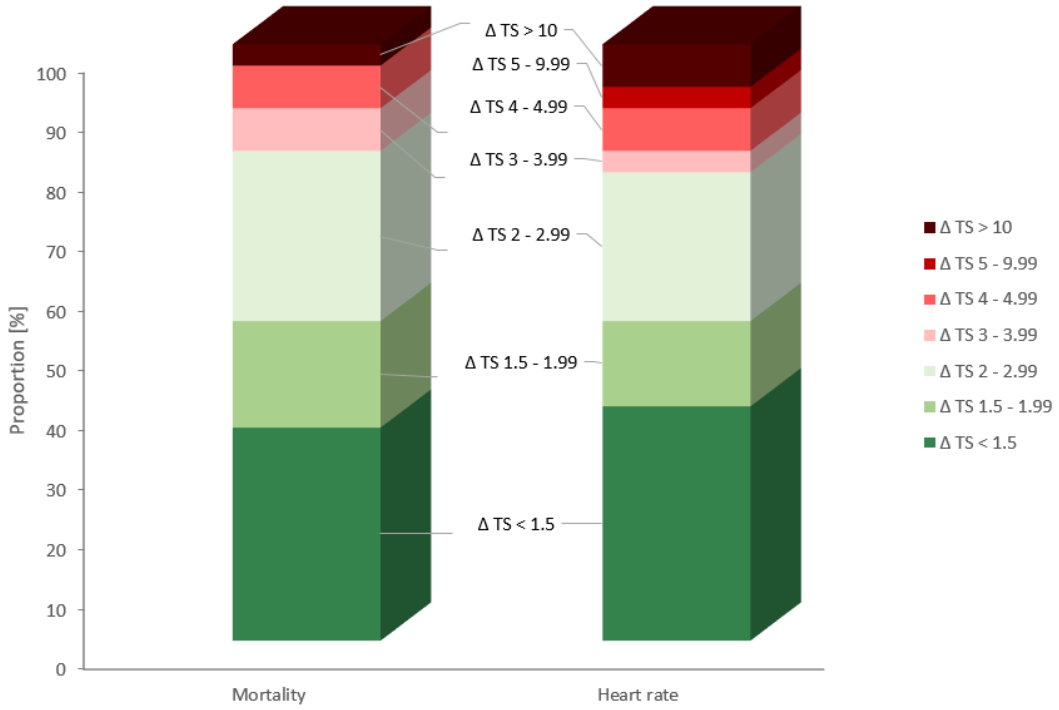
		Δ D	Δ LC_{50}	Δ EC_{20}	Δ $TS_{D/LC}$	Δ $TS_{D/EC}$	E	better predictor
	pH 8 vs. 9	8.13	13.50	3.32	1.66	2.44	1.47	mortality
Metoprolol	**pH 5 vs. 6**	1.35	2.12	4.24	1.57	3.14	2.00	mortality
	pH 6 vs. 8	26.92	41.16	36.61	1.53	1.36	1.12	heart rate
	pH 8 vs. 9	8.32	6.83	3.11	1.22	2.67	2.19	mortality
Tetracaine	**pH 5 vs. 6**	13.49	2.87	3.00	4.70	4.50	1.04	heart rate
	pH 6 vs. 8	66.07	15.04	24.01	4.39	2.75	1.60	heart rate
	pH 8 vs. 9	2.88	1.01	2.90	2.87	1.01	2.84	heart rate
Tramadol	**pH 6 vs. 8**	61.66	67.21	76.96	1.09	1.25	1.15	mortality
	pH 8 vs. 9	6.61	2.51	5.04	2.63	1.31	2.01	heart rate
Mean					2.60	3.59	1.80	

Factorial predicted (Δ D) and observed (Δ LC50 [mortality at 96 hpf], Δ EC20 [heart rate at 48 hpf]) toxicity shifts, as well as the deviation of observed from predicted shift ratio: Δ TSD/LC [mortality], Δ TSD/EC [heart rate] and the extent (E) to which the toxicity parameters 'mortality' and 'heart rate' deviate from each other; values for Δ TSD/LC and Δ TSD/EC given in green/red indicate an overestimation (green) or underestimation (red) of toxicity by log D.
Source: Own depiction

To picture, how nicely mortality and heart rate reflected the log D prediction, on the one hand, and to assess the differences between both parameters statistically, on the other hand, each Δ $TS_{D/LC}$/Δ $TS_{D/EC}$ value was assigned to one of seven categories [C1-C7] ranging from Δ TS < 1.5 to Δ TS > 10 (C1: Δ TS < 1.5; C2: Δ TS = 1.5-1.99; C3: Δ TS = 2-2.99; C4: Δ TS = 3-3.99; C5: Δ TS = 4-4.99; C6: Δ TS = 5-9.99; C7: > 10). Then the respective proportion of each category is calculated and given as percentage (Figure 5.25). Finally, the percentage distribution of the categories per endpoint was used to statistically assess the potential difference between the two toxicity parameters, mortality and heart rate, via likelihood ratio $\chi 2$ test using *JMP® 15.1.0 (SAS Institute Inc.)*.

In more than 50 % of the cases, in both mortality and heart rate, Δ TS was below factor 2 and in about 80 % of the cases still below factor 3, meaning that usually the observed toxicity deviated from the log D prediction less than threefold. Besides, the statistical analysis confirmed that there was no significant difference in Δ TS between heart rate and mortality *(likelihood ratio $\chi 2$, $df = 6$, $n = 56$, $\chi 2 = 2.292$, $p = 0.810$)*.

Figure 5.25: Comparison between the toxicity parameters mortality and heart rate

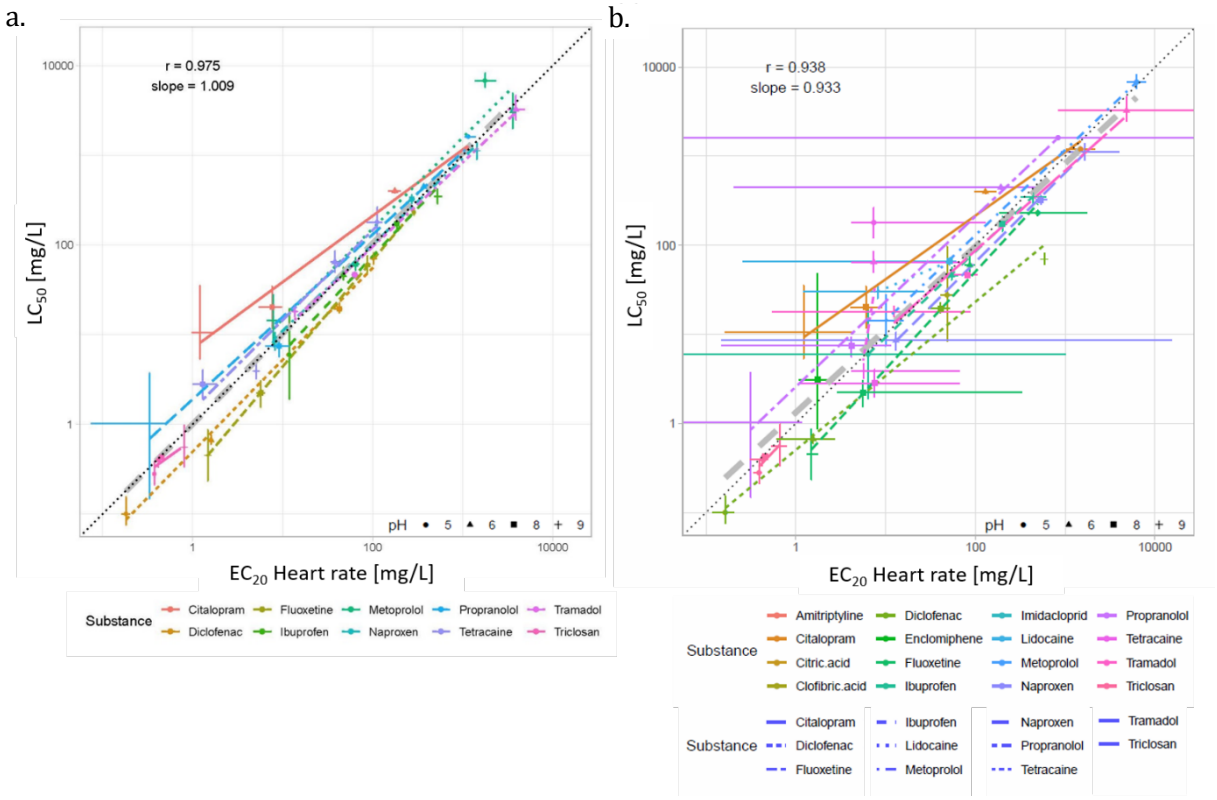


Comparison between the toxicity parameters mortality at 96 hpf and heart rate at 48 hpf concerning their factorial deviation from predicted (log D) toxicity shift expressed as proportion of Δ TS ranging from marginal deviations below factor 1.5 to considerable deviations greater factor 10. Results for both parameters did not differ significantly from each other (likelihood ratio $\chi 2$, df = 6, n = 56, $\chi 2$ = 2.292, p = 0.810).
Source: Own depiction

Additional modelling confirmed the finding for the ten specific compounds (Figure 5.26a), as well as for all substances tested (Figure 5.26b) and proved a very consistent relationship between mortality and heart rate.

Thus, since heart rate at 48 hpf has proven to be a good predictor for mortality occurring until 96 hpf, it may serve as an early warning parameter in the future, implementing the principle of the '3 Rs' and expand established test protocols by making them more realistic, quicker and more ethically justifiable.

Figure 5.26: Modelled relationship between mortality and heart rate



Modelled relationship between mortality at 96 hpf (LC_{50}) and heart rate at 48 hpf (EC_{20}) for a. the ten specific substances and b. for all substances.
Source: Own depiction

5.5 FETs carried out by UBA with *Danio rerio* for 12 other substances

FETs were not conducted due to work and time limitations.

6 WP 5 – Part B: Results for the chemical analyses

6.1 General remarks about the FET LC_{50} exposure

6.1.1 Analysed substances

Several substances included in the FET trials were not separately exposed to their LC_{50} concentrations, because they were cancelled in the process. Cancellations were mainly due to low toxicity while already reaching the solubility limits. Compounds included in the FET but excluded from the chemical analyses were the cholesterol-lowering agent clofibric acid, the herbicide glyphosate, the insecticide imidacloprid and citric acid.

6.1.2 Sample quantities and concentrations

In order to be able to provide the required sample quantities for chemical analysis, embryos were exposed in a separate trial. For each pH, 120 embryos were exposed ideally resulting in a sample size of 60 embryos that survived until 96 hpf.

The concentrations used for the exposure were based on the LC_{50} values determined in the fish embryo test. In some cases, the concentrations used were slightly below the calculated LC_{50}. Due to the biological variability, induced mortality at the LC_{50} was occasionally > 50 % leading to an insufficient amount of sample, thus a lower concentration was used subsequently (Table 6.1).

6.1.3 Sampling of dead embryos

In the beginning, the idea was to determine a threshold concentration, at which the embryos detoxification system is overwhelmed, and they die; a concept that is defined as “lethal body burden” [LBB]. For most of the substances, dead embryos were collected additionally. The samples of dead embryos obtained for the first four substances were pooled to increase the mass per sample. As it was considered not optimal to mix embryos from different time points and thus from different developmental stages, embryos were frozen separately for each time point. That led to single or very few individuals per Eppendorf vessel (Eppi) commonly, which in turn was disadvantageous for the chemical analysis due to too small quantities.

Eventually, after obtaining the results for the first several substances, which were not convincing, it was refrained from that idea entirely.

6.1.4 Fresh weight of the samples

Since the Eppendorf vials were not weighed for the first trials, including the compounds diclofenac, ibuprofen, propranolol, enclomiphene, citalopram and triclosan, sample weight had to be extrapolated. For the extrapolation, the fresh weight was determined exemplarily for the sertraline samples by weighing the empty and filled vial. The average weight of an embryo was calculated from the fresh weights and the corresponding number of embryos, which was 0.242 ± 0.059 mg. Based on this average weight, the fresh weight of the previous samples was extrapolated according to the number of individuals per vial. For all further samples of substances subsequently tested to sertraline, the empty and fill weight of the individual vials was determined and the fresh weight was calculated according to these data (equation 6.1 below).

$$Weight\ of\ ZFE\ sample = Weight_{\ Filled\ Eppendorf} - Weight_{\ Empty\ Eppendorf} \quad [6.1]$$

6.1.5 Washing step

As the method of sampling the embryos was not yet technically perfected in the beginning, the samples of the first four tested substances (diclofenac, triclosan, citalopram and propranolol) were not washed prior to freezing. Thus, these samples needed a washing step after thawing and before proceeding with the actual chemical analysis itself. It was assumed that the washing step after thawing is not ideal and may adversely affect the result, therefore, affected samples were repeated to assure a reliable outcome.

For some of these substances, additional pH levels were tested afterwards, so that the sample preparation already included washing before freezing, thus, it was not necessary to repeat every pH level for all four substances. The results of the aforementioned "wash" samples are not reported in this document.

6.2 Material and methods of FET chemical analyses

6.2.1 Chemical and Reagents

All the analytical standards used were of high-purity grade. Many of them were purchased from Sigma-Aldrich (Steinheim, Germany). The standards Sertraline HCl, Fluoxetine, Ketotifen, Metoprolol, Naproxen and Tramadol were purchased from Analytical Standard Solutions (A2S) (France). Moreover, the standards Acetylsalicylic acid, Lidocaine and Pentachlorophenol were purchased from HPC Standards GmbH (Borsdorf, Germany). The standard Tamoxifen which was used as IS, was purchased from Toronto Research Chemicals (TRC)(Canada). The isotope-labelled internal standards (IS), Triclosan-D3, Citalopram-D4, Propranolol-D7, Sertraline-D3, Tetracaine-D6, Ibuprofen-D3, Lidocaine-D10, Metoprolol-D7 and Fluoxetine-D5 were purchased from Analytical Standard Solutions (A2S) (France). Furthermore, the internal standards Diclofenac-D4, Naproxen-D3 and Acetylsalicylic acid-D4 were purchased from HPC Standards GmbH (Borsdorf, Germany). The IS, Amitriptyline-D3 and was purchased from Cerilliant (Round Rock, TX) and Tramadol-D6 was purchased from Toronto Research Chemicals (TRC)(Canada). Stock standard solutions of individual compounds (1000 µg mL^{-1}) were prepared in MeOH and stored at −20 °C in amber glass bottles to prevent photo-degradation.

Regarding the materials used for the sample preparation, bulk beads, 1.4 mm (zirconium oxide) purchased from Bertin Technologies, (France) and regenerated cellulose syringe RC filters (pore size 0.2 µm, diameter 15mm) purchased from Phenomenex (Torrance, CA, USA) were used.

All the solvents used for the sample preparation as well as the LC-QTOF-MS analysis were UPLC-MS grade. Methanol (MeOH) was purchased from Merck (Darmstadt, Germany) and distilled water (H_2O) was provided by a Milli-Q purification apparatus (Direct-Q UV; Millipore, Bedford, MA, USA). ACN was supplied from Merck (Darmstadt, Germany). The additives of the mobile phases, ammonium formate (≥ 99.0%), ammonium acetate (99%), and formic acid (99%) were all purchased from Fluka (Buchs, Switzerland).

6.2.2 Sample preparation

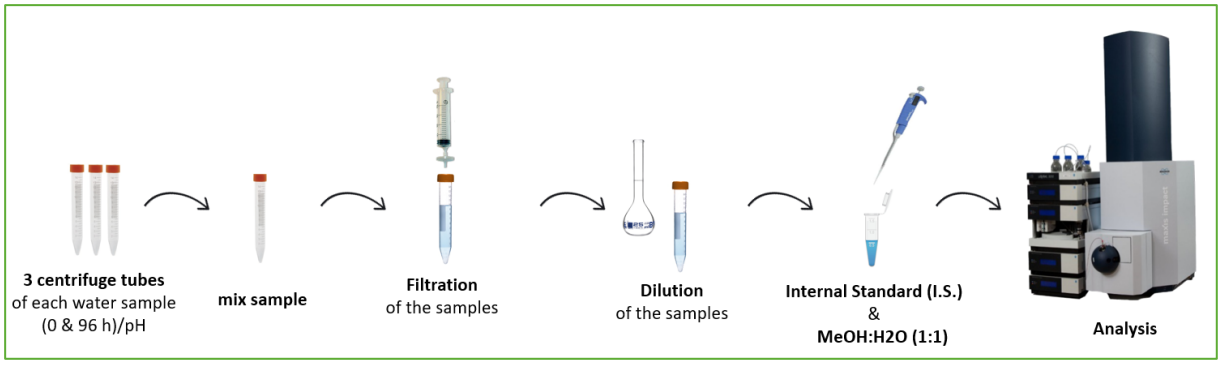
6.2.2.1 Water samples from exposure experiments

For each exposure water sample at each pH value and state (i.e., t=0 or 96h), three separate centrifuge tubes were available. Hence from each tube of the same sample (pH and state), equal parts of water sample were mixed, filtered, and then transferred into a new centrifuge tube. These "mix samples" we used for further analysis. The Figure 6.1 below illustrates the steps of the applied sample preparation procedure for the water samples.

Regarding the water sample preparation, preliminary test analyses were performed, based on the theoretical concentrations ($C_{theoretical}$ of each sample) of each respective sample. The aim was to include the final concentration of the diluted water mix samples in the linear dynamic range of the instrument.

After completion of the above-mentioned preliminary tests, the diluted samples were analysed and the "optimal dilution level", for each substance and pH value, was determined experimentally. Afterwards, the water mix samples were diluted according to the "optimal dilution level" of each substance, to proceed with the quantification experiment. The diluted samples were transferred into glass vials and stable isotope-labelled internal standards were added. The IS were used to account for potential insufficiencies of the whole analytical procedure (sample preparation and instrumental analysis). The sample preparation procedure was repeated six times for each water sample of the exposure experiments to Sertraline, Amitriptyline, Tetracaine and Ibuprofen prior to LC-ESI-QTOF analysis, to increase the reliability and accuracy of the results. The six replicates were measured for each sample and the potential outliers were assessed through Q-test.

Figure 6.4: Sample preparation of the water samples from exposure experiments



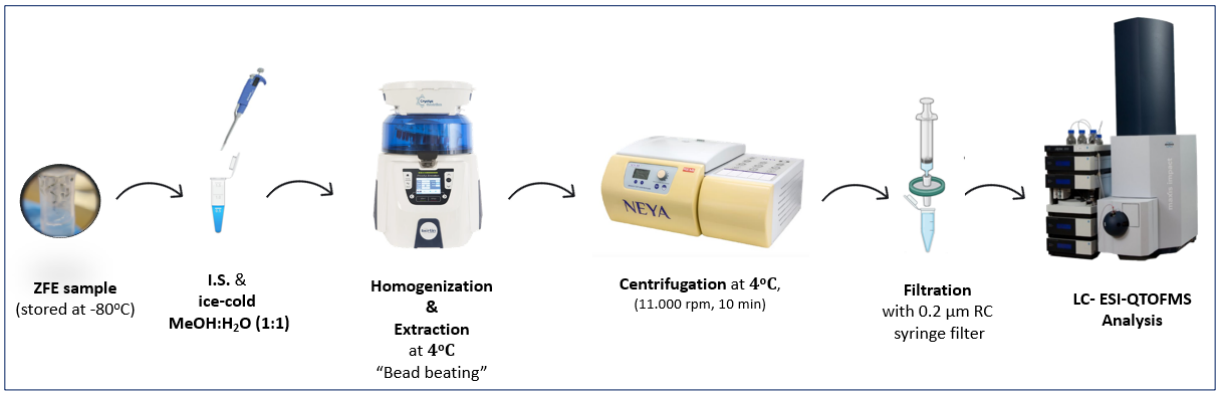
Source: Own depiction

6.2.2.2 ZFE samples from exposure experiments

The ZFE samples were delivered in Eppendorf tubes and stored at -80°C until sample preparation. Sample preparation was applied to the zebrafish embryo samples and LC-ESI-QTOF-MS was employed for the analysis. The extraction procedure was based on previously reported methods (Brox et al. 2014; Aceña et al. 2017; Damalas et al. 2018) and was slightly adjusted for this study.

Concerning the steps of the sample preparation, the ZFE samples were spiked with isotope-labelled internal standards (prepared in ice-cold methanol) of each analyte, to account for potential insufficiencies during the sample preparation. Samples were then pooled with 1.4 mm bulk beads (zirconium oxide) and subsequently, ice-cold mixture of the extraction solvents MeOH: H_2O (1:1 v/v) was added. The final volume of the extraction solvent was 500 µL. Homogenization and extraction of the samples were performed on a "bead beating" Precellys Evolution 24 homogenizer equipped with a Cryolis Evolution cooler. The homogenizer operated at 8200 rpm for three cycles of 15 s each, with a 60s break between each cycle. The homogenized samples were centrifugated in a precooled (4 °C) centrifuge NEYA 16R (Remi Neya Centrifuges, Italy) for 10 minutes at 4 °°C and 11.000 rpm. The supernatants were collected and then transferred to Eppendorf tubes. Finally, the extracts were filtered through a 0.2 µm RC syringe filter and then transferred to glass vials for immediate analysis. The sample preparation of the ZFE samples is presented in Figure 6.2 below:

Figure 6.2: Sample preparation of zebrafish embryos from exposure experiments



Source: Own depiction

Initial analyses of the ZFE extracts (1 ZFE sample-extract/pH per substance) were performed, to investigate if their concentration levels were included in the linear dynamic range of the instrument. The objective was to ensure quantification accuracy. The extracts that were out of the linear dynamic range were diluted. The diluted ZFE extracts were analysed as well, using LC-HRMS.

As mentioned above, a washing step was added in the sample treatment protocol before proceeding with the chemical analysis, for the first four tested substances (diclofenac, triclosan, citalopram and propranolol). This step was performed to ensure that the potential remaining water from the exposure experiments has been removed from the Eppendorf tubes. Initially, the potentially remaining droplets of the exposure solution were removed from the Eppendorf tubes using a pipette. Afterwards, the embryos were washed with 500 µL refrigerated ultrapure water prior to their extraction. The water was carefully collected and transferred to Eppendorf tubes, constituting the "wash" sample. Finally, the "wash" samples were filtered through a 0.2 µm RC syringe filter and they were transferred to glass vials for LC-HRMS analysis. However, the exposure experiments of these samples were repeated (including the washing before freezing), to assure a reliable outcome. The sample preparation was performed without the washing step, as described above for the rest of the substances (Figure 6.2).

6.2.3 Instrumental analysis

LC-ESI-QTOF Analysis

The analysis of the exposure water samples and ZFE extracts was carried out using an LC-ESI-QTOF system. Ultrahigh-performance liquid chromatography (UHPLC) system with an HPG-

3400 pump (Dionex Ultimate 3000 RSLC, Thermo Fischer Scientific, Dreieich, Germany) coupled to a QTOF mass spectrometer (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used. The samples were analyzed with reversed-phase liquid chromatography (RPLC) in both positive and negative ionization modes. Chromatographic separation was performed using an Acclaim RSLC C18 column (2.1 x 100 mm, 2.2 µm) from Thermo Fischer Scientific (Dreieich, Germany) preceded by a guard column of the same packaging material, thermostated at 30 oC. For positive ionization mode, the mobile phases were water/methanol, 90/10 (aqueous solvent) and methanol (organic solvent), both amended with 5mM ammonium formate and 0.01% formic acid. For negative ionization mode, the mobile phases consisted of water/methanol, 90/10 (aqueous solvent) and methanol (organic solvent), both acidified with 5mM ammonium acetate. The gradient elution program was the same for both ionization modes, starting with 1% B with a flow rate of 0.2 mL min^{-1} for 1 min and it increases to 39 % in 2 min (flow rate 0.2 mL min^{-1}), and then to 99.9 % (flow rate 0.4 mL min^{-1}) in the following 11 min. Then it keeps constant for 2 min (flow rate 0.48 mL min^{-1}), then initial conditions were restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.2 mL min^{-1}. The injection volume was set up to 5 µL. The gradient elution program was the same for both ionization modes and is presented in Table 6.2 below.

Table 6.2: Gradient elution program of reversed-phase liquid chromatography (RPLC)

Time (min)	Flow rate (mL min^{-1})	Aqueous solvent %	Organic solvent %
0	0.2	99.0	1.0
1	0.2	99.0	1.0
3	0.2	61.0	39.0
14	0.4	0.1	99.9
16	0.48	0.1	99.9
16.1	0.48	99.0	1.0
19.1	0.2	99.0	1.0
20.0	0.2	99.0	1.0

Source: Own depiction

The QTOF system was equipped with an electrospray ionization interface (ESI), operating in positive and negative modes. The operation parameters of ESI were the following: capillary voltage, 2500 V for positive and 3000 V for negative mode; end plate offset, 500 V; nebulizer pressure, 2 bar (N2); drying gas, 8 L min^{-1} (N2); and drying temperature, 200 °C. The QTOF MS system was operated in data-independent (broadband collision-induced dissociation (bbCID)) acquisition, as well as in data-dependent (Auto MS/MS) acquisition mode and records spectra over the range m/z 30–1000, with a scan rate of 2 Hz. A QTOFMS external calibration was performed daily with a sodium formate solution, and a segment (0.1–0.25 min) in every chromatogram was used for internal calibration, using a calibrant injection at the beginning of each run. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of water/isopropanol (1:1). The instrument provided a typical resolving power (FWHM) between 36,000-40,000 during calibration (m/z 226.1593, 430.9137 and 702.8636). Data processing was implemented with *Data Analysis* 5.1 and *TASQ* 2.1 software (Bruker Daltonics, Bremen, Germany).

LC-MS/MS Analysis

For the analysis of the water and ZFE samples from the exposure experiment to 2,3-Dichlorophenol (DCP) as well as for the analysis of the ZFE extracts from the exposure experiment to Diclofenac at pH 5 and pH 6, an LC-MS/MS system was used. The LC-MS/MS system was comprised of a Thermo Scientific (San Jose, CA, USA) liquid chromatographic system consisting of a degasser, a UHPLC Accella pump, incorporating a column thermostat and an autosampler, interfaced to a Thermo Scientific (TSQ) Quantum Access mass analyzer. Chromatographic separation was performed using a Waters Atlantis T3 (100 mm × 2.1 mm, 3 µm) chromatographic column, preceded by a pre-column of the same packaging material. The column was thermostated at 25 °C, and the full loop injection volume of the extract was set at 10 µL. Chromatographic separation was carried out using a gradient elution program. The mobile phase consisted of water containing 1 mM ammonium formate (solvent A), methanol (solvent B), and acetonitrile (solvent C), with a flow rate of 100 µL/min. The gradient elution started with 25% (v/v) MeOH and increased linearly to 95% MeOH in 10 min which was held for 9.0 min (until 19.0 min), reverted to 25% MeOH and re-equilibrated for 5.0 min (from 20.0 to 25.0 min) at 20% MeOH (total run time of 25 min) at 5% ACN for a total run time of 25.0 min. The gradient elution program is presented in Table 6.3 below.

Table 6.3: Gradient elution program

Time (min)	Flow rate (mL min^{-1})	Aqueous solvent (A) %	Organic solvent (B) %	Organic solvent (C) %
0	0.1	70.0	25.0	5
10.0	0.1	0.0	95.0	5
19.0	0.1	0.0	95.0	5
19.5	0.2	70.0	25.0	5
23.5	0.2	70.0	25.0	5
23.6	0.1	70.0	25.0	5
25.0	0.1	70.0	25.0	5

Source: Own depiction

The MS analysis was performed with electrospray ionization (ESI) interface in the negative ion mode with a capillary voltage of 2500 V. The sheath gas (N_2) and the auxiliary gas (N_2) flow rates were set at 20 and 10 arbitrary units, respectively. The ion transfer capillary temperature was 270 °C. Data acquisition and instrument control were performed using Xcalibur software, Version 2.3 (Thermo Fisher).

The MRM (Multiple Reaction Monitoring) and the optimal operating conditions for the tandem mass spectrometric analysis of the examined contaminants, have been determined and reported in a previous study (Dasenaki & Thomaidis, 2015).

However, optimization of the collision energy and tube lens voltage values was performed for each compound separately by direct infusion of individual standard solutions. The optimized values are reported in Table 6.4 below.

Table 6.4: Precursor – product ions, collision energies and tube lens voltage values

Compound	ESI	Precursor Ion (m/z)	Product Ion (m/z)	Tube Lens Offset (V)	Collision Energy (V)
Diclofenac	-	294	250.1	-31.0	15
			214.2	-31.0	24
Diclofenac-D4	-	298	254.2	-69.1	15
			217.1	-69.1	23
Dichlorophenol	-	161	125.2	-52.8	17
			89.1	-52.8	30
Tetrachlorophenol	-	231	195.2	-49.3	20
			192.8	-46.1	24

Source: Own depiction

6.2.4 Identification procedure

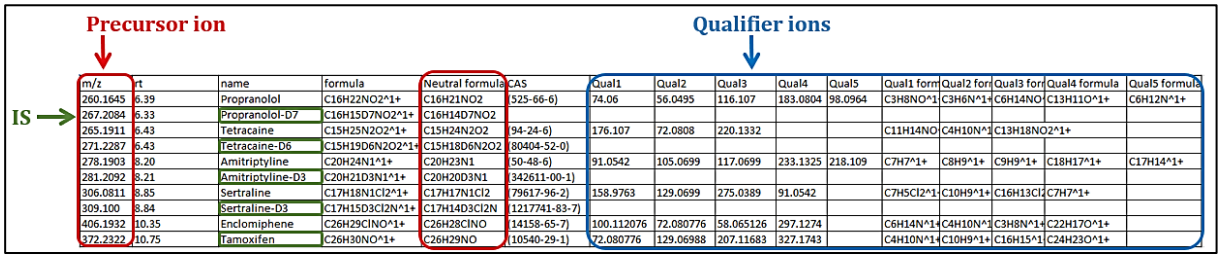
For the identification of the different substances, reference standard solutions were analyzed utilizing LC-ESI-QTOFMS. Processing of the raw data was carried out with the software tools Data Analysis 5.1 and TASQ CLIENT 2.1.

The following steps were applied to the raw data of the reference standard solutions, to obtain the analytical evidence that was mandatory for the identification of each substance in the respective samples:

- Mass calibration of the raw data files was performed with a calibration solution to minimize potential mass errors.
- Determination of the Retention time (RT) of each analyte, by creating its extracted ion chromatogram (EIC) with a mass window of ± 5 mDa.
- Isotopic pattern fitting evaluation (*mSigma value is a measurement of the fit between the measured and the theoretical isotopic pattern. The lowest the value of mSigma the highest the isotopic fitting).
- MS and MS/MS spectra processing – determination of the precursor and qualifier ions of each analyte. For the determination of the qualifier ions, data-dependent acquisition mode was used.

An in-house database containing all the substances was compiled. This database contained information over the RT, precursor, and qualifier ions (fragments) of the parent compounds. In the following figure (Figure 6.3), the data contained in the in-house database for the substances detected in positive ionization mode, are presented:

Figure 6.3: Analytical evidence data included in the in-house database for the identification of the tested compounds



m/z	rt	name	formula	Neutral formula	CAS	Qual1	Qual2	Qual3	Qual4	Qual5	Qual1 form	Qual2 for	Qual3 for	Qual4 formula	Qual5 formula
260.1645	6.39	Propranolol	C16H22NO2^1+	C16H21NO2	(525-66-6)	74.06	56.0495	116.107	183.0804	98.0964	C3H8NO^1	C3H6N^1+	C6H14NO	C13H11O^1+	C6H12N^1+
267.2084	6.33	Propranolol-D7	C16H15D7NO2^1+	C16H14D7NO2											
265.1911	6.43	Tetracaine	C15H25N2O2^1+	C15H24N2O2	(94-24-6)	176.107	72.0808	220.1332			C11H14NO	C4H10N^1	C13H18NO2^1+		
271.2287	6.43	Tetracaine-D6	C15H19D6N2O2^1+	C15H18D6N2O2	(80404-52-0)										
278.1903	8.20	Amitriptyline	C20H24N1^1+	C20H23N1	(50-48-6)	91.0542	105.0699	117.0699	233.1325	218.109	C7H7^1+	C8H9^1+	C9H9^1+	C18H17^1+	C17H14^1+
281.2092	8.21	Amitriptyline-D3	C20H21D3N1^1+	C20H20D3N1	(342611-00-1)										
306.0811	8.85	Sertraline	C17H18N1Cl2^1+	C17H17N1Cl2	(79617-96-2)	158.9763	129.0699	275.0389	91.0542		C7H5Cl2^1+	C10H9^1+	C16H13Cl2	C7H7^1+	
309.100	8.84	Sertraline-D3	C17H15D3Cl2N^1+	C17H14D3Cl2N	(1217741-83-7)										
406.1932	10.35	Enclomiphene	C26H29ClNO^1+	C26H28ClNO	(14158-65-7)	100.112076	72.080776	58.065126	297.1274		C6H14N^1+	C4H10N^1	C3H8N^1+	C22H17O^1+	
372.2322	10.75	Tamoxifen	C26H30NO^1+	C26H29NO	(10540-29-1)	72.080776	129.06988	207.11683	327.1743		C4H10N^1+	C10H9^1+	C16H15^1	C24H23O^1+	

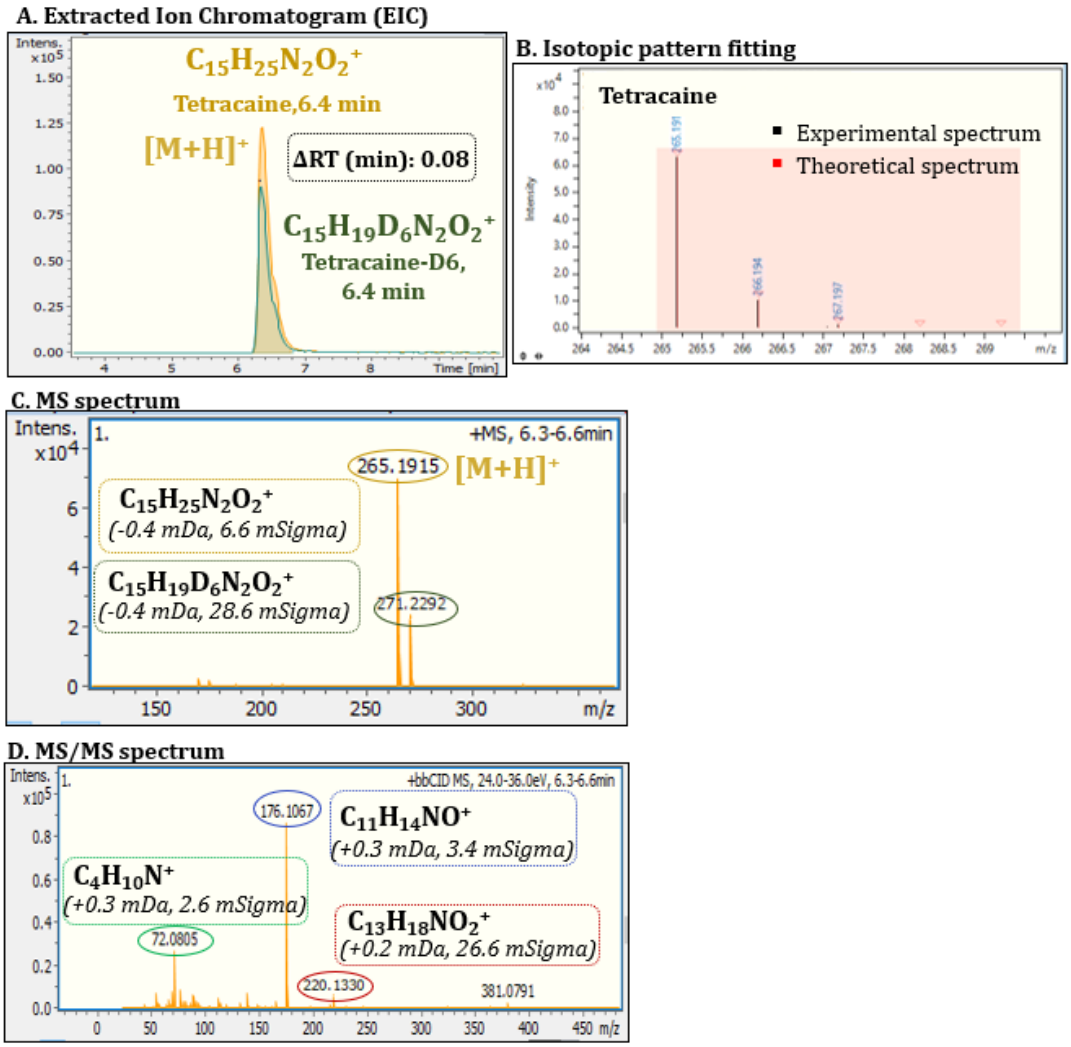
Source: Own depiction

Samples were screened utilizing the in-house built database. The identification of the substances was based on the following criteria:

- **Retention time shift** (ΔRT: 0-0.2 min), which refers to the difference between the experimental and the theoretical retention time.
- **Mass accuracy:** < 5 mDa, which refers to the difference between the experimental and the theoretical accurate mass in mDa or ppm.
- **Isotopic pattern fitting - mSigma**: <200, which refers to the conformity of fit between the theoretical and the experimental isotopic pattern.
- **> 2 qualifier ions**, the presence of qualifier ions apart from the precursor ion, was considered as a mandatory identification criterion, increasing the selectivity of the analytical procedure. The potential detection of additional qualifier ions enhances the identification confidence.

In the following figure (Figure 6.4), the identification procedure for the substance Tetracaine in water samples is exemplified:

Figure 6.4: Identification workflow for the substance Tetracaine



Identification workflow for the substance Tetracaine.
A: Extracted ion chromatogram (EIC) of the Tetracaine and the Tetracaine-IS (Tetracaine-D6) and retention time difference between the experimental and the theoretical retention time (ΔRT). B: Isotopic pattern fitting of Tetracaine. The theoretical spectrum is presented with black color and the corresponding experimental spectra with red C: MS spectrum of the molecular ion of Tetracaine ($[M+H]^+$). Principal ion: yellow color, Ion of IS: green color (Information in brackets: mass accuracy, isotopic pattern fitting) D: MS/MS spectrum of Tetracaine. Qualifier ions are presented in frames with blue, green, and red color. 3 qualifier ions are detected (Information in brackets: mass accuracy, isotopic pattern fitting).
Source: Own depiction

Regarding the procedure performed on the raw data from LC-MS/MS, the MRM transition with the highest intensity was used for quantification (quantifier), whereas the other transition was used for confirmation (qualifier) for each compound. The analytes were considered confirmed in the samples if the retention time did not differ by more than ± 0.4 min from that of the reference standard. Identification should be based on chromatographic peaks observed in the extracted ion chromatograms of two or more ions that are specific for the analyte. The peaks must have a similar peak shape, overlap with each other, and the quantifier/qualifier ratio in the extracted samples was within ±20 % of the ratio in the reference standards from the same sequence. Calibration standards were prepared at six different concentrations, to cover the respective dynamic range for all analytes. Linear calibration curves typically displayed correlation coefficients greater than 0.99.

6.2.5 Quantification procedure

The instrument provides a linear signal response at a certain concentration range for each analyte. Reference standard solutions were used, for the determination of this concentration range for each substance. Calibration curves were constructed with reference standard solutions at different concentration levels. The quantification of all the substances was performed by using the aforementioned reference standard calibration curves. The stable isotopically labelled internal standards (IS) were used to achieve reliable and accurate quantitative results. The IS were used for correcting potential insufficiencies of the sample preparation, ion suppression phenomena and for improving the quantification confidence. For that reason, relative areas have been used, namely, the absolute area of each substance has been divided with the area of the respective IS (Equation [6.2]).

$$Relative\ Area = {Absolute\ Area\ of\ Analyte}/{Area\ of\ IS} \qquad [6.2]$$

The calibration curves were constructed using the linear regression model. The regression lines (Equation [6.3]) were determined by the least-squares method, and were of the form:

$$y = (a \pm S_a) * C + (b \pm S_b) \qquad [6.3]$$

in which,

y: relative peak area of each analyte

a: the slope,

b: the intercept

C: concentration of analyte

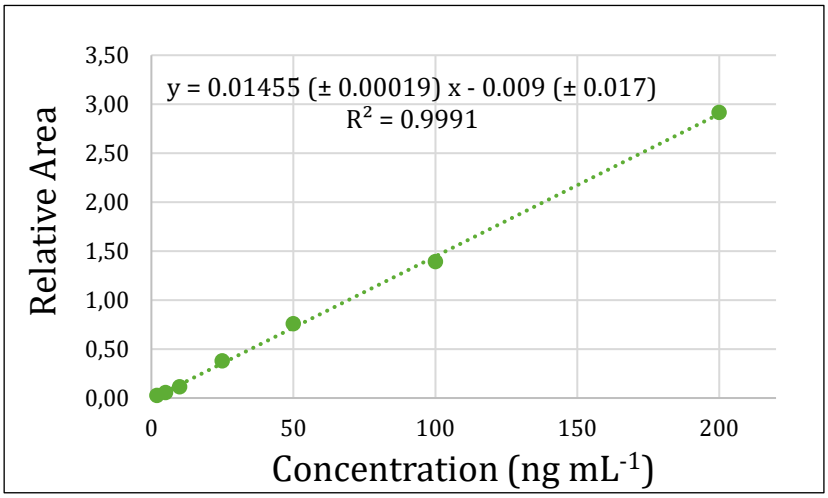
Sb: standard deviation of the intercept

Sa: standard deviation of the slope

The concentration of each analyte in the samples was determined by using the corresponding equation [6.3] with the relative peak areas (y).

In the following figure (Figure 6.5), the calibration curve of Tetracaine at seven different concentration levels ($C_{standard}$ (ng mL^{-1}): 2, 5, 10, 25, 50, 100, 200) is presented. For each one of the above-mentioned substances, regression trendlines were determined. The regression trendlines were used for the quantification of each substance in water and ZFE samples.

Figure 6.5: Calibration curve of reference standard Tetracaine



Source: Own depiction

Concerning the ZFE extracts, the reported concentrations (ng mL^{-1}) refer to 0.5 mL of MeOH/H_2O (1:1 v/v) mixture that was used for the extraction. For the estimation of the respective ZFE mass (ng) contained in the 0.5 mL of extract, Equation [6.4] was used:

$$M(ng) = C(ng\ mL^{-1}) * 0.5\ mL \qquad [6.4]$$

The exact weight of the ZFE contained in each delivered Eppendorf was used for the calculation of the internal concentration (C_{int}). The Cint was calculated based on the following equation (Equation [6.5]):

$$C_{int}\ (mg\ kg^{-1}) = C_{int}\ (ng\ mg^{-1}) = {M\ (ng)}/{Weight\ of\ sample\ (mg)} \qquad [6.5]$$

The bioconcentration factor (BCF) of each compound was calculated, in order to evaluate the extent of bioaccumulation. For the calculation of the BCF (L kg^{-1}), the following equation (Equation [6.6]) was used:

$$BCF = \frac{C_{int}}{C_{exp}} \qquad [6.6]$$

C_{int} (mg kg^{-1}): concentration in the organism

C_{exp} (mg L^{-1}): concentration in the exposure medium

The concentrations of the water samples from the start and the end of the exposure experiments were estimated and used to calculate the average exposure concentration for water samples. The calculated average exposure concentration corresponds to the exposure concentration (C_{exp}) which used for the calculations of the bioconcentration factors.

6.3 Results of the chemical analyses

The results from the analysis of the water and ZFE samples from the exposure experiments of ZFE to pharmaceuticals and pesticides are presented separately below. The concentration levels (ng mL^{-1}) of each sample were determined ($C_{measured}$) and the standard deviation (SD) was also

calculated. Regarding the ZFE samples, the internal concentrations were determined and are reported in the following tables for each one of the tested substances. The C_{int} are presented using bar charts in the following figures.

The overall results from the chemical analyses of FET samples including the measured concentration of the water samples from the start and end of the exposure experiments (see Appendix, Table A5), as well as the internal concentrations of zebrafish embryos and the respective calculated bioconcentration factors (BCF) (see Appendix, Table A6) of all the different substances are presented in the Appendix below.

6.3.1 Pharmaceuticals

6.3.1.1 NSAIDs

Acetylsalicylic acid

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Acetylsalicylic acid water samples, sampled at the Start and the End of the exposure experiments at the different pH values (5, 6 and 8), were determined (Table 6.5). Different pH values are indicated with different colours.

Table 6.5: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Acetylsalicylic acid exposure experiments

Water Acetylsalicylic acid	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	**6747 ± 472**	$18*10^3$
pH 5 END	**254 ± 18**	
pH 6 START	**$(36.5 ± 2.6)*10^3$**	$90*10^3$
pH 6 END	**$(37.8 ± 2.6)*10^3$**	
pH 8 START	**1028 ± 72**	$2800*10^3$
pH 8 END	**248 ± 17**	

Source: Own depiction

Concerning the results of the analysis of the water samples reported in Table 6.5, the $C_{measured}$ at pH 5 and at pH 6 were lower (almost three times) compared to the theoretical concentration. The $C_{measured}$ of the END water samples from the exposure experiment at pH 5 was lower than the theoretical one and the Start water samples. The END water samples were not diluted at all. Furthermore, the $C_{measured}$ of the END water samples from the exposure experiment at pH 6 was higher compared to the $C_{measured}$ of the Start water samples. However, the measured concentrations were at the same range, if the SD of the $C_{measured}$ is taken into consideration. The $C_{measured}$ at pH 8 were lower compared to the $C_{theoretical}$.

It should be noted, that before the sample preparation, a small amount of solid was observed into the falcon tubes. The water sample at pH 8 were not diluted at all. Therefore, this could not be attributed to a potential mistake during the sample preparation.

ZFE Samples

- Internal Concentration

The internal concentrations (C_{int} ± SD (mg kg^{-1})) of Acetylsalicylic acid in the extracts of the ZFE samples was determined and presented in Table 6.6 below. Different pH values are indicated with different colors.

Table 6.6: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Acetylsalicylic acid exposure experiments

	ZFE Acetylsalicylic acid					
	Eppi No.	Status	Embryos No.	Labelling	Weight of sample (mg)	C_{int} (mg kg^{-1}) ± SD
pH 5	1	dead	13	pH 5 1 ASA	5.1	<LOD
	2	alive	20	pH 5 2 ASA	3.9	<LOD
	3	alive	20	pH 5 3 ASA	10.7	<LOD
	4	alive	20	pH 5 4 ASA	21.8	<LOD
	5	alive	17	pH 5 5 ASA	7.4	<LOD
pH 6	1	dead	11	pH 6 1 ASA	1.8	<LOD
	2	alive	18	pH 6 2 ASA	5.4	<LOD
	3	alive	18	pH 6 3 ASA	4.2	<LOD
pH 8	1	alive	20	pH 8 ASA 2800 mg/L 20* (1)	10.1	<LOD
	2	alive	20	pH 8 ASA 2800 mg/L 20* (2)	12.6	<LOD
	3	alive	20	pH 8 ASA 2800 mg/L 20* (3)	12.9	<LOD

* The limit of detection (LOD) was estimated by using the data from the calibration curve of the substance Acetylsalicylic acid (LOD= 53.1 ng mL^{-1}).

Source: Own depiction

Diclofenac

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Diclofenac water samples, sampled at the Start and the End of the exposure experiments at the different pH values (5, 6, 8 and 9), were determined (Table 6.7). Different pH values are indicated with different colours.

Table 6.7: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Diclofenac exposure experiments

Water Diclofenac	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	34.1 ± 2.0	50
pH 5 END	15.66 ± 0.94	
pH 6 START	302 ± 18	400
pH 6 END	237 ± 14	
pH 8 START	(11.45 ± 0.80) * 10^3	17 * 10^3
pH 8 END	(14.5 ± 1.0) * 10^3	

Water Diclofenac	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 9 START	(37.0 ± 2.6) * 10^3	70 * 10^3
pH 9 END	(29.5 ± 2.1) * 10^3	

Source: Own depiction

Concerning the results from the analysis of the water samples (reported in Table 6.7), the $C_{measured}$ of the END water sample was higher compared to the $C_{measured}$ of the START water sample, for the exposure experiments at pH 8. On the contrary, the $C_{measured}$ of the END water samples at pH 5 was significantly lower compared to the respective START samples.

As far as the water samples at pH 9 is concerned, $C_{measured}$ were lower (≃ 2 times) compared to the theoretical concentration ($C_{theoretical}$= 70 * 10^3 ng mL^{-1}).

ZFE Samples

- Internal Concentration

The internal concentrations (C_{int} ± SD (mg kg^{-1})) of Diclofenac in the extracts of the ZFE samples was determined and presented in Table 6.8 below. Different pH values are indicated with different colors.

Table 6.8: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Diclofenac exposure experiments

	ZFE Diclofenac					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**alive**	20	Diclo pH 5 0.05 mg/L 20* 1	5.700	**<LOD**
	2	**alive**	20	Diclo pH 5 0.05 mg/L 20* 2	5.600	**<LOD**
	3	**alive**	20	Diclo pH 5 0.05 mg/L 20* 3	5.700	**<LOD**
pH 6	6	**alive**	15	Diclo pH 6 0.4 mg/L 15* 6	4.100	**1.823 ± 0.088**
	7	**alive**	15	Diclo pH 6 0.4 mg/L 15* 7	4.300	**1.107 ± 0.053**
	8	**alive**	14	Diclo pH 6 0.4 mg/L 14* 8	3.900	**<LOD**
pH 8	5	**alive**	15	Diclo pH 8 17 mg/L 15* 5	4.800	**12.55 ± 0.58**
	6	**alive**	16	Diclo pH 8 17 mg/L 16* 6	4.700	**8.18 ± 0.38**
	7	**alive**	15	Diclo pH 8 17 mg/L 15* 7	4.600	**11.64 ± 0.54**
pH 9	1	**alive**	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 1	4.500	**29.4 ± 1.4**
	2	**alive**	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 2	4.700	**27.1 ± 1.3**
	3	**alive**	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 3	4.200	**30.0 ± 1.4**
	4	**alive**	16	Diclo pH 9 70 mg/L 16 alive 96 hpf 4	4.500	**24.8 ± 1.2**

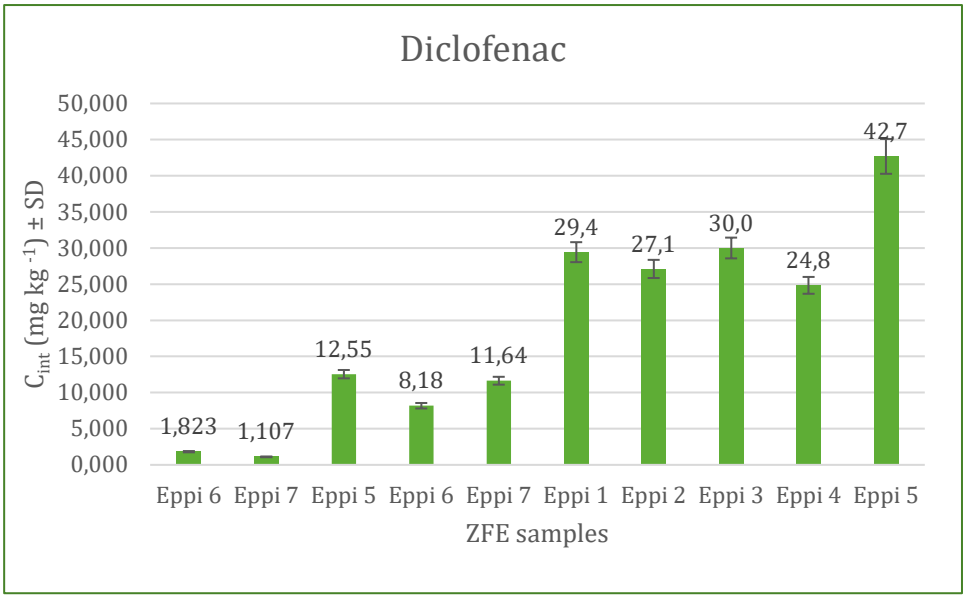
	ZFE Diclofenac					
	5	**dead**	16	Diclo pH 9 70 mg/L 16 dead 96 hpf 5	2.600	**42.7 ± 2.4**

* The limit of detection (LOD) was estimated by using the data from the calibration curve of the substance Diclofenac (LOD= 8.4 ng mL^{-1}).

Source: Own depiction

In Figure below, the C_{int} are presented for each pH value using bar charts. Different pH values are indicated with different colors.

Figure 6.6: Internal concentrations (C_{int} (mg kg^{-1})) of Diclofenac in the ZFE samples



Source: Own depiction

The internal concentrations (mg kg^{-1}) from the analysis of the ZFE samples were at the same range for most of the samples (presented in Figure 6.6). Most of the ZFE samples at pH 6 had lower internal concentrations in comparison with the rest of the samples at the other pH values (pH 8 and pH 9). Concerning the results of the ZFE samples at pH 9, the weight of Eppendorf tube number 5 was quite small (2.6 mg) in comparison with the number of embryos in comparison with the number of embryos (Embryos No= 16) contained in the Eppendorf tube and the weight of the rest of the samples at pH 9. This probably leads to such a high C_{int} in comparison with the C_{int} of the rest of the ZFE samples at pH 9. If the weight of the Eppi No 5 was according to the No of embryos (≃ 4.2 mg), the internal concentration would be at the same range (C_{int}≃26 mg kg^{-1}).

Ibuprofen

Water Samples

Concentration levels ($C_{measured\ Average}$ ± SD (ng mL^{-1})) of Ibuprofen water samples, sampled at the Start and the End of the exposure experiments at four different pH values (5, 6, 8 and 9), were determined (Table 6.9). Different pH values are indicated with different colors.

Table 6.9: Measured concentration levels (ng mL^{-1}) of water samples from Ibuprofen exposure experiments

Water Ibuprofen	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	520 ± 36	8700
pH 5 END	328 ± 16	
pH 6 START	3731 ± 283	45200
pH 6 END	2824 ± 249	
pH 8 START	(109 ± 14) *10^3	150 *10^3
pH 8 END	(130.5 ± 5.6 *10^3	
pH 9 START	(178 ± 14) *10^3	300 *10^3
pH 9 END	(127 ± 10) *10^3	

Source: Own depiction

Concerning the results of the analysis of the water samples reported in Table 6.9, the $C_{measured}$ of the END water sample at pH 8 was higher compared to the $C_{measured}$ of the START water sample. In addition, the $C_{measured}$ of the START water samples at pH 5 and pH 6 was higher compared to the $C_{measured}$ of the END water samples. Regarding the water samples at pH 9, the $C_{measured}$ were lower (≃ 2 times) compared to the theoretical concentration ($C_{theoretical}$= 300 * 10^3 ng mL^{-1}). As far as the water samples at pH 5 and pH 6 is concerned, $C_{measured}$ were significantly lower (*almost 15 and 10 times respectively*) compared to the theoretical concentrations. The water samples at pH 6 were diluted only 2 times (*in the vial prior to analysis*), whereas the water samples at pH 5 were not diluted at all. Therefore, the fact that $C_{measured}$ was lower than the $C_{theoretical}$, could not be attributed to potential mistake during sample preparation. Potential incomplete dissolution of the analytes during exposure experiments may be a possible explanation for this issue.

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Ibuprofen in the extracts of the ZFE samples were determined (Table 6.10). Different pH values are indicated with different colors.

Table 6.10: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Ibuprofen exposure experiments

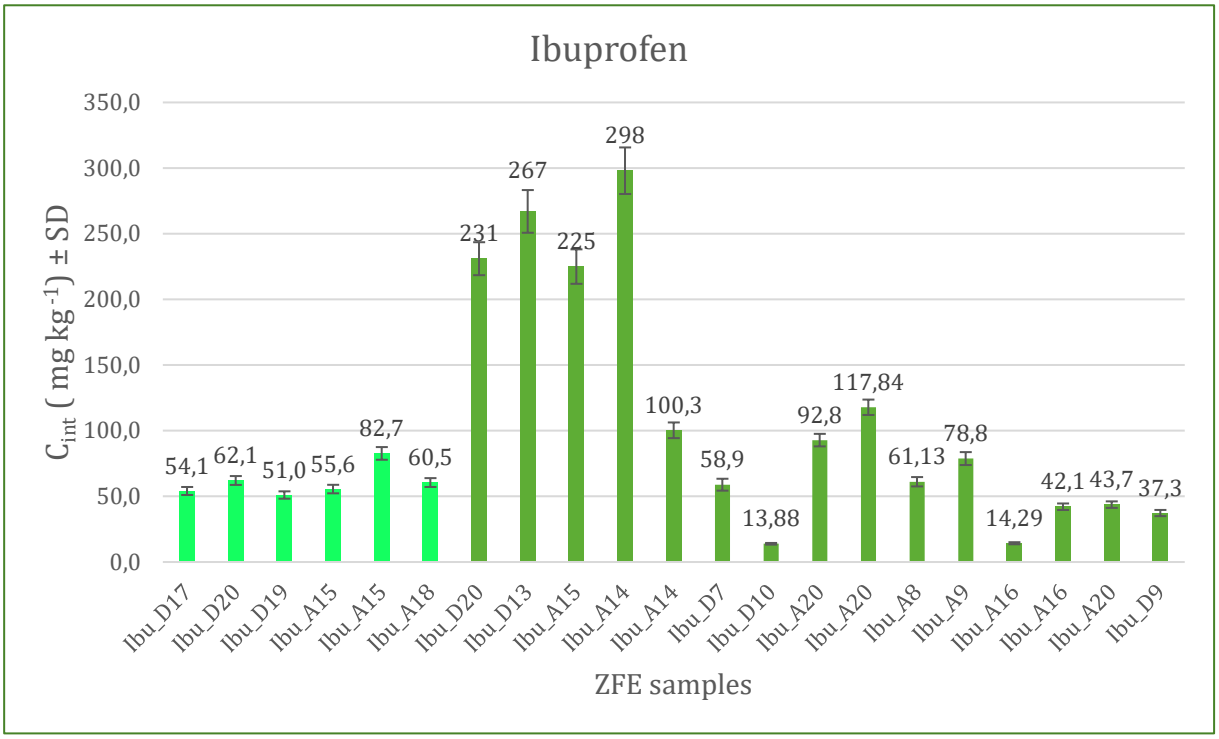
	ZFE Ibuprofen					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**dead**	17	Ibu pH 5 D17	4.1164	**54.1 ± 3.1**
	2	**dead**	20	Ibu pH 5 D20	4.8428	**62.1 ± 3.4**
	3	**dead**	19	Ibu pH 5 D19	4.6007	**51.0 ± 2.8**
	1	**alive**	15	Ibu pH 5 A15	3.6321	**55.6 ± 3.2**
	2	**alive**	15	Ibu pH 5 A15	3.6321	**82.7 ± 4.8**

	ZFE Ibuprofen					
	3	**alive**	18	Ibu pH 5 A18	4.3585	**60.5 ± 3.4**
pH 6	1	**dead**	20	Ibu pH 6 D20	4.8428	**231 ± 13**
	2	**dead**	13	Ibu pH 6 D13	3.1478	**267 ± 16**
	1	**alive**	15	Ibu pH 6 A15	3.6321	**225 ± 13**
	2	**alive**	14	Ibu pH 6 A14	3.3900	**298 ± 18**
	3	**alive**	14	Ibu pH 6 A14	3.3900	**100.3 ± 6.0**
pH 8	3	**dead**	7	Ibu pH 8 #7 tot 3	1.6950	**58.9 ± 4.5**
	4	**dead**	10	Ibu pH 8 #10 tot 4	16.900	**13.88 ± 0.63**
	6	**alive**	20	Ibu pH 8 #20 leb.6	6.4000	**92.8 ± 4.8**
	7	**alive**	20	Ibu pH 8 #20 leb.7	7.8000	**117.8 ± 5.9**
	8	**alive**	15	Ibu pH 8 #15 leb.8	3.7000	**61.1 ± 3.6**
	9	**alive**	14	Ibu pH 8 #14 leb.9	2.9000	**78.8 ± 4.9**
pH 9	1	**alive**	16	Ibu pH 9 300 mg/L 16 alive 96 hpf 1	0.2750	**14.29 ± 0.79**
	2	**alive**	16	Ibu pH 9 300 mg/L 16 alive 96 hpf 2	0.2188	**42.1 ± 2.5**
	3	**alive**	20	Ibu pH 9 300 mg/L 20 alive 96 hpf 3	0.2000	**43.7 ± 2.5**
	4	**dead**	9	Ibu pH 9 300 mg/L 9 dead 96 hpf 4	0.3111	**37.3 ± 2.4**

Source: Own depiction

In the figure (Figure 6.7) below, the C_{int} are presented for each pH value (5, 6, 8 and 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.7: Internal concentrations (C_{int} (mg kg^{-1})) of Ibuprofen in the ZFE samples



Source: Own depiction

Regarding the results of ZFE samples of the exposure experiments (presented on Table 6.10), the C_{int} (mg kg^{-1}) were in the same range for most of the samples. Most of the ZFE samples at pH 6 had higher (almost 3 times) internal concentration in comparison with the rest of the samples (presented in Figure 6.7). For the estimation weight of the ZFE samples of Ibuprofen at pH 8, the difference between the filled and the empty Eppi was used. These values were used for the determination of the C_{int}. It's worth mentioning that the weight of the Eppi number 4 (*Ibu_D10_pH 8*) was quite large (16.9 mg) in comparison with the number of embryos (Embryos No=10) contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples at pH 8.

Naproxen

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Naproxen water samples, sampled at the Start and the End of the exposure experiments at three different pH values (6, 8, 9), were determined (Table 6.11). Different pH values are indicated with different colors.

Table 6.11: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Naproxen exposure experiments

Water Naproxen	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 6 START	5660 ± 566	8000
pH 6 END	5216 ± 522	
pH 8 START	(147 ± 15) * 10^3	325 * 10^3

Water Naproxen	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 8 END	(163 ± 16) * 10^3	
pH 9 START	(543 ± 54) * 10^3	900 * 10^3
pH 9 END	(478 ± 48) * 10^3	

Source: Own depiction

Concerning the results from the analysis of the water samples (reported in Table 6.11), the $C_{measured}$ of the END water sample was higher compared to the $C_{measured}$ of the START water sample, for the exposure experiments at pH 8. Furthermore, the $C_{measured}$ of the water sample of the exposure experiments at pH 8, were lower (≃ 2 times) compared to the theoretical concentration ($C_{theoretical}$= 325 * 10^3 ng mL^{-1}). The exposure concentration of Naproxen was higher at pH 9 and for this reason, more stages of dilution were required. This can explain the fact that the standard deviation of the $C_{measured}$ at pH 9 was higher compared to the SD of the $C_{measured}$ at the other two pH values.

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Naproxen in the extracts of the ZFE samples was determined (Table 6.12). Different pH values are indicated with different colors.

Table 6.12: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Naproxen exposure experiments

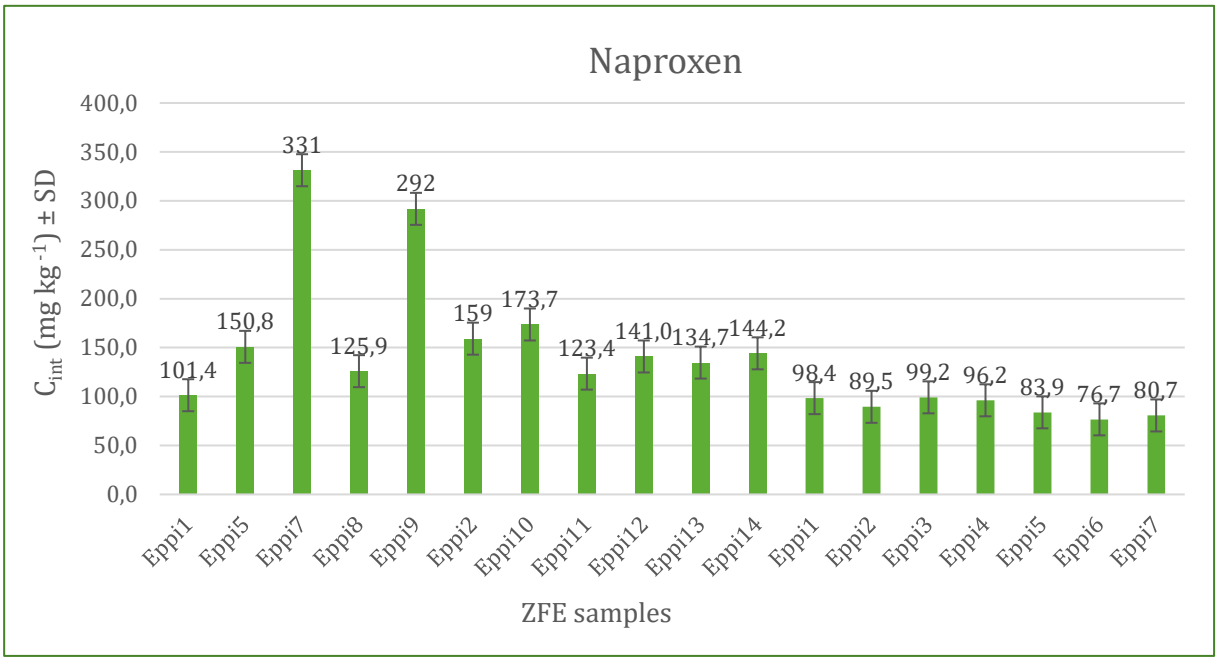
	ZFE Naproxen					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 6	**1**	**dead**	3	pH 6 Naproxen † 72 hpf no. 3	1.6000	**101.4 ± 7.5**
	5	**dead**	6	pH 6 Naproxen † 96 hpf no. 6	3.0000	**150.8 ± 8.5**
	7	**alive**	20	pH 6 Naproxen * 96 hpf no. 20	6.6000	**331 ± 15**
	8	**alive**	20	pH 6 Naproxen * 96 hpf no. 20	13.400	**125.9 ± 5.2**
	9	**alive**	16	pH 6 Naproxen * 96 hpf no. 16	6.9000	**292 ± 13**
pH 8	**2**	**dead**	1	pH 8 Naproxen † 72 hpf no. 1	0.9000	**159 ± 16**
	10	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.2000	**173.7 ± 8.4**
	11	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.5000	**123.4 ± 5.9**
	12	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.1000	**141.0 ± 6.8**

	ZFE Naproxen					
	13	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.3000	**134.7 ± 6.5**
	14	**alive**	15	pH 8 Naproxen * 96 hpf no. 15	3.8000	**144.2 ± 7.6**
pH 9	**1**	**dead**	3	pH 9 Naproxen † 96 hpf no.3	1.6000	**98.4 ± 7.3**
	2	**alive**	20	pH 9 Naproxen * 96 hpf no.20	4.6000	**89.5± 4.5**
	3	**alive**	20	pH 9 Naproxen * 96 hpf no.20	5.3000	**99.2 ± 4.8**
	4	**alive**	20	pH 9 Naproxen * 96 hpf no.20	4.6000	**96.2 ± 4.8**
	5	**alive**	20	pH 9 Naproxen * 96 hpf no.20	5.3000	**83.9 ± 4.0**
	6	**alive**	20	pH 9 Naproxen * 96 hpf no.20	5.6000	**76.7 ± 3.6**
	7	**alive**	13	pH 9 Naproxen * 96 hpf no.13	3.3000	**80.7 ± 4.4**

Source: Own depiction

In Figure 6.8 below, the C_{int} are presented for each pH value (6, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.8: Internal concentrations (C_{int} (mg kg^{-1})) of Naproxen in the ZFE samples



Source: Own depiction

The internal concentrations (mg kg^{-1}) from the analysis of the ZFE samples were at the same range for most of the samples (presented in Figure 6.8). Regarding the results of the ZFE samples, the C_{int} of the two ZFE samples at pH 6 differed from the overall results. Concerning the results of the ZFE samples at pH 6, the weight of Eppendorf tube number 8 was quite high (13.4

mg) in comparison with the number of embryos (Embryos No= 20) contained in the Eppendorf tube and the weight of the rest of the samples with 20 ZFE/Eppendorf. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples at pH 6.

6.3.1.2 Anaesthetics

Lidocaine

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Lidocaine water samples, sampled at the Start and the End of the exposure experiments at three different pH values (5, 8 and 9), were determined (Table 6.13).

Table 6.13: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Lidocaine exposure experiments

Water Lidocaine	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	(521 ± 16) * 10^3	2000 * 10^3
pH 5 END	(618 ± 19) * 10^3	
pH 8 START	(18.69 ± 0.58) * 10^3	50 * 10^3
pH 8 END	(17.28 ± 0.54) * 10^3	
pH 9 START	(4.57 ± 0.14) * 10^3	10 * 10^3
pH 9 END	(3.97 ± 0.12) * 10^3	

Source: Own depiction

Concerning the results of the analysis of the water samples reported in Table 6.13, of the END water sample at pH 5 was higher compared to the $C_{measured}$ of the START water sample and the $C_{measured}$ were lower (≃ 4 times) compared to the theoretical concentration ($C_{theoretical}$= 2000 * 10^3 ng mL^{-1}). As far as the water samples at pH 8 and pH 9 is concerned, the $C_{measured}$ were significantly lower (*almost 2 times*) compared to the theoretical concentrations.

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Lidocaine in the extracts of the ZFE samples was determined (Table 6.14). Different pH values are indicated with different colors.

Table 6.14: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Lidocaine exposure experiments

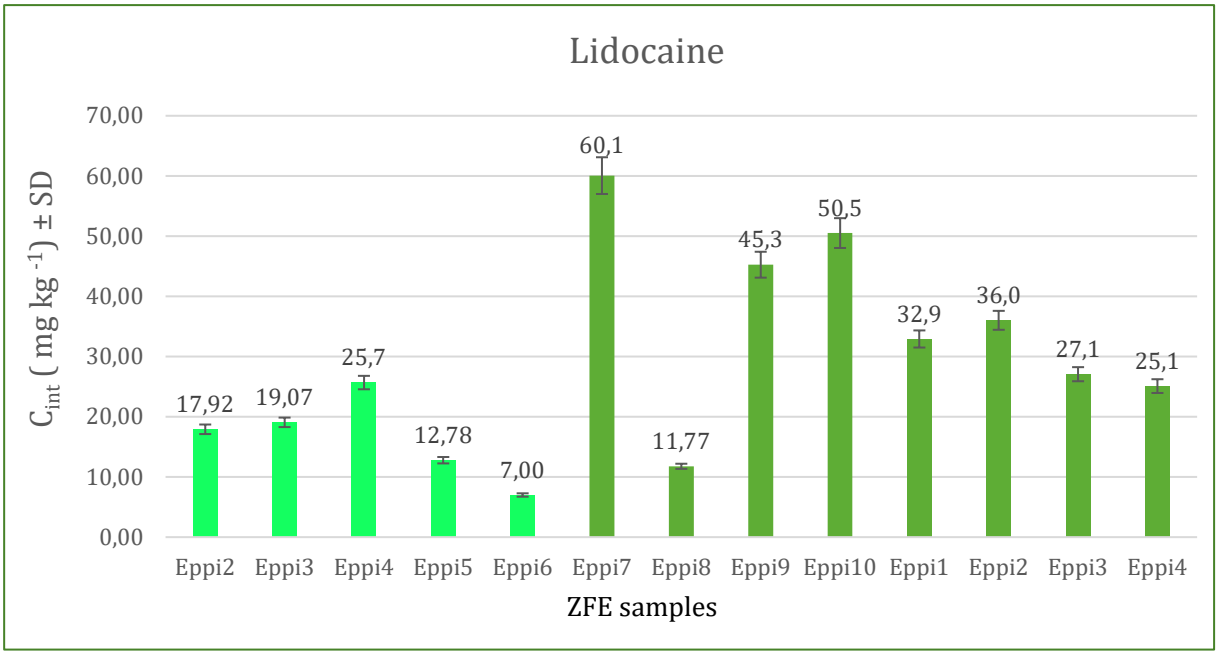
	ZFE Lidocaine					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	2	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 2	4.5000	**17.92 ± 0.79**
	3	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 3	5.8000	**19.07 ± 0.79**

	ZFE Lidocaine					
	4	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 4	4.8000	**25.7 ± 1.1**
	5	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 5	5.6000	**12.78 ± 0.53**
	6	**alive**	17	Lido pH 5 2000 mg/L 96 h 15 alive 6	6.6000	**7.00 ± 0.28**
pH 8	7	**alive**	15	Lid pH 8 50 mg/L 96 h 15 alive 7	3.0000	**60.1 ± 3.1**
	8	**alive**	15	Lid pH 8 50 mg/L 96 h 15 alive 8	11.7000	**11.77 ± 0.43**
	9	**alive**	16	Lid pH 8 50 mg/L 96 h 15 alive 9	3.6000	**45.3 ± 2.2**
	10	**dead**	15	Lid pH 8 50 mg/L 96 h 15 dead 10	3.3000	**50.5 ± 2.5**
pH 9	1	**alive**	15	pH 9 10 mg/L 96 h 15 alive 1	4.9000	**32.9 ± 1.4**
	2	**alive**	15	pH 9 10 mg/L 96 h 15 alive 2	4.7000	**36.0 ± 1.6**
	3	**alive**	15	pH 9 10 mg/L 96 h 15 alive 3	4.9000	**27.1 ± 1.2**
	4	**dead**	15	pH 9 10 mg/L 96 h 15 dead 4	4.1000	**25.1 ± 1.1**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (5, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.9: Internal concentrations (C_{int} (mg kg^{-1})) of Lidocaine in the ZFE samples



Source: Own depiction

The internal concentrations (mg kg^{-1}) from the analysis of the ZFE samples were at the same range for most of the samples (presented in Figure 6.9). Most of the ZFE samples at pH 5 had lower internal concentrations in comparison with the rest of the samples at the other pH values (pH 8 and pH 9). Concerning the results of the ZFE samples at pH 8, the weight of Eppi number 8 was quite large (11.7 mg) in comparison with the number of embryos (Embryos No= 15) contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples. If the weight was according to the No of embryos (≃ 3 mg) the internal concentration would be at the same range (C_{int}=46 mg kg^{-1}) of the rest of the ZFE samples at pH 8.

Tetracaine

Water Samples

Concentration levels ($C_{measured\ Average}$ ± SD (ng mL^{-1})) of Tetracaine water samples, sampled at the Start and the End of the exposure experiments at four different pH values (5, 6, 8 and 9), were determined (Table 6.15). Different pH values are indicated with different colors.

Table 6.15: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Tetracaine exposure experiments

Water Tetracaine	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	(158 ± 14) *10^3	170.13 *10^3
pH 5 END	(152 ± 14) *10^3	
pH 6 START	(56.0 ± 3.2) *10^3	59.2 *10^3
pH 6 END	(49.8 ± 1.4) *10^3	
pH 8 START	2406 ± 49	3900
pH 8 END	1705 ± 37	
pH 9 START	2186 ± 48	3000
pH 9 END	93.8 ± 2.1	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of Tetracaine (reported in Table 6.15), at the different pH values (pH 5, pH 6, pH 8 and pH 9), were at the same range with the theoretical ones. Furthermore, the $C_{measured}$ of the START water samples were higher compared to the $C_{measured}$ of the END water samples, for the exposure experiments at all pH values. As far as the END water sample from the exposure experiment at pH 9 is concerned, $C_{measured}$ was significantly lower (almost 30 times) compared to the theoretical concentrations ($C_{theoretical}$= 3 * 10^3 ng mL^{-1}). The water samples at pH 9 was diluted only 2 times, in the vial prior to analysis. Therefore, the fact that $C_{measured}$ was lower than the Ctheoretical, could not be attributed to potential mistake during sample preparation. The SD of the $C_{measured}$ at pH 5 and pH 6 was higher compared with the SD of the $C_{measured}$ at pH 8 and pH 9. The exposure concentrations of were higher at pH 5 and pH 6 and for this reason more stages of dilution were required. Consequently, the standard deviation was higher at pH 5 and at pH 6.

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Tetracaine in the extracts of the ZFE

samples was determined (Table 6.16). Different pH values are indicated with different colors.

Table 6.16: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Tetracaine exposure experiments

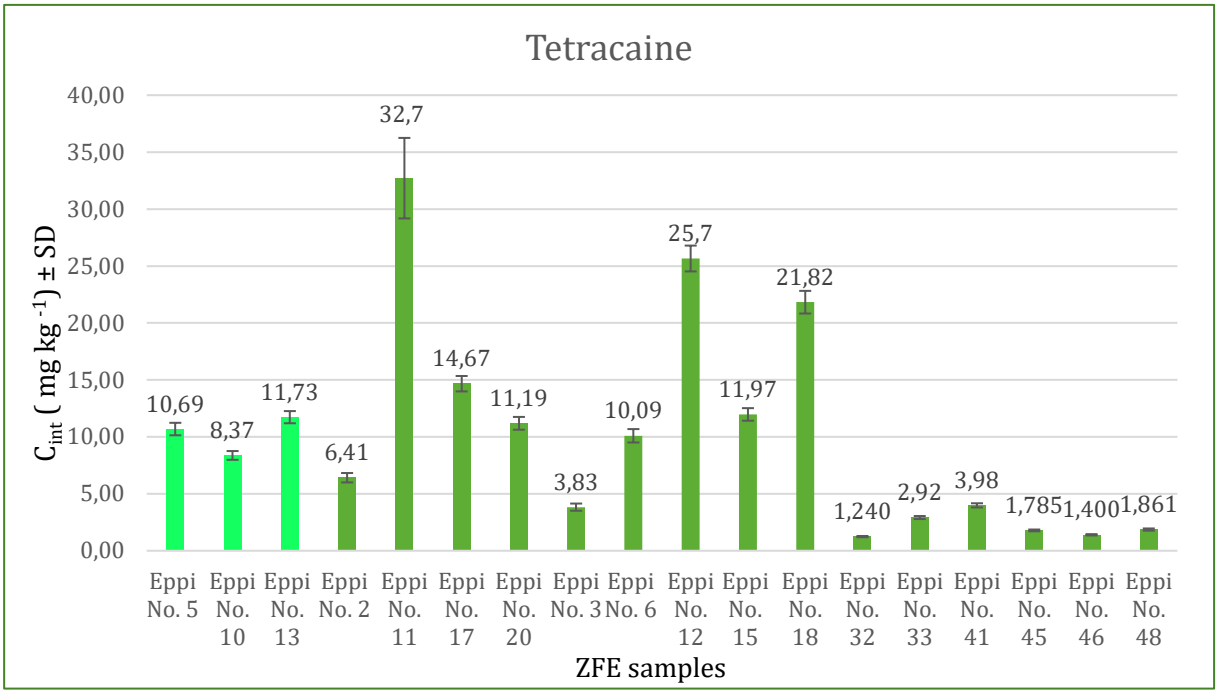
	ZFE Tetracaine					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**dead**	1	Nr. 1 pH 5 1.0051g+Tc 1Embryo	0.242	**< LOD**
	5	**dead**	14	Nr. 5 pH 5 +14 Embryos Tc	3.390	**10.69 ± 0.55**
	10	**alive**	20	Nr. 10 pH 5 Alive Tc 20 Embryos	4.843	**8.37 ± 0.39**
	13	**alive**	21	Nr. 13 pH 5 Alive 21 Embryos Tc	5.085	**11.73 ± 0.53**
pH 6	2	**dead**	8	Nr. 2 1.005g+Tc 8 Embryos	1.937	**6.41 ± 0.41**
	11	**alive**	3	Nr. 11 pH 6 <3 3 Embryos Tc	0.726	**30.7 ± 3.5**
	17	**alive**	20	Nr. 17 pH 6 Alive Tc 1.0044 g 20 Embryos	4.843	**14.67 ± 0.68**
	20	**dead**	15	Nr. 20 pH6 + 15 Embryos Tc	3.632	**11.19 ± 0.56**
pH 8	3	**dead**	5	Nr. 3 pH 8 1.0075 g + Tc 5 Embryos	1.211	**3.83 ± 0.32**
	6	**dead**	10	Nr. 6 pH 8 + 10 Embryos Tc	2.421	**10.09 ± 0.59**
	12	**alive**	20	Nr. 12 pH 8 <3 20 Embryos 1.071 g Tc	4.843	**24.6 ± 1.1**
	15	**alive**	20	Nr. 15 pH 8 <3 1.0035 20 Embryos Tc	4.843	**11.97 ± 0.55**
	18	**alive**	21	Nr. 18 pH 8 <3 21 Embryos 1.0035 g Tc	5.085	**21.82 ± 0.99**
pH 9	27	**dead**	2	Tetra 3 mg/L pH9 72hpf t2 27	1.800	**< LOD**
	32	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 32	5.900	**1.240 ± 0.055**
	33	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 33	5.100	**2.92 ± 0.13**
	41	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 41	4.900	**3.98 ± 0.18**
	43	**dead**	1	Tetra 3 mg/L pH9 48hpf t1 43	1.800	**< LOD**
	45	**alive**	15	Tetra 3 mg/L pH9 96hpf *15 45	6.100	**1.785 ± 0.078**

	ZFE Tetracaine					
	46	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 46	9.200	**1.400 ± 0.057**
	48	**alive**	8	Tetra 3 mg/L pH9 96hpf *8 48	3.400	**1.861 ± 0.095**

Source: Own depiction

In the Figure 6.10 below, the C_{int} are presented for the different pH values (5, 6, 8 and 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.10: Internal concentrations (C_{int} (mg kg^{-1})) of Tetracaine in the ZFE samples



Source: Own depiction

Regarding the results of ZFE samples of the exposure experiments (presented on Table 6.16), the C_{int} (mg kg^{-1}) were in the same range for most of the samples. Most of the ZFE samples at pH 9 had lower internal concentrations in comparison with the rest of the samples at the other pH values (pH 5,6 and pH 8). Concerning the results of the ZFE samples at pH 9, the weight of Eppendorf tube number 46 was quite large (9.2 mg) in comparison with the number of embryos (Embryos No= 20) contained in the Eppendorf tube. The other ZFE samples at pH 9 contained 20 ZFE per each Eppendorf, weighted approximately 5-6 mg. Concerning the results of the analysis of the ZFE samples reported in Table 6.16, the C_{int} (mg kg^{-1}) of some of the ZFE samples were lower than the limit of detection. These samples contained small quantity of dead embryos (1 or 2 ZFE per sample) and therefore it was difficult to be detected.

6.3.1.3 Opioids

Tramadol

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Tramadol water samples, sampled at the Start and the End of the exposure experiments at three different pH values (6, 8, 9), were determined (presented in Table 6.17). Different pH values are indicated with different colours.

Table 6.17: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Tramadol exposure experiments

Water Tramadol	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 6 START	(56.0 ± 3.2) *10^3	59.2 *10^3
pH 6 END	(49.8 ± 1.4) *10^3	
pH 8 START	2406 ± 49	3900
pH 8 END	1705 ± 37	
pH 9 START	2186 ± 48	3000
pH 9 END	93.8 ± 2.1	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of Tramadol (reported in Table above), at the different pH values (pH 6, pH 8 and pH 9), were lower (almost 3 times) than the theoretical one. Furthermore, the $C_{measured}$ of the START water samples were lower compared to the $C_{measured}$ of the END water samples, for the exposure experiments at all pH values. The SD of the $C_{measured}$ at pH 6 was higher compared with the SD of the $C_{measured}$ at pH 8 and pH 9. The exposure concentrations of were higher at pH 6 and for this reason more stages of dilution were required. Consequently, the standard deviation was higher at pH 6.

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Tramadol in the extracts of the ZFE samples was determined (Table 6.18). Different pH values are indicated with different colors.

Table 6.18: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Tramadol exposure experiments

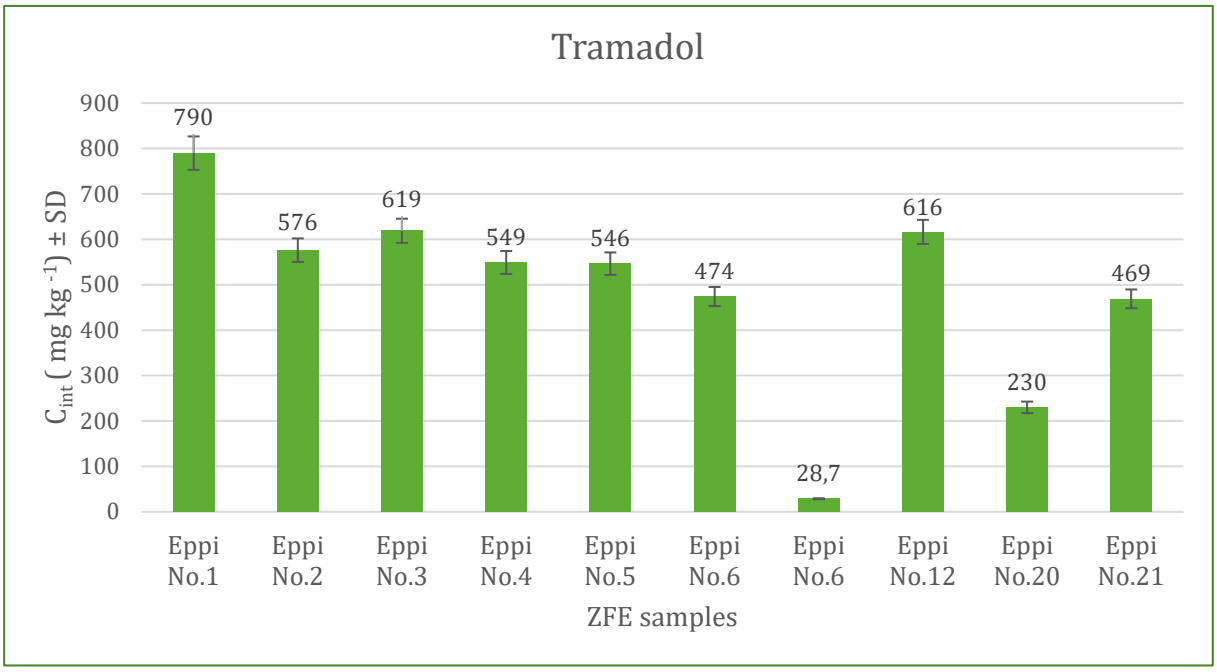
	ZFE Tramadol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 6	1	**alive**	15	Tram pH 6 2750 mg/L 96 h 15 alive 1	3.900	**790 ± 37**
	2	**alive**	15	Tram pH 6 2750 mg/L 96 h 15 alive 2	4.400	**576 ± 26**
	3	**alive**	15	Tram pH 6 2750 mg/L 96 h 15 alive 3	5.100	**619 ± 27**
pH 8	4	**alive**	15	Tram pH 8 40 mg/L 96 h 15 alive 4	4.100	**549 ± 25**
	5	**alive**	15	Tram pH 8 40 mg/L 96 h 15 alive 5	4.300	**546 ± 25**
	6	**alive**	15	Tram pH 8 40 mg/L 96 h 15 alive 6	4.600	**474 ± 21**
	6	**dead**	2	Tram 15 mg/L pH9 t2 6	9.600	**28.7 ± 1.1**

	ZFE Tramadol					
pH 9	12	**alive**	15	Tram 15 mg/L pH9 *15 12	5.100	**616 ± 27**
	20	**dead**	6	Tram 15 mg/L 20t pH9 20	2.500	**230 ± 13**
	21	**alive**	15	Tram 15 mg/L pH9 *15 21	4.700	**469 ± 21**

Source: Own depiction

Figure 6.11 below, the $C_{in}t$ are presented for each pH value (6, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.11: Internal concentrations (C_{int} (mg kg^{-1})) of Tramadol in the ZFE samples



Source: Own depiction

The internal concentrations (mg kg^{-1}) from the analysis of the ZFE samples were at the same range for most of the samples (presented in Figure 6.11). Concerning the results of the ZFE samples at pH 9, the weight of Eppendorf tube no 6 was quite large (9.6 mg) for only 2 zebrafish embryos included. This probably leads to such a low Cint in comparison with the C_{int} of the rest of the ZFE samples at the three pH values.

6.3.1.4 Antihistamines

Ketotifen

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Ketotifen water samples, sampled at the Start and the End of the exposure experiments at pH 9, were determined (Table 6.19). Different pH values are indicated with different colors.

Table 6.19: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Ketotifen exposure experiments

Water Ketotifen	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 9 START	709 ± 16	1500

Water Ketotifen	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng $mL^{-1)}$
pH 9 END	587 ± 13	

Source: Own depiction

Concerning the results of the analysis of the water samples reported in table above from Ketotifen exposure experiments the $C_{measured}$ at pH 9 were lower (≃ 2 times) compared to the theoretical concentration ($C_{theoretical}$= 1.500 ng mL^{-1}).

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Ketotifen in the extracts of the ZFE samples was determined (Table 6.20).

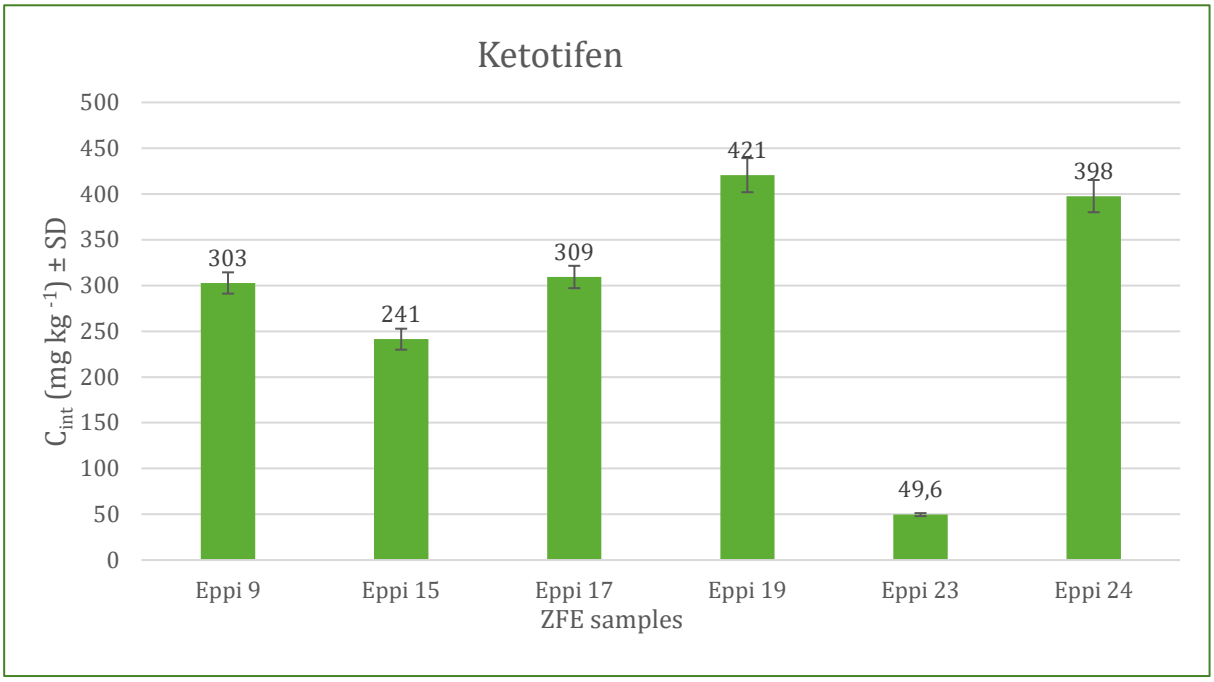
Table 6.20: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Ketotifen exposure experiments

	ZFE Ketotifen					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 9	9	**alive**	20	Ket 1,5 mg/L pH9 *20 96hpf 9	4.1000	**303 ± 12**
	15	**alive**	12	Ket 1,5 mg/L pH9 *12 96hpf 15	2.5000	**241 ± 11**
	17	**alive**	15	Ket 1,5 mg/L pH9 *15 96hpf 17	3.8000	**309 ± 12**
	19	**alive**	15	Ket 1,5 mg/L pH9 96hpf *15 19	2.9000	**421 ± 19**
	23	**dead**	7	Ketotifen 1,5 mg/L t7 pH9 96hpf 23	6.6000	**49.6 ± 1.6**
	24	**alive**	15	Ket 1,5 mg/L pH9 96hpf *15 24	2.9000	**398 ± 18**

Source: Own depiction

In Figure 6.12 below, the internal concentrations (C_{int} (mg kg^{-1})) of Ketotifen in the ZFE samples at pH 9 are presented.

Figure 6.12: Internal concentrations (C_{int} (mg kg^{-1})) of Ketotifen in the ZFE samples



Source: Own depiction

Concerning the results of the ZFE samples at pH 9, the internal concentrations (mg kg^{-1}) from the analysis of the ZFE samples were at the same range (presented in Figure 6.12). The weight of Eppi number 23 was quite large (6.6 mg) in comparison with the number of embryos (Embryos No= 7) contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples. If the weight was according to the No of embryos (≃ 1.4 mg) the internal concentration would be at the same range (C_{int}=243 mg kg^{-1}) of the rest of the ZFE samples at pH 9.

6.3.1.5 Beta blockers

Metoprolol

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Metoprolol water samples, sampled at the Start and the End of the exposure experiments at four different pH values (5, 6, 8 and 9), were determined (Table 6.13). Different pH values are indicated with different colours.

Table 6.21: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Metoprolol exposure experiments

Water Metoprolol	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	(9507 ± 665) * 10^3	6600 * 10^3
pH 5 END	(8689 ± 608) * 10^3	
pH 6 START	(2297 ± 161) * 10^3	3180 * 10^3
pH 6 END	(3707 ± 259) * 10^3	

Water Metoprolol	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 8 START	(77.1 ± 5.4) * 10^3	70 * 10^3
pH 8 END	(74.0 ± 5.2) * 10^3	
pH 9 START	(11.60 ± 0.81) * 10^3	10 * 10^3
pH 9 END	(10.39 ± 0.73) * 10^3	

Source: Own depiction

It's important to note that Metoprolol had high sensitivity (at level of ng mL^{-1}). Therefore, the substance Metoprolol has been easily detected in the water samples. The $C_{measured}$ of the water samples from the exposure experiments of metoprolol (reported in Table 6.21), at the different pH values (pH 6, pH 8 and pH 9), were at the same range with the theoretical ones. On contrary, the $C_{measured}$, at pH 5 was higher (almost 2 times) compared to the $C_{theoretical.}$ Furthermore, the $C_{measured}$ of the END water samples at pH 6 was higher compared to the $C_{measured}$ of the START water samples. The SD of the $C_{measured}$ at pH 5 was higher compared with the SD of the $C_{measured}$ at the other pH values. The exposure concentrations were higher at pH 5 and for this reason more stages of dilution were required. Consequently, the standard deviation was higher at pH 5.

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Metoprolol in the extracts of the ZFE samples was determined (Table 6.22). Different pH values are indicated with different colors.

Table 6.22: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Metoprolol exposure experiments

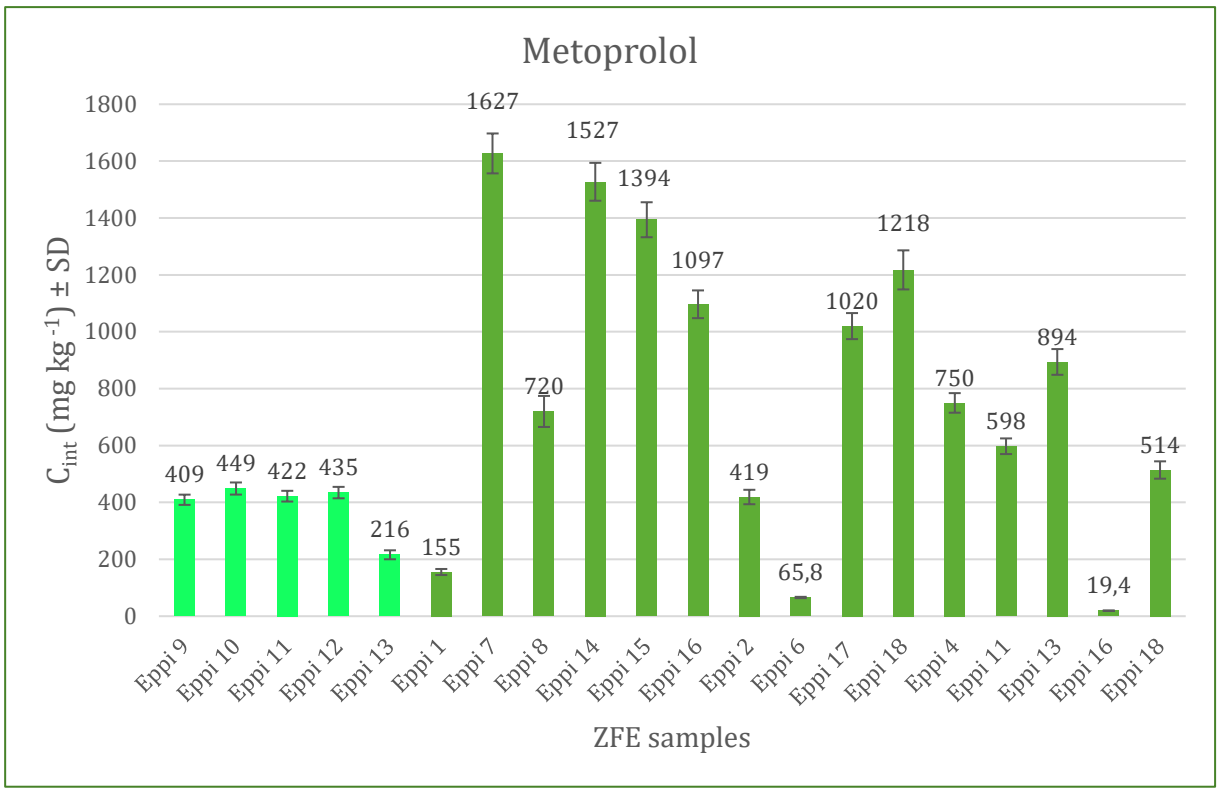
	ZFE Metoprolol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	9	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	5.8000	**409 ± 18**
	10	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	4.3000	**449 ± 21**
	11	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	5.4000	**422 ± 19**
	12	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	4.7000	**435 ± 20**
	13	**alive**	3	pH 5 Metoprolol * 96 hpf no. 3	1.5000	**216 ± 16**
pH 6	1	**dead**	1	pH 6 Metoprolol † 72 hpf no.1	1.7000	**155 ± 11**
	7	**dead**	20	pH 6 Metoprolol † 96 hpf no.20	6.3000	**1627 ± 70**
	8	**dead**	2	pH 6 Metoprolol † 96 hpf no.20	1.4000	**720 ± 55**

	ZFE Metoprolol					
	14	**alive**	20	pH 6 Metoprolol * 96 hpf no. 20	6.0000	**1527 ± 67**
	15	**alive**	20	pH 6 Metoprolol * 96 hpf no. 20	5.7000	**1394 ± 62**
pH 8	16	**alive**	17	pH 6 Metoprolol * 96 hpf no. 17	5.5000	**1097 ± 49**
	2	**dead**	6	pH 8 Metoprolol † 72 hpf no.6	2.2000	**419 ± 25**
	6	**dead**	14	pH 8 Metoprolol † 96 hpf no.14	56.7000	**65.8 ± 2.3**
	17	**alive**	20	pH 8 Metoprolol * 96 hpf no. 20	5.3000	**1020 ± 46**
pH 9	18	**alive**	8	pH 8 Metoprolol * 96 hpf no. 8	2.6000	**1218 ± 69**
	4	**alive**	15	Met 10mg/L *15 pH9 4	4.8000	**748 ± 34**
	11	**alive**	15	Met 10mg/L pH9 *15 11	4.8000	**598 ± 28**
	13	**dead**	18	Met 10mg/L 18t pH9 13	3.5000	**894 ± 45**
	16	**dead**	1	Met 10mg/L 16t pH9 16	2.8000	**19.4 ± 1.1**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (5, 6, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.13: Internal concentrations (C_{int} (mg kg^{-1})) of Metoprolol in the ZFE samples



Source: Own depiction

Concerning the results of ZFE samples of the exposure experiments to Metoprolol, the C_{int} (mg kg^{-1}) were in the same range for most of the samples (presented in Figure 6.13). Most of the ZFE samples at pH 5 had lower internal concentrations in comparison with the rest of the samples at the other pH values (pH 6, 8 and pH 9). Regarding the results of the ZFE samples at pH 8, the weight of Eppendorf tube number 6 was quite large (56.7 mg) in comparison with the number of embryos (Embryos No= 14) contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples. If the weight was according to the No of embryos (≃3.7 mg) the internal concentration would be at the same range ((C_{int}=1005 mg kg^{-1}). In addition, the ZFE sample (Eppi No 16) from the exposure experiment at pH 9 was only one dead embryo and maybe this is the reason why the C_{int} is lower than the C_{int} of the rest of the ZFE samples at pH 9.

Propranolol

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Propranolol water samples, sampled at the Start and the End of the exposure experiments at four different pH values (5, 6, 8 and 9), were determined (Table 6.23). Different pH values are indicated with different colours.

Table 6.23: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Propranolol exposure experiments

Water Propranolol	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	(921 ± 74) * 10^3	1600 * 10^3
pH 5 END	(846 ± 68) * 10^3	
pH 6 START	(428 ± 34) * 10^3	440 * 10^3
pH 6 END	(472 ± 38) * 10^3	
pH 8 START	(6.01 ± 0.48) * 10^3	9* 10^3
pH 8 END	(5.42 ± 0.43) * 10^3	
pH 9 START	413 ± 33	600
pH 9 END	241 ± 19	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of Propranolol (reported in Table 6.23), at pH 5 were lower (≃ 2 times) than the theoretical one. Furthermore, the $C_{measured}$ of the START water samples were higher compared to the $C_{measured}$ of the END water samples, for the exposure experiments at pH 5, 8 and 9. The $C_{measured}$ of the END water sample at pH 6 was higher compared to the $C_{measured}$ of the START water sample (presented in Table 6.23). However, the $_{measured}$ concentrations of the END and the START water samples were at the same range, if the SD of the $C_{measured}$ at pH 6 is taken into consideration. The SD of the $C_{measured}$ at pH 5 and pH 6 was higher compared with the SD of the $C_{measured}$ at pH 8 and pH 9. The exposure concentrations of were higher at pH 5 and pH 6 and for this reason more stages of dilution were required. Consequently, the standard deviation was higher at pH 5 and at pH 6.

ZFE Samples

- Internal Concentration
  The internal concentration (C_{int} ± SD (mg kg^{-1})) of Propranolol in the extracts of the ZFE samples was determined (Table 6.24). Different pH values are indicated with different colors.

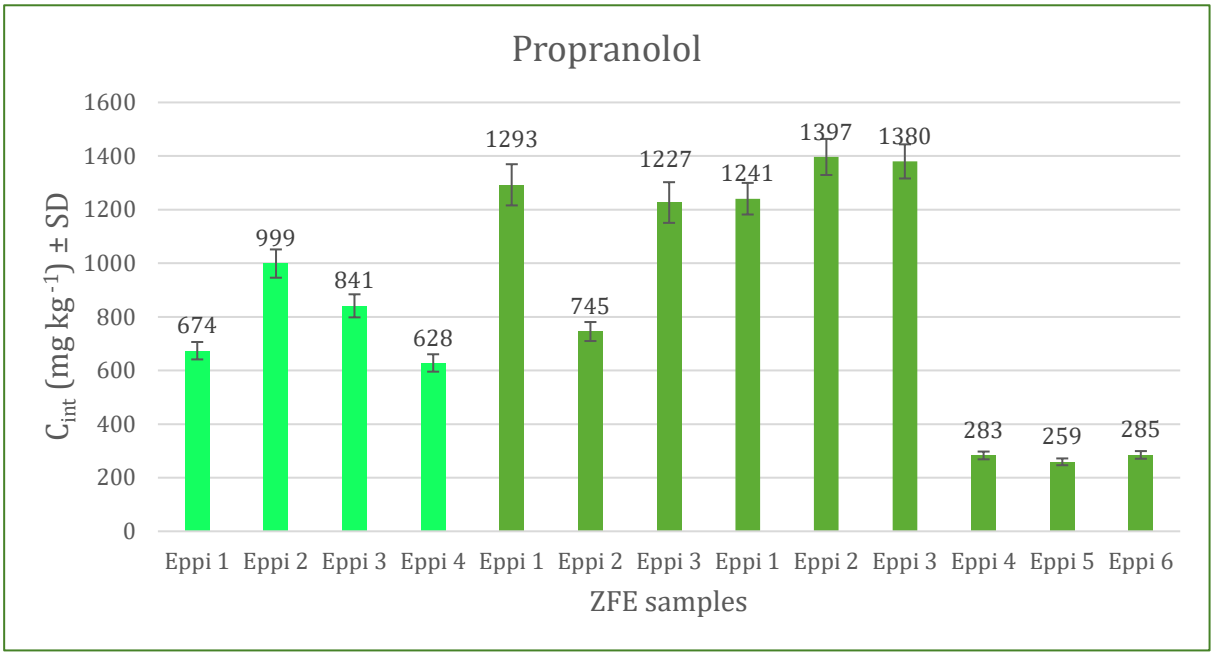
Table 6.24: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Propranolol exposure experiments

	ZFE Propranolol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**alive**	15	Prop pH 5 1600 mg/L 15 alive 96 hpf 1	4.1000	**674 ± 32**
	2	**alive**	15	Prop pH 5 1600 mg/L 15 alive 96 hpf 2	3.1000	**999 ± 53**
	3	**alive**	13	Prop pH 5 1600 mg/L 13 alive 96 hpf 3	3.4000	**841 ± 43**
	4	**dead**	14	Prop pH 5 1600 mg/L 14 dead 96 hpf 4	3.3000	**628 ± 32**
pH 6	1	**alive**	9	Prop pH 6 440 mg/L 9* 1	2.3000	**1293 ± 77**
	2	**alive**	9	Prop pH 6 440 mg/L 9* 2	4.3000	**745 ± 35**
	3	**alive**	8	Prop pH 6 440 mg/L 8* 3	2.1000	**1227 ± 76**
pH 8	1	**alive**	20	Prop pH 8 9 mg/L 20* 1	4.3000	**1241 ± 59**
	2	**alive**	20	Prop pH 8 9 mg/L 20* 2	4.1000	**1397 ± 67**
	3	**alive**	20	Prop pH 8 9 mg/L 20* 3	4.8000	**1380 ± 64**
pH 9	4	**alive**	16	Prop pH 9 0.6 mg/L 16* 4	3.4000	**283 ± 14**
	5	**alive**	16	Prop pH 9 0.6 mg/L 16* 5	3.9000	**259 ± 13**
	6	**alive**	15	Prop pH 9 0.6 mg/L 15* 6	3.6000	**285 ± 14**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value using bar charts. Different pH values are indicated with different colors.

Figure 6.14: Internal concentrations (C_{int} (mg kg^{-1})) of Propranolol in the ZFE samples



Source: Own depiction

Regarding the results of ZFE samples of the exposure experiments to Propranolol, the C_{int} (mg kg^{-1}) were in the same range for most of the samples (presented in Figure 6.14). Most of the ZFE samples at pH 9 had lower internal concentrations in comparison with the rest of the samples at the other pH values (pH 5,6 and pH 8). Regarding the results of the ZFE samples at pH 6, the weight of Eppi number 2 was quite large (4.3 mg) in comparison with the number of embryos (Embryos No=9) contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples. If the weight was according to the No of embryos (≃2.2 mg) the internal concentration would be at the same range ((C_{int}=1457 mg kg^{-1}).

6.3.1.6 Estrogen receptor modulator

Enclomiphene

Water Samples

Concentration levels ($C_{measured}$ (ng mL^{-1})) of Enclomiphene water samples, sampled at the Start and the End of the exposure experiments at two different pH values (8, 9), were determined (Table 6.25). Different pH values are indicated with different colors.

Table 6.25: Measured concentration levels (ng mL^{-1}) of water samples from Enclomiphene exposure experiments

Water Enclomiphene	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 8 START	< LOQ	3100
pH 8 END	< LOQ	
pH 9 START	< LOQ	3100
pH 9 END	< LOQ	

Source: Own depiction

Concerning the results of the analysis of the water samples reported in Table 6.25, the $C_{measured}$ (ng mL^{-1})) of all the water samples (START and END) were lower than the limit of quantification (LOQ= 114 ng mL^{-1}). The $C_{measured}$ of the water samples at pH 8 and pH 9 were significantly lower (at least 30 times) compared to the theoretical concentrations (C= 3.1 mg L^{-1}), since the water samples of both pH values were not diluted at all. Therefore, the fact that $C_{measured}$ was lower than the $C_{theoretical}$, could not be attributed to a potential mistake during sample preparation. It's important to note that the Enclomiphene had great sensitivity (at the level of ng mL^{-1}). Therefore, the substance Enclomiphene should have been easily detected in the water samples which were not diluted at all (*since theoretically, the concentration was at the level of mg L^{-1}*).

ZFE Samples

- Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Enclomiphene in the extracts of the ZFE samples were determined (Table 6.26). Different pH values are indicated with different colors.

Table 6.26: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Enclomiphene exposure experiments

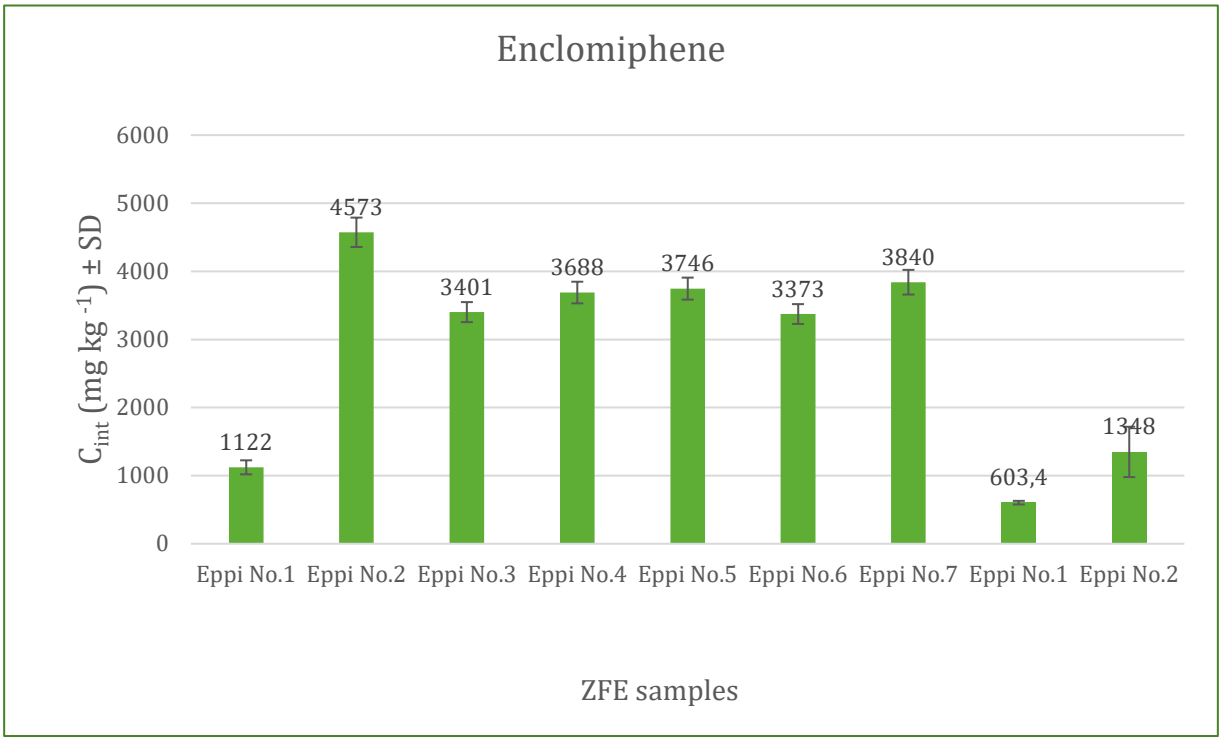
	ZFE Enclomiphene					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 8	1	**dead**	4	Enclo pH 8 D4	0.969	**1122 ±103**
	2	**alive**	15	Enclo pH 8 A15	3.632	**4573 ± 216**
	3	**alive**	20	Enclo pH 8 A20	4.843	**3401 ± 147**
	4	**alive**	20	Enclo pH 8 A20	4.843	**3688 ± 159**
	5	**alive**	20	Enclo pH 8 A20	4.843	**3746 ± 162**
	6	**alive**	20	Enclo pH 8 A20	4.843	**3373 ± 146**

	ZFE Enclomiphene					
	7	**alive**	15	Enclo pH 8 A15	3.632	**3840 ± 181**
pH 9	1	**dead**	19	Enclo pH 9 D19	4.601	**603 ± 26**
	2	**dead**	1	Enclo pH 9 D1	0.242	**1348 ± 369**

Source: Own depiction

In the figure (Figure 6.15) below, the C_{int} are presented for each pH value (8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.15: Internal concentration (C_{int} (mg kg^{-1})) of Enclomiphene in the ZFE samples.



Source: Own depiction

As far as the results of ZFE samples of the Enclomiphene exposure experiments (presented in Table 6.26) is concerned, the C_{int} (mg kg^{-1}) were at the same range for most of the samples, especially at the samples at pH 8. Regarding the ZFE samples at pH 9, there wasn't a representative picture of the C_{int} (mg kg^{-1}), since there were not enough ZFE samples of the exposure experiments at this pH value. In addition, it's worth mentioning that the C_{int} was lower in the "dead" ZFE samples (Eppi 1 at pH8 and both Eppendorf at pH 9) in comparison with the rest of the samples (presented in Figure 6.15).

6.3.1.7 Anti-depressants

Amitriptyline

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Amitriptyline water samples, sampled at the Start and the End of the exposure experiments at four different pH values (5, 6, 8, 9), were determined (Table 6.27). Different pH values are indicated with different colours.

Table 6.27: Measured concentration levels (ng mL^{-1}) of water samples from Amitriptyline exposure experiments.

Water Amitriptyline	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	(110.9 ± 7.8) * 10^3	225 * 10^3
pH 5 END	(107.0 ± 7.5) * 10^3	
pH 6 START	(58.4 ± 5.6) * 10^3	89160
pH 6 END	(60.9 ± 1.7) * 10^3	
pH 8 START	82.2 ± 7.1	2100
pH 8 END	72.5 ± 6.0	
pH 9 START	25.4 ± 2.0	1600
pH 9 END	19.86 ± 0.94	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of Amitriptyline (reported in Table), at pH 5 , pH 8 and pH 9, were lower (≃ 2, 25 and 65 times respectively) compared to the theoretical concentrations. The water sample at pH 8 was diluted only 2 times (*in the vial prior to analysis),* whereas the water sample at pH 9 was not diluted at all. Therefore, the fact that $C_{measured}$ was significantly lower than the $C_{theoretical}$, could not be attributed to a potential mistake during sample preparation. The $C_{measured}$ of the END water sample at pH 6 was higher compared to the $C_{measured}$ of the START water sample (presented in Table). However, the measured concentrations of the END and the START water samples were at the same range, if the SD of the $C_{measured}$ at pH 6 is taken into consideration. The SD of the $C_{measured}$ at pH 5 and pH6 were higher compared with the SD of the Cmeasured at the other two pH values. The exposure concentrations of Amitriptyline were higher at these pH values and for this reason more stages of dilution were required. The analytical error appears to increase when more dilution steps are performed. This can explain the fact that the standard deviation was higher at pH 5 and pH6 compared to the SD at the other two pH values.

ZFE Samples

► Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Amitriptyline in the extracts of the ZFE samples was determined (Table 6.28). Different pH values are indicated with different colors.

Table 6.28: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Amitriptyline exposure experiments.

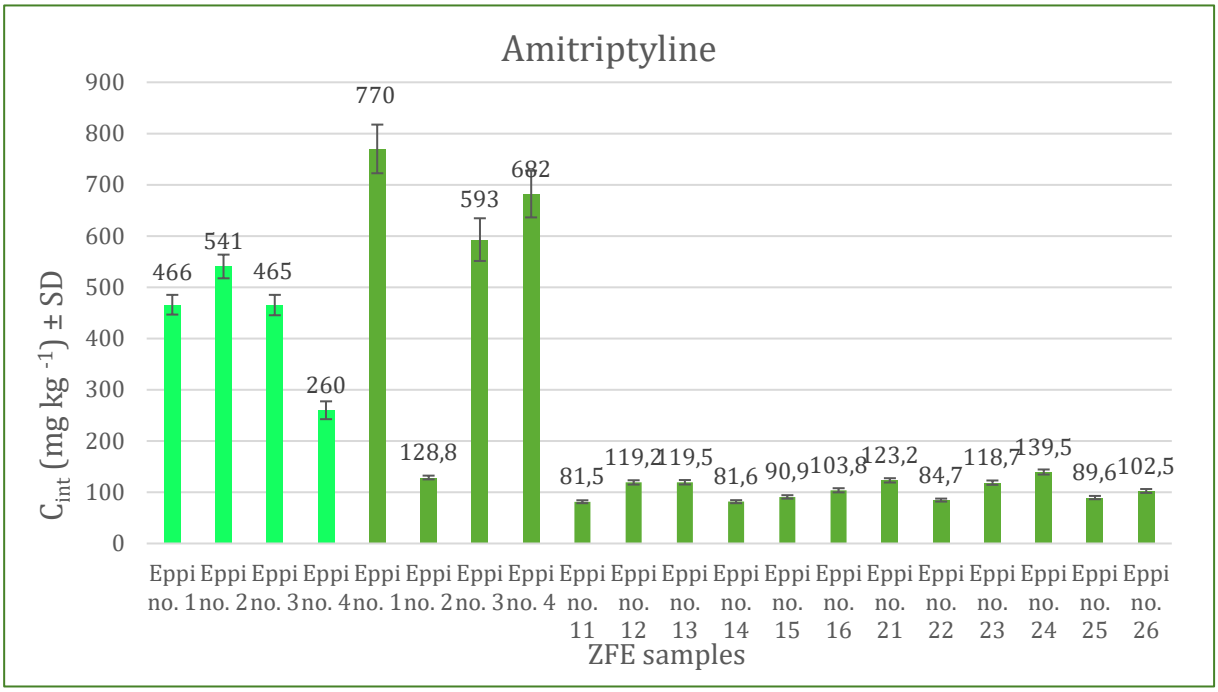
	ZFE Amitriptyline					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**alive**	15	Ami pH 5 225 mg/L 15 alive 96 hpf 1	3.600	**466 ± 19**
	2	**alive**	15	Ami pH 5 225 mg/L 15 alive 96 hpf 2	3.300	**541 ± 23**

	ZFE Amitriptyline					
	3	**alive**	15	Ami pH 5 225 mg/L 15 alive 96 hpf 3	3.300	**465 ± 20**
	4	**dead**	4	Ami pH 5 225 mg/L 4 dead 96 hpf 4	1.400	**260 ± 17**
pH 6	1	**dead**	12	Am pH 6 12x dead 48 h 1	1.600	**770 ± 47**
	2	**dead**	9	Am pH 6 2x dead 96 h 2	16.10	**128.7 ± 3.7**
	3	**dead**	2	Am pH 6 2x dead 96 h 3	1.300	**593 ± 42**
	4	**alive**	4	Am pH 6 4x alive 96 h 4	1.400	**682 ± 46**
pH 8	11	**alive**	20	Am pH 8 20x alive 96 h 11	5.900	**81.5 ± 2.8**
	12	**alive**	20	Am pH 8 20x alive 96 h 12	5.600	**119.2 ± 4.2**
	13	**alive**	20	Am pH 8 20x alive 96 h 13	5.100	**119.5 ± 4.4**
	14	**alive**	20	Am pH 8 20x alive 96 h 14	5.200	**81.6 ± 3.0**
	15	**alive**	20	Am pH 8 20x alive 96 h 15	4.900	**90.9 ± 3.4**
	16	**alive**	19	Am pH 8 19x alive 96 h 16	4.100	**103.8 ± 4.1**
pH 9	21	**alive**	20	Am pH 9 20x alive 96 h 21	6.400	**123.2 ± 4.2**
	22	**dead**	21	Am pH 9 21x dead 96 h 22	6.400	**84.7 ± 2.9**
	23	**alive**	20	Am pH 9 20x alive 96 h 23	5.500	**118.7 ± 4.2**
	24	**alive**	20	Am pH 9 20x alive 96 h 24	5.600	**139.5 ± 4.9**
	25	**alive**	15	Am pH 9 15x alive 96 h 25	5.300	**89.6 ± 3.2**
	26	**alive**	15	Am pH 9 15x alive 96 h 26	4.900	**102.5 ± 3.8**

Source: Own depiction

In figure below, the C_{int} are presented for each pH value (5, 6, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.16: Internal concentration (C_{int} (mg kg^{-1})) of Amitriptyline in the ZFE samples.



Source: Own depiction

Concerning the results from the analysis of the ZFE samples, the internal concentrations (mg kg^{-1}) were at the same range for most of the samples (presented in Figure 6.16). To estimate the weight of the ZFE samples of Amitriptyline, the difference between the filled and the empty Eppi was used. Even small variations at the weight of the samples seem to affect the C^{int}. Furthermore, the weight of the Eppi number 2 was quite high (16.1 mg) in comparison with the respective number of embryos (Embryos No= 9) contained into the Eppendorf tube. This probably leads to such a low C^{int} in comparison with the C^{int} of the rest of the ZFE samples at pH 6. If the weight was according to the No of embryos (≃ 2-3mg), the internal concentration would be at the same range ((C^{int}=455 ±12 mg kg^{-1}) with the rest samples at pH 6. It's worth noting that the internal concentration for almost all the ZFE samples at pH 5 and pH 6 were significantly higher (almost 4-6 times) compared to the C^{int} of the ZFE samples at pH 8 and pH 9 (presented in Figure 6.16).

Citalopram

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Citalopram water samples, sampled at the Start and the End of the exposure experiments at the different pH values (5, 6, 8, 9), were determined (Table 6.29). Different pH values are indicated with different colours.

Table 6.29: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Citalopram exposure experiments.

Water Citalopram	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	(1132 ± 136) * 10^3	1180 * 10^3
pH 5 END	(1196 ± 143) * 10^3	
pH 6 START	(381 ± 34) * 10^3	380 * 10^3
pH 6 END	(392 ± 35) * 10^3	
pH 8 START	(19.9 ± 1.8) * 10^3	24 * 10^3

Water Citalopram	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 8 END	(26.4 ± 2.4) * 10^3	
pH 9 START	4243 ± 509	5000
pH 9 END	3219 ± 386	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of Citalopram (reported in Table 6.29), at the different pH values (pH 5, pH 6, pH 8 and pH 9), were at the same range with the theoretical ones. Furthermore, the $C_{measured}$ of the START water samples were lower compared to the $C_{measured}$ of the END water samples, for the exposure experiments at pH 5, pH6 and pH8. However, for pH 5 and pH6 they were at the same range, if the SD is taken into consideration. The SD of the $C_{measured}$ at pH 5 and pH 6 was higher compared with the SD of the $C_{measured}$ at pH 8 and pH 9. The exposure concentrations of were higher at pH 5 and pH 6 and for this reason more stages of dilution were required. Consequently, the standard deviation was higher at pH 5 and at pH 6.

ZFE Samples

- Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Citalopram in the extracts of the ZFE samples was determined (Table 6.30). Different pH values are indicated with different colors.

Table 6.30: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Citalopram exposure experiments.

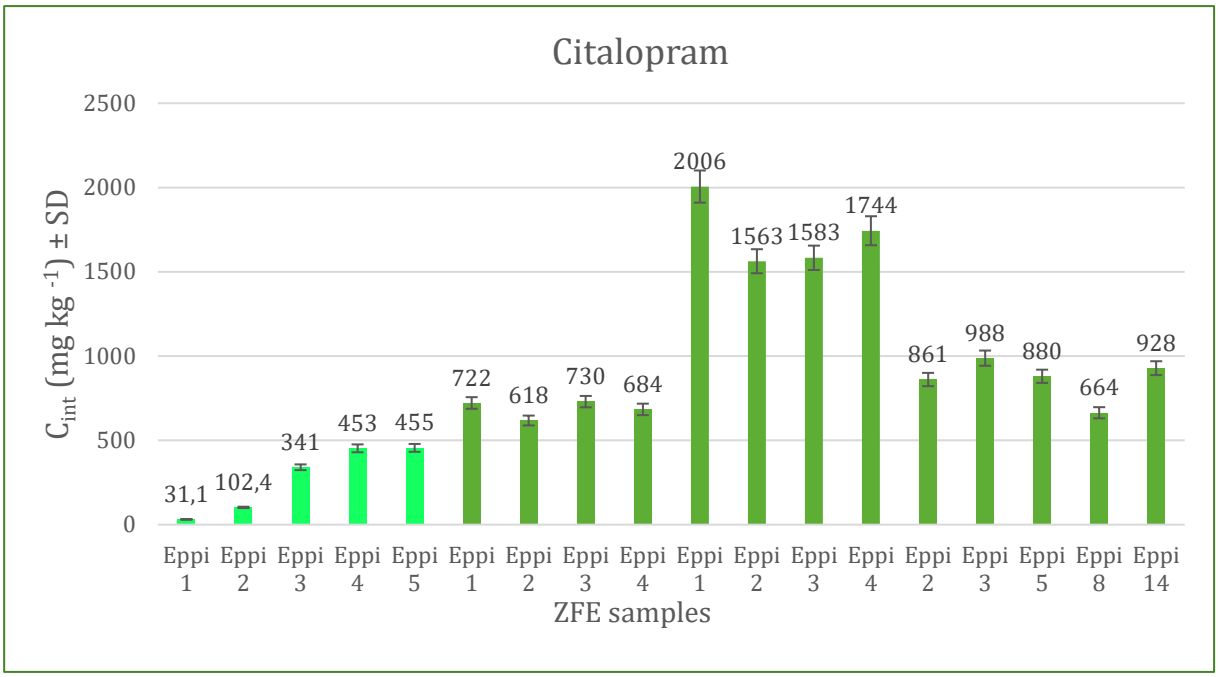
	ZFE Citalopram					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**dead**	1	Cit pH 5 1180 mg/L 1 dead 96 hpf 1	2.200	**31.1 ± 1.9**
	2	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 2	12.400	**102.4 ± 4.0**
	3	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 3	3.700	**341 ± 17**
	4	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 4	3.300	**453 ± 23**
	5	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 5	3.400	**455 ± 23**
pH 6	1	**alive**	20	Cit. pH 6 380 mg/L 20* 1	4.200	**722 ± 35**
	2	**alive**	20	Cit. pH 6 380 mg/L 20* 2	4.400	**618 ± 29**
	3	**alive**	20	Cit. pH 6 380 mg/L 20* 3	4.800	**730 ± 34**
	4	**alive**	15	Cit. pH 6 380 mg/L 15* 4	3.700	**684± 34**
pH 8	1	**alive**	20	Cit. pH 8 20 mg/L 20* 1	4.300	**2006 ± 95**
	2	**alive**	20	Cit. pH 8 20 mg/L 20* 2	5.000	**1563 ± 71**

	ZFE Citalopram					
	3	**alive**	20	Cit. pH 8 20 mg/L 20* 3	5.000	**1583 ± 72**
	4	**alive**	15	Cit. pH 8 20 mg/L 15* 4	3.800	**1744 ± 86**
pH 9	2	**alive**	16	Cit 5mg/L pH9 *16 2	4.900	**861 ± 39**
	3	**alive**	15	Cit 5mg/L pH9 *15 3	5.000	**988 ± 45**
	5	**alive**	15	Cit 5mg/L pH9 *15 5	5.500	**880 ± 39**
	8	**dead**	10	Cit 5mg/L pH9 t10 8	3.700	**664 ± 33**
	14	**alive**	17	Cit 5mg/L pH9 *17 14	5.600	**928 ± 41**

Source: Own depiction

In figure below, the C_{int} are presented for each pH value (5, 6, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.17: Internal concentration (C_{int} (mg kg^{-1})) of Citalopram in the ZFE samples.



Source: Own depiction

Regarding the results of ZFE samples of the exposure experiments (presented on Table 6.30), the C_{int} (mg kg^{-1}) were in the same range for most of the samples (presented in Figure 6.17). Most of the ZFE samples at pH 5 had lower internal concentrations whereas at pH 8 had higher C_{int} ,in comparison with the rest of the samples at the other pH values. Regarding the results of the ZFE samples at pH 5, the weight of Eppi number 1 was quite large for 1 embryo contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples at pH 5. If the weight of the Eppi No 1 was 0.22 mg (1 ZFE), the internal concentration would be at the same range (C_{int}=311 mg kg^{-1}). Furthermore, the weight of Eppi number 2 at pH 5, The weight was quite large (12.4 mg) in comparison with the number of embryos (Embryos No= 15) contained in the Eppendorf tube. This probably leads to such a low C_{int}. If the weight of the Eppi No 2 was according to the No of embryos (≃3.3 mg), the internal concentration would be at the same range ((C_{int}≃380 mg kg^{-1}).

Fluoxetine

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Fluoxetine water samples, sampled at the Start and the End of the exposure experiments at the different pH values (5, 6, 8, 9), were determined (Table 6.31). Different pH values are indicated with different colours.

Table 6.31: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Fluoxetine exposure experiments

Water Fluoxetine	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	(152 ± 11) * 10^3	220 * 10^3
pH 5 END	(215 ± 15) * 10^3	
pH 6 START	(33.8 ± 2.4) * 10^3	55 * 10^3
pH 6 END	(34.8 ± 2.4) * 10^3	
pH 8 START	1106 ± 77	2000
pH 8 END	758 ± 53	
pH 9 START	174 ± 12	500
pH 9 END	83 ± 6	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of Fluoxetine (reported in Table 6.31), at the different pH values at pH 5 and pH 6 were at the same range with the theoretical ones. The $C_{measured}$ of the water samples from the exposure experiments at pH 8 and pH 9 were lower (almost 2 and 5 times respectively) than the theoretical one. Furthermore, the $C_{measured}$ of the START water samples were lower compared to the $C_{measured}$ of the END water samples, for the exposure experiments at pH 5 and pH 6. However, for pH 6 they were at the same range, if the SD is taken into consideration. The SD of the $C_{measured}$ at pH 5 was higher compared with the SD of the $C_{measured}$ at the other pH values. The exposure concentrations of were higher at pH 5 and for this reason more stages of dilution were required.

ZFE Samples

- Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Fluoxetine in the extracts of the ZFE samples was determined (Table 6.32). Different pH values are indicated with different colors.

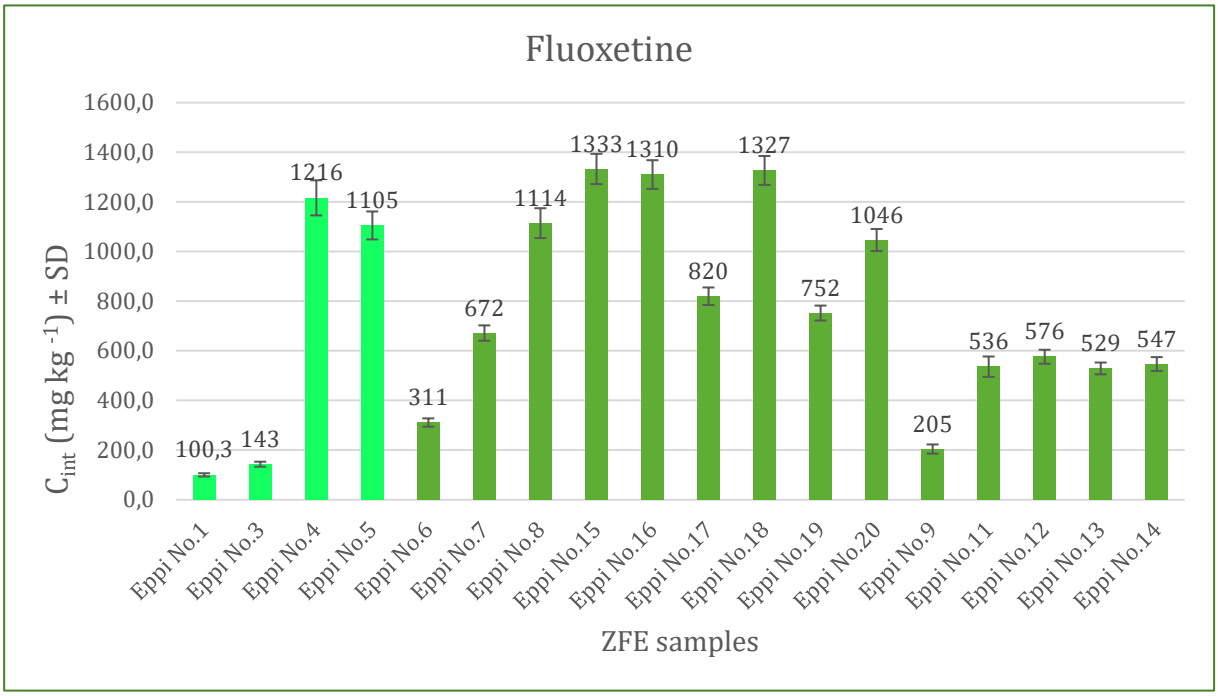
Table 6.32: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Fluoxetine exposure experiments

	ZFE Fluoxetine					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**dead**	1	pH5 Fluo 1	1.700	**100.3 ± 6.6**
	3	**dead**	2	pH5 Fluo 3	1.500	**143 ± 10**
	4	**alive**	11	pH5 Fluo * 4	2.200	**1216 ± 71**
	5	**alive**	13	pH5 Fluo * 5	3.000	**1105 ± 56**
pH 6	6	**dead**	5	pH6 Fluo 6	2.600	**311 ± 17**
	7	**alive**	15	pH6 Fluo * 7	4.000	**672 ± 31**
	8	**alive**	18	pH6 Fluo * 8	2.600	**1114 ± 60**
pH 8	15	**alive**	16	pH8 Fluo * 15	4.100	**1333 ± 61**
	16	**alive**	18	pH8 Fluo * 16	4.600	**1310 ± 58**
	17	**alive**	20	pH8 Fluo * 17	5.000	**820 ± 35**
	18	**alive**	20	pH8 Fluo * 18	4.700	**1327 ± 58**
	19	**alive**	17	pH8 Fluo * 19	6.800	**752 ± 30**
	20	**alive**	14	pH8 Fluo * 20	5.300	**1046 ± 44**
pH 9	9	**dead**	3	pH9 Fluo 9	1.000	**205 ± 18**
	11	**dead**	7	pH9 Fluo 11	1.300	**536 ± 41**
	12	**alive**	16	pH9 Fluo * 12	3.400	**576 ± 28**
	13	**alive**	17	pH9 Fluo * 13	4.300	**529 ± 24**
	14	**alive**	13	pH9 Fluo * 14	3.000	**547 ± 28**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (5, 6, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.18: Internal concentration (C_{int} (mg kg^{-1})) of Fluoxetine in the ZFE samples



Source: Own depiction

As far as the results of ZFE samples of the Fluoxetine exposure experiments (presented in Table 6.32) is concerned, the C_{int} (mg kg^{-1}) were at the same range for most of the samples, especially at pH 5, pH 6 and pH 8. Most of the ZFE samples at pH 9 had lower internal concentrations in comparison with the rest of the samples at the other pH values. It's worth mentioning that the C_{int} was lower in the "dead" ZFE samples (Eppi 1 and 3 at pH 5 and Eppi 6 at pH 6) in comparison with the rest of the samples (presented in Figure).

Sertraline

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Sertraline water samples, sampled at the Start and the End of the exposure experiments at three different pH values (6, 8, 9), were determined (Table 6.33). Different pH values are indicated with different colors.

Table 6.33: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Sertraline exposure experiments

Water Sertraline	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 6 START	2867± 109	10500
pH 6 END	3379 ± 466	
pH 8 START	306 ± 15	1100
pH 8 END	69.3 ± 6.0	
pH 9 START	114.9 ± 3.5	100
pH 9 END	83.5 ± 4.8	

Source: Own depiction

Concerning the results from the analysis of the water samples (reported in Table 6.33), the $C_{measured}$ of the END water sample was higher compared to the $C_{measured}$ of the START water sample, for the exposure experiments at pH 6. On the contrary, the $C_{measured}$ of the END water samples at pH 8 and pH 9 was significantly lower compared to the respective START samples. As far as the water samples at pH 6 and pH 8 is concerned, $C_{measured}$ were lower ($\simeq$ 3 times) compared to the theoretical concentration. Moreover, the $C_{measured}$ of the START water sample, for the exposure experiments at pH 9 was slightly higher compared to the theoretical one. The exposure concentration of Sertraline was higher at pH 6 and for this reason, more stages of dilution were required. This can explain the fact that the standard deviation of the $C_{measured}$ at pH 6 was higher compared to the SD of the $C_{measured}$ at the other two pH values (pH 8 and pH 9).

ZFE Samples

► Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Sertraline in the extracts of the ZFE samples was determined (Table 6.34). Different pH values are indicated with different colors.

Table 6.34: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Sertraline exposure experiments

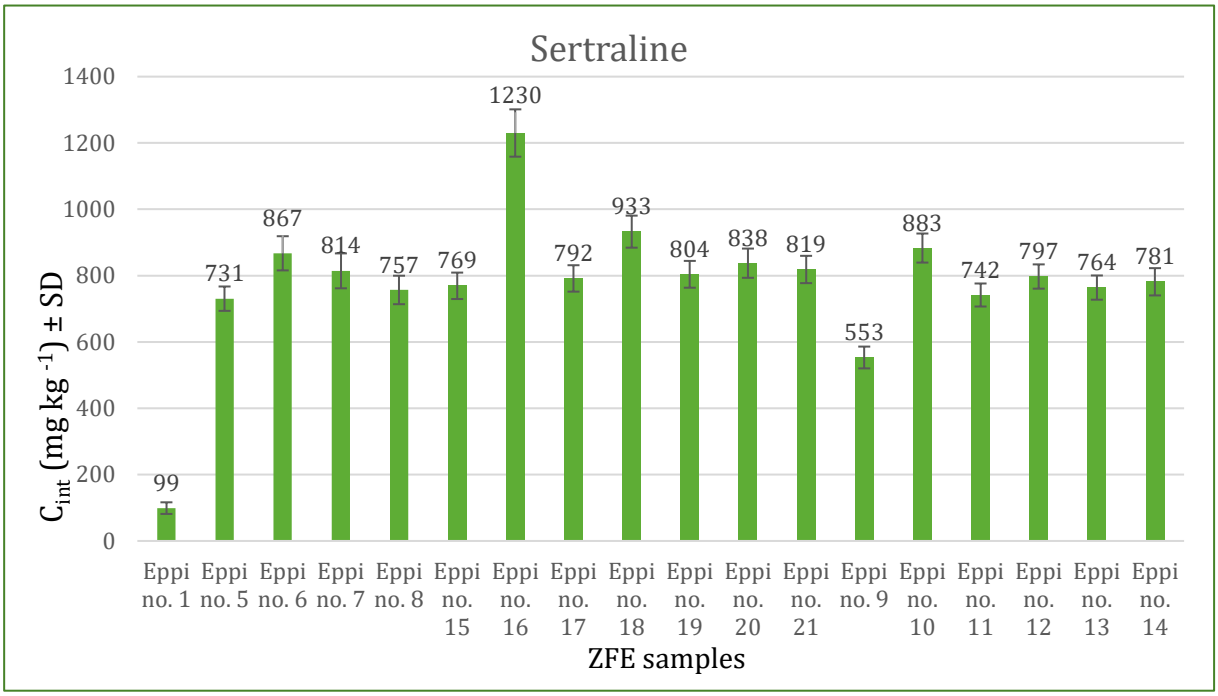
	ZFE Sertraline					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 6	1	**dead**	1	1.PHION pH 6 Sertralin 72h #1	0.400	**99 ± 18**
	5	**alive**	10	5.PHION pH 6 Sertralin 96h lebend #10	2.900	**731 ± 37**
	6	**alive**	16	6.PHION pH 6 lebend Sertralin #16	2.000	**867 ± 52**
	7	**alive**	8	7.PHION pH 6 Sertralin lebend #8 96h	1.700	**814 ± 53**
	8	**alive**	11	8.PHION pH 6 leb.Sertralin 96h #11	2.200	**757 ± 43**
pH 8	15	**alive**	12	15.PHION pH 8 Sertralin 96h #12 lebend	2.700	**769 ± 40**
	16	**alive**	12	16.PHION pH 8 Sertralin 96h #12 lebend	2.100	**1230 ± 71**
	17	**alive**	12	17.PHION pH 8 Sertralin 96h #12 lebend	2.900	**792 ± 40**
	18	**alive**	12	18.PHION pH 8 Sertralin 96h #12 lebend	2.700	**933 ± 48**
	19	**alive**	12	19.PHION pH 8 Sertralin 96h #12 lebend	2.900	**804 ± 40**
	20	**alive**	12	20.PHION pH 8 Sertralin 96h #12 lebend	2.600	**838 ± 44**

	ZFE Sertraline					
	21	**alive**	15	21.PHION pH 8 Sertralin 96h #15 lebend	2.900	**819 ± 41**
pH 9	9	**dead**	8	9. PHION pH 9 tot Sertralin #8 96h	2.000	**553 ± 33**
	10	**alive**	11	10.PHION pH 9 Sertralin lebend 96h #11	3.000	**883 ± 44**
	11	**alive**	13	11.PHION pH 9 lebend Sertralin 96h #13	3.500	**742 ± 35**
	12	**alive**	13	12.PHION pH 9 lebend Sertralin 96h #13	3.700	**797 ± 37**
	13	**alive**	13	13.PHION pH 9 Sertralin lebend 96h #13	3.300	**764 ± 37**
	14	**alive**	9	14.PHION pH 9 Sertralin lebend 96h #9	2.600	**781 ± 41**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (6, 8, 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.19: Internal concentration (C_{int} (mg kg^{-1})) of Sertraline in the ZFE samples



Source: Own depiction

The internal concentrations (mg kg^{-1}) from the analysis of the ZFE samples were at the same range for most of the samples (presented in Figure 6.19). Regarding the results of the ZFE samples, the C_{int} of the two ZFE samples differed from the overall samples. Firstly, the C_{int} at Eppi 1, from exposure experiments at pH 6 was lower in comparison with the rest of the samples at pH 6. The weight of Eppi number 1 was quite large 1 embryo contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples at pH 6. Moreover, concerning the results of the ZFE samples at pH 8, the internal concentration at

Eppi number 16 was higher in comparison with the rest of the samples (presented in Table 6.34). The weight of the Eppi 16 was lower in comparison with the other samples, that contained the same number of embryos (Embryos No=12). If the weight of the Eppi 16 was approximately 2.700 mg (*namely, similar to the rest of the samples with 12 ZFE/ Eppi*), the C_{int} would be in the same range (C_{int}=957 ± 21 mg kg^{-1}) as the rest of the samples at pH 8. Small variations at the weight of samples seems to affect the C_{int}.

6.3.2 Pesticides

6.3.2.1 Chlorophenols

2,3-Dichlorophenol (DCP)

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of 2,3-Dichlorophenol water samples, sampled at the Start and the End of the exposure experiments at three different pH values (5, 6 and8), were determined (Table 6.35). Different pH values are indicated with different colours.

Table 6.35: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from 2,3-Dichlorophenol exposure experiments

Water 2,3-Dichlorophenol	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	1278 ±69	6000
pH 5 END	336 ± 18	
pH 6 START	2072 ± 112	5000
pH 6 END	311 ± 17	
pH 8 START	2221 ± 120	7500
pH 8 END	2600 ± 140	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of 2,3-Dichlorophenol, at the different pH values (pH 5, pH 6 and pH 8), were lower (almost three times) compared to the $C_{theoretical}$. The DCP had high sensitivity (at the level of ng mL^{-1}). Therefore, the substance DCP should have been easily detected in the water samples which were diluted $\simeq$ 12 times. Furthermore, the $C_{measured}$ of the END water samples were lower compared to the $C_{measured}$ of the START water samples, for the exposure experiments at pH 5 and pH6. It also important to mention that the $C_{measured}$ of the END water samples at pH 5 and pH 6 was really low in comparison with the $C_{theoretical}$.

ZFE Samples

► Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of 2,3-Dichlorophenol in the extracts of the ZFE samples was determined (Table 6.36). Different pH values are indicated with different colors.

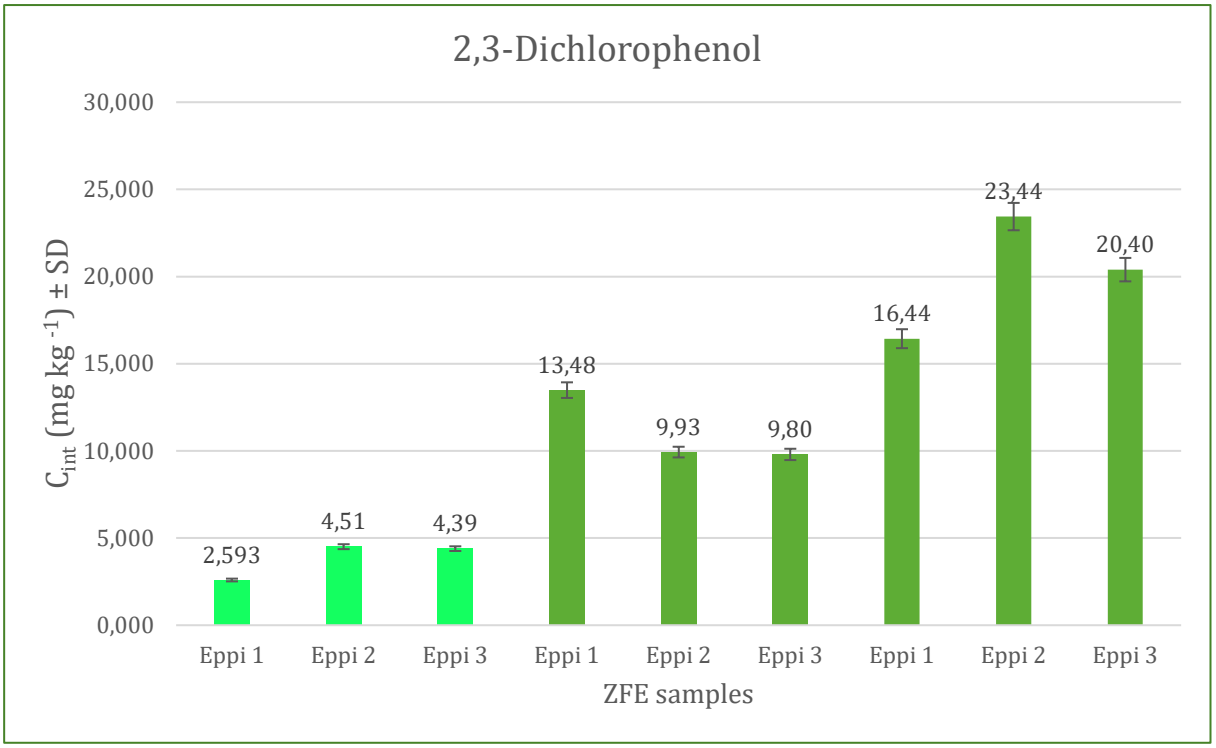
Table 6.36: Measured internal concentrations (mg kg^{-1}) of ZFE samples from 2,3-Dichlorophenol exposure experiments

	ZFE 2,3-Dichlorophenol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**alive**	19	DCP pH5 6 mg/L 19* (1)	17.40	**2.593 ± 0.079**
	2	**alive**	20	DCP pH5 6 mg/L 20* (2)	14.50	**4.51 ± 0.14**
	3	**alive**	20	DCP pH5 6 mg/L 20* (3)	17.10	**4.39 ± 0.13**
pH 6	1	**alive**	20	DCP pH6 5 mg/L 20* (1)	9.70	**13.48 ± 0.45**
	2	**alive**	19	DCP pH6 5 mg/L 19* (2)	15.90	**9.93 ± 0.31**
	3	**alive**	20	DCP pH6 5 mg/L 20* (3)	9.90	**9.80 ± 0.32**
pH 8	1	**alive**	20	DCP pH8 7.5 mg/L 20* (1)	9.90	**16.44 ± 0.54**
	2	**alive**	20	DCP pH8 7.5 mg/L 20* (2)	9.30	**23.44 ± 0.78**
	3	**alive**	20	DCP pH8 7.5 mg/L 20* (3)	10.10	**20.40 ± 0.67**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (5, 6, 8) using bar charts. Different pH values are indicated with different colors.

Figure 6.20: Internal concentration (C_{int} (mg kg^{-1})) of 2,3-Dichlorophenol in the ZFE samples



Source: Own depiction

As far as the results of ZFE samples of the 2,3-Dichlorophenol exposure experiments (presented in Table) is concerned, the C_{int} (mg kg^{-1}) were at the same range for most of the samples, especially at pH 6 and pH 8. Most of the ZFE samples at pH 5 had lower internal concentrations

in comparison with the rest of the samples at the other pH values. It's worth mentioning that the weight of the ZFE samples at pH 5 was higher compared with the weight of the same amount of ZFE (20 embryos) in the samples at pH 6 and pH 8. Maybe this leads to such a low C_{int} in comparison with the C_{int} of the rest of the ZFE samples at pH 6 and pH 8.

2,4,5- Trichlorophenol (TPC)

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of 2,4,5-Trichlorophenol water samples, sampled at the Start and the End of the exposure experiments at three different pH values (5, 6, 8), were determined (Table 6.37). Different pH values are indicated with different colours.

Table 6.37: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from 2,4,5-Trichlorophenol exposure experiments

Water 2,4,5-Trichlorophenol	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	294 ± 15	575
pH 5 END	< LOD	
pH 6 START	174.2 ± 9.1	500
pH 6 END	< LOD	
pH 8 START	711 ± 37	1000
pH 8 END	473 ± 25	

Source: Own depiction

Concerning the results of the analysis of the water samples reported in Table 6.37, the $C_{measured}$ (ng mL^{-1})) of END the water samples (START and END) were lower than the limit of detection (LOD= 114 ng mL^{-1}). These END water samples of both pH values were not diluted at all. Therefore, this could not be attributed to a potential mistake during sample preparation. It's important to note that the TCP had high sensitivity (at the level of ng mL^{-1}). Therefore, the substance TCP should have been easily detected in the water samples since they were not diluted at all. Furthermore, the $C_{measured}$ at pH 5 and at pH 6 were lower (almost two times) compared to the $C_{theoretical}$.

ZFE Samples

- Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of 2,4,5-Trichlorophenol in the extracts of the ZFE samples was determined (Table 6.38). Different pH values are indicated with different colors.

Table 6.38: Measured internal concentrations (mg kg^{-1}) of ZFE samples from 2,4,5-Trichlorophenol exposure experiments

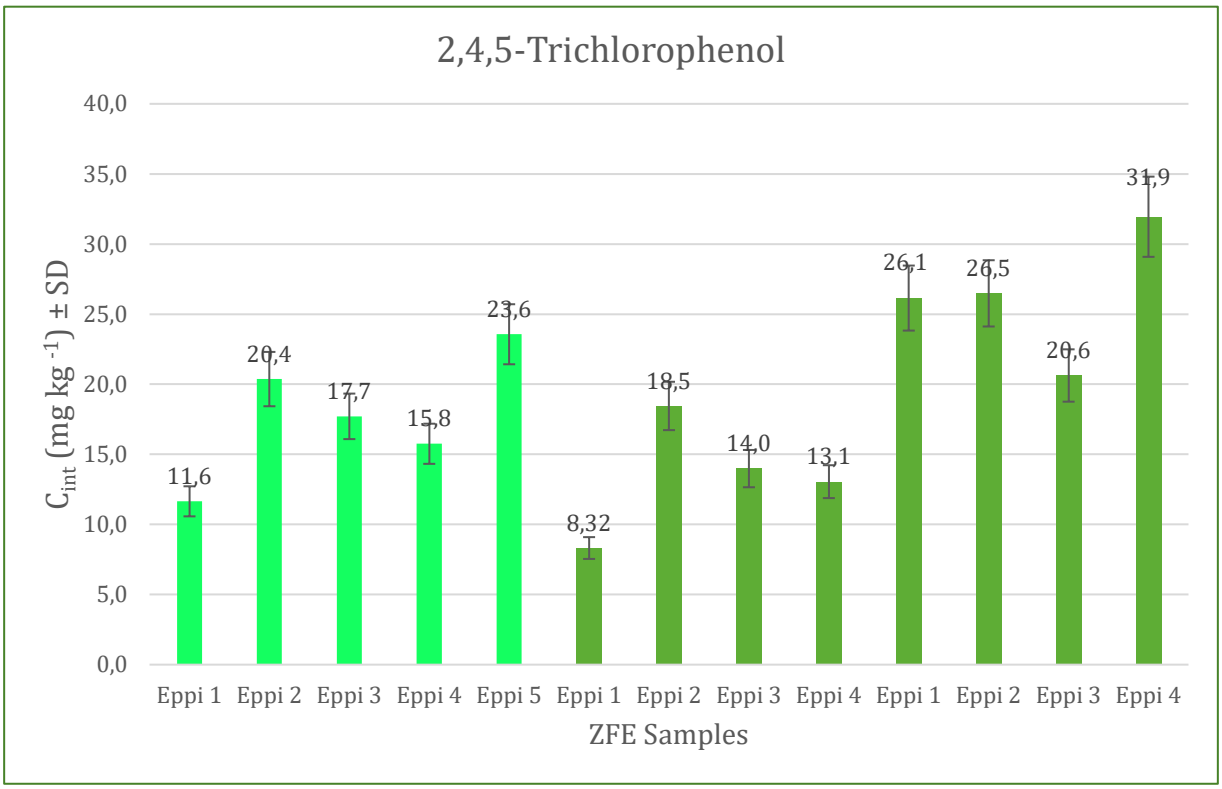
	ZFE 2,4,5-Trichlorophenol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**alive**	20	1 TCP pH5 0,575 mg/l *20	3.700	**11.6 ± 1.1**
	2	**alive**	16	2 TCP pH5 0,575 mg/l *16	3.100	**20.4 ± 1.9**

	ZFE 2,4,5-Trichlorophenol					
	3	**alive**	20	3 TCP pH5 0,575 mg/l *20	3.800	**17.7 ± 1.6**
	4	**alive**	20	4 TCP pH5 0,575 mg/l *20	4.000	**15.8 ± 1.4**
	5	**alive**	20	5 TCP pH5 0,575 mg/l *20	4.000	**23.6 ± 2.1**
pH 6	1	**alive**	15	1 TCP pH6 0,5 mg/l *15	3.400	**8.32 ± 0.78**
	2	**alive**	15	2 TCP pH6 0,5 mg/l *15	3.400	**18.5 ± 1.7**
	3	**alive**	15	3 TCP pH6 0,5 mg/l *15	3.100	**14.0 ± 1.3**
	4	**alive**	20	4 TCP pH6 0,5 mg/l *20	4.300	**13.1 ± 1.2**
pH 8	1	**alive**	20	1 TCP pH8 1 mg/l *20	4.700	**26.1 ± 2.3**
	2	**alive**	20	2 TCP pH8 1 mg/l *20	4.500	**26.5 ± 2.4**
	3	**alive**	20	3 TCP pH8 1 mg/l *20	4.100	**20.6 ± 1.9**
	4	**alive**	20	4 TCP pH8 1 mg/l *20	4.400	**31.9 ± 2.9**

Source: Own depiction

In figure below, the C_{int} are presented for each pH value (5,6, 8) using bar charts. Different pH values are indicated with different colors.

Figure 6.21: Internal concentration (C_{int} (mg kg^{-1})) of 2,4,5-Trichlorophenol in the ZFE samples



Source: Own depiction

The internal concentrations (mg kg^{-1}) from the analysis of the ZFE samples were at the same range for most of the samples (presented in Figure).

Pentachlorophenol (PCP)

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Pentachlorophenol water samples, sampled at the Start and the End of the exposure experiments at three different pH values (5, 6, 8), were determined (Table 6.39). Different pH values are indicated with different colours.

Table 6.39: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Pentachlorophenol exposure experiments

Water Pentachlorophenol	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	13.69 ± 0.46	50
pH 5 END	< LOD	
pH 6 START	27.97 ± 0.95	52
pH 6 END	< LOD	
pH 8 START	145.9 ± 4.9	260
pH 8 END	118.6 ± 4.0	

Source: Own depiction

Regarding the results of the analysis of the water samples reported in Table 6.39, the $C_{measured}$ (ng mL^{-1})) of END the water samples (START and END) were lower than the limit of detection (*LOD= 9 ng mL^{-1}*). These END water samples of both pH values were not diluted at all. It's important to note that the PCP had high sensitivity (at the level of ng mL^{-1}). Therefore, the substance PCP should have been easily detected in the water samples since the samples were not diluted at all. So, this could not be attributed to a potential mistake during sample preparation. Furthermore, the $C_{measured}$ were lower (almost two times) for the water samples at pH 6 and pH 8 and almost 4 times lower for the water samples at pH 5, compared to the $C_{theoretical}$.

ZFE Samples

► Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Pentachlorophenol in the extracts of the ZFE samples was determined (Table 6.40). Different pH values are indicated with different colors.

Table 6.40: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Pentachlorophenol exposure experiments

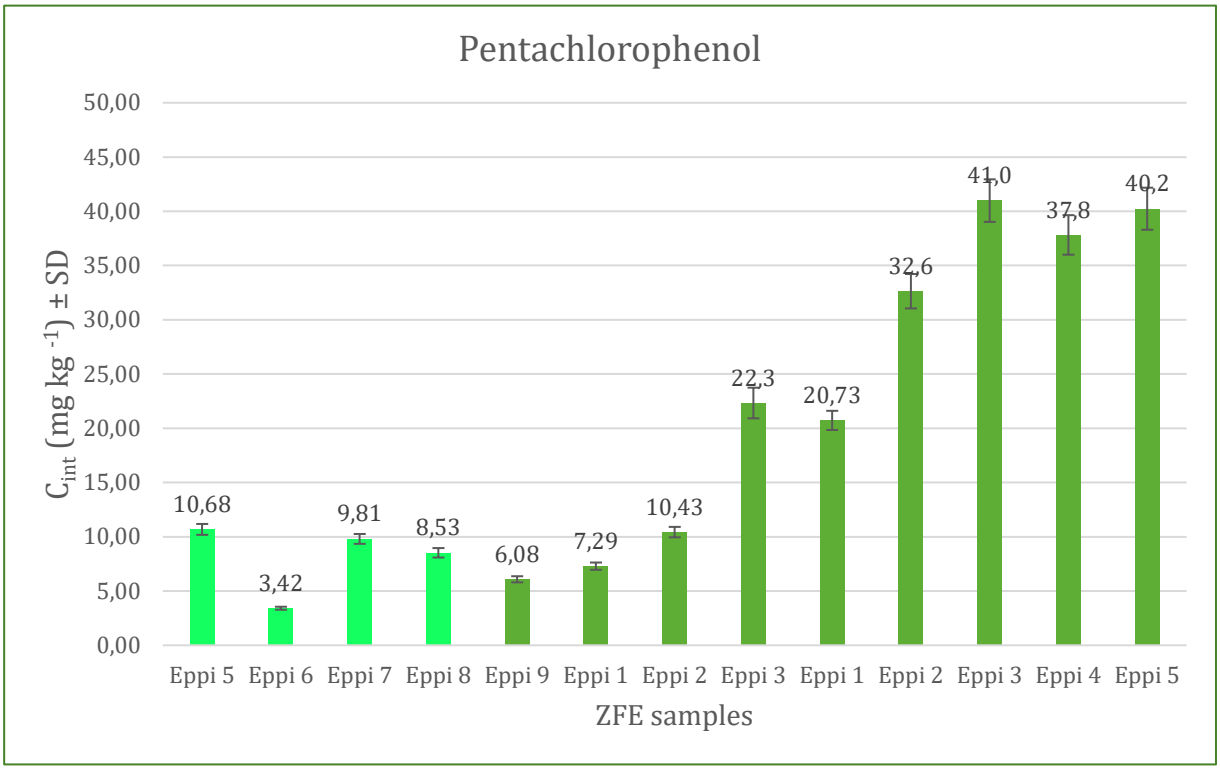
	ZFE Pentachlorophenol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	5	**alive**	20	PCP pH 5 0.05 mg/L 20* 5	4.500	**10.68 ± 0.50**
	6	**alive**	20	PCP pH 5 0.05 mg/L 20* 6	8.700	**3.42 ± 0.14**
	7	**alive**	20	PCP pH 5 0.05 mg/L 20* 7	4.700	**9.81 ± 0.45**
	8	**alive**	15	PCP pH 5 0.05 mg/L 15* 8	3.400	**8.53 ± 0.44**
pH 6	1	**alive**	20	PCP pH 6 0.052 mg/L 20* 1	4.700	**6.08 ± 0.28**
	2	**alive**	20	PCP pH 6 0.052 mg/L 20* 2	4.800	**7.29 ± 0.34**

	ZFE Pentachlorophenol					
	3	**alive**	20	PCP pH 6 0.052 mg/L 20* 3	4.800	**10.43 ± 0.48**
pH 8	1	**dead**	6	PCP pH 8 0.26 mg/L 6† 72 hpf 1	2.000	**22.3 ± 1.4**
	2	**alive**	15	PCP pH 8 0.26 mg/L 15* 96 hpf 2	6.700	**20.73 ± 0.88**
	3	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 3	4.000	**32.6 ± 1.6**
	4	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 4	4.200	**41.0 ± 2.0**
	5	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 5	4.100	**37.8 ± 1.8**
	6	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 6	4.100	**40.2 ± 1.9**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (5,6, 8) using bar charts. Different pH values are indicated with different colors.

Figure 6.22: Internal concentration (C_{int} (mg kg^{-1})) of Pentachlorophenol in the ZFE samples



Source: Own depiction

Regarding the results of ZFE samples of the exposure experiments (presented on Table 6.40), the C_{int} (mg kg^{-1}) were in the same range for most of the samples, especially at pH 5 and pH 6 (presented in Figure 6.22). Most of the ZFE samples at pH 8 had higher internal concentrations in comparison with the rest of the samples at the other pH values. Regarding the results of the ZFE samples at pH 8, the weight of Eppendorf tube number 2 was quite large in comparison with

the number of embryos (Embryos No= 15) contained in the Eppendorf tube and the weight of the rest of the samples with 20 ZFE per Eppendorf (≃4.1 mg). This probably leads to lower C_{int} in comparison with the rest internal concentrations of the ZFE samples at pH 8.

6.3.2.2 Herbicides

Bromoxynil

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Bromoxynil water samples, sampled at the Start and the End of the exposure experiments at three different pH values (5, 6, 8), were determined (Table 6.41). Different pH values are indicated with different colors.

Table 6.41: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Bromoxynil exposure experiments

Water Bromoxynil	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	144.3 ± 8.7	150
pH 5 END	87.7 ± 5.3	
pH 6 START	136.0 ± 8.2	200
pH 6 END	126.4 ± 7.6	
pH 8 START	(14.5 ± 1.3)*10^3	15*10^3
pH 8 END	(14.7 ± 1.3)*10^3	

Source: Own depiction

The $C_{measured}$ of the water samples from the exposure experiments of Bromoxynil (reported in Table 6.41), at the different pH values (pH 5, pH 6, pH 8), were at the same range with the theoretical ones. Furthermore, the $C_{measured}$ of the START water samples were higher compared to the $C_{measured}$ of the END water samples, for the exposure experiments at pH 5 and pH6. In addition, the $C_{measured}$ of the START water samples were lower compared to the $C_{measured}$ of the END water samples at pH 8. However, the $_{measured}$ concentrations were at the same range if the SD is taken into consideration. The SD of the $C_{measured}$ at pH 8 was higher compared with the SD of the $C_{measured}$ at the other pH values. The exposure concentrations of were higher at pH 8 and for this reason more stages of dilution were required and consequently, the standard deviation was higher at this pH value.

ZFE Samples

► Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Bromoxynil in the extracts of the ZFE samples was determined (Table 6.42). Different pH values are indicated with different colors.

Table 6.42: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Bromoxynil exposure experiments

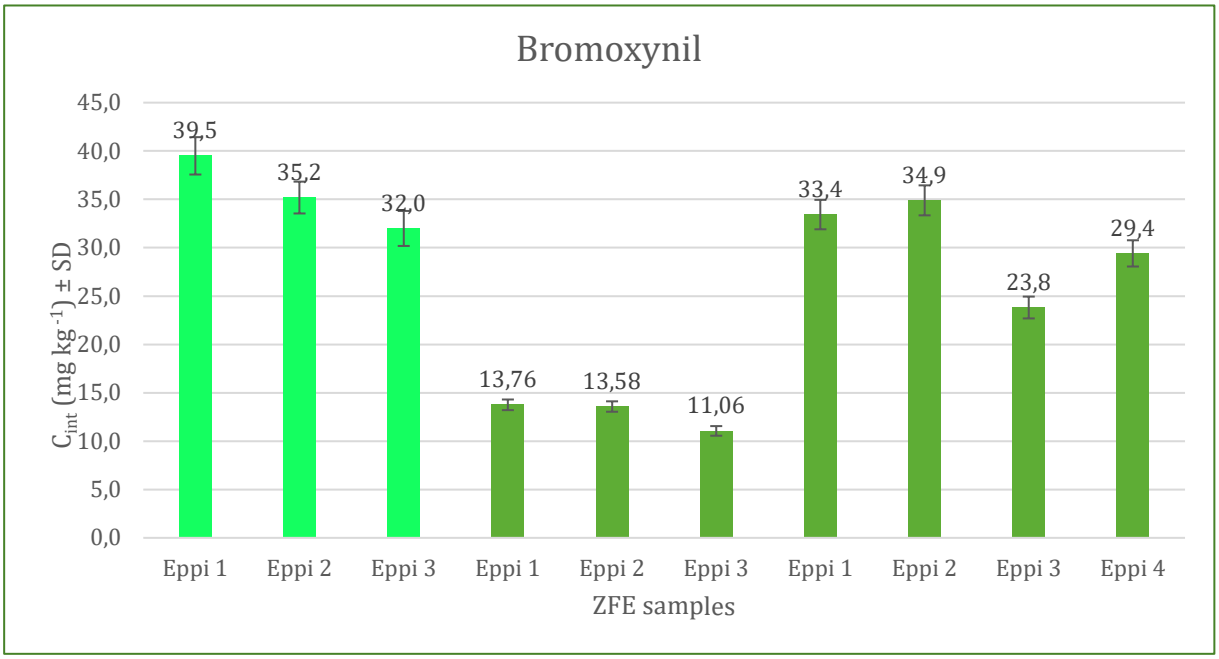
	ZFE Bromoxynil					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
	1	**alive**	20	Brom. pH5 0,15 mg/L 1 *20	2.700	**39.5 ± 1.9**

	ZFE Bromoxynil					
pH 5	2	**alive**	20	Brom. pH5 0,15 mg/L 2 *20	3.000	**35.2 ± 1.6**
	3	**alive**	15	Brom. pH5 0,15 mg/L 3 *15	2.000	**32.0 ± 1.8**
pH 6	1	**alive**	20	Brom. pH6 0,2 mg/L 1 *20	4.600	**13.76 ± 0.55**
	2	**alive**	20	Brom. pH6 0,2 mg/L 2 *20	4.700	**13.58 ± 0.54**
	3	**alive**	13	Brom. pH6 0,2 mg/L 3 *13	3.300	**11.06 ± 0.50**
pH 8	1	**alive**	20	Brom. pH8 15 mg/L 1 *20	3.200	**33.4 ± 1.5**
	2	**alive**	20	Brom. pH8 15 mg/L 2 *20	3.400	**34.9 ± 1.6**
	3	**alive**	18	Brom. pH8 15 mg/L 3 *18	2.900	**23.8 ± 1.1**
	4	**alive**	20	Brom. pH8 15 mg/L 4 *20	3.100	**29.4 ± 1.4**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (5,6, 8) using bar charts. Different pH values are indicated with different colors.

Figure 6.23: Internal concentration (C_{int} (mg kg^{-1})) of Bromoxynil in the ZFE samples



Source: Own depiction

Concerning the results of ZFE samples of the exposure experiments to Bromoxynil, the C_{int} (mg kg^{-1}) were in the same range for most of the samples, especially at pH 5 and pH 8 (presented in Figure 6.23). Most of the ZFE samples at pH 6 had lower internal concentrations in comparison with the rest of the samples at the other pH values.

6.3.2.3 Anti-microbial agents

Triclosan

Water Samples

Concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Triclosan water samples, sampled at the Start and the End of the exposure experiments at three different pH values (5, 6, 8 and 9), were determined (Table 6.43). Different pH values are indicated with different colors.

Table 6.43: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Triclosan exposure experiments

Water Triclosan	$C_{measured}$ (ng mL^{-1}) ± SD	$C_{theoretical}$ (ng mL^{-1})
pH 5 START	21.6 ± 1.5	200
pH 5 END	< LOD	
pH 6 START	37.0 ± 2.6	300
pH 6 END	< LOD	
pH 8 START	70.9 ± 5.0	350
pH 8 END	< LOD	
pH 9 START	264 ± 18	600
pH 9 END	21.3 ± 1.5	

Source: Own depiction

Regarding the results of the analysis of the water samples reported in Table 6.43, the $C_{measured}$ (ng mL^{-1})) of END the water samples (START and END) at pH 5,6 and 8 were lower than the limit of detection (*LOD= 8 ng mL^{-1}*). The water samples from Triclosan exposure experiments were not diluted at all prior to analysis. It's important to note that the Triclosan had great sensitivity (at the level of ng mL^{-1}). Therefore, the substance Triclosan should have been easily detected in the water samples since the samples were not diluted at all. So, this could not be attributed to a potential mistake during sample preparation. Furthermore, the $C_{measured}$ were lower (almost 10 times) for the water samples at pH 5 and pH 6, and almost 3-4 times lower for the water samples at pH 8 and pH 9, compared to the $C_{theoretical}$.

ZFE Samples

- Internal Concentration

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Triclosan in the extracts of the ZFE samples was determined (Table 6.44). Different pH values are indicated with different colors.

Table 6.44: Measured internal concentrations (mg kg^{-1}) of ZFE samples from Triclosan exposure experiments

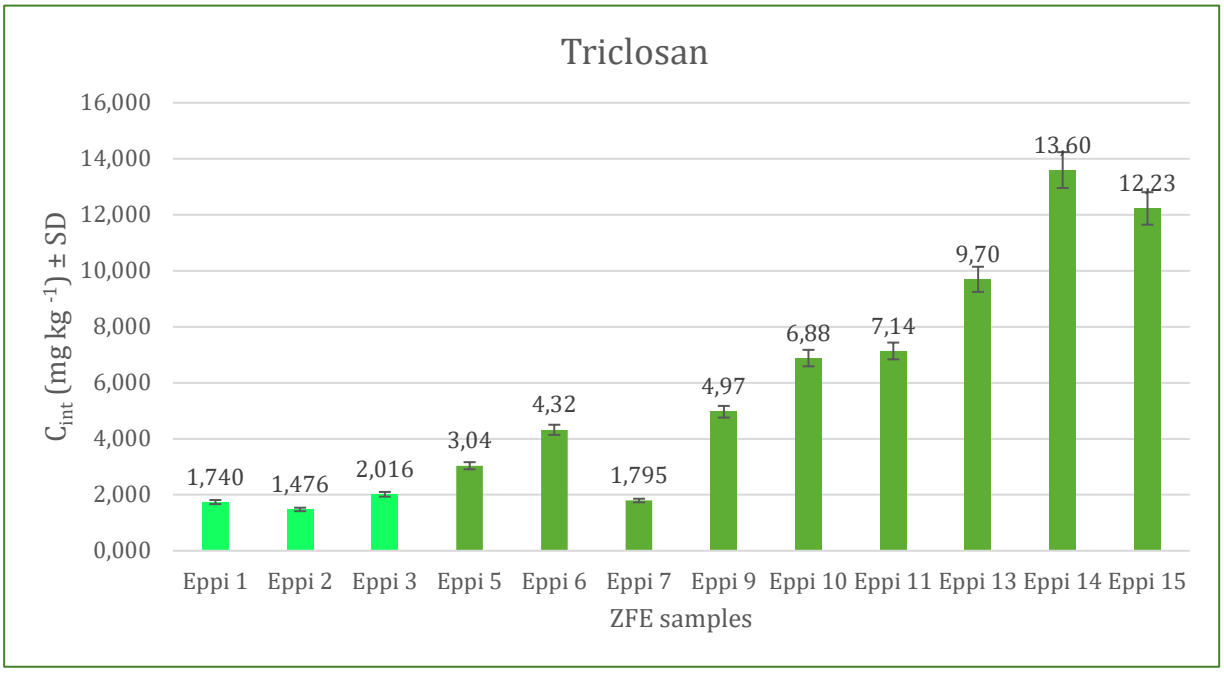
	ZFE Triclosan					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**C_{int} (mg kg^{-1}) ± SD**
pH 5	1	**alive**	20	Triclo pH 5 0.2 mg/L 20* 1	6.000	**1.740 ± 0.073**
	2	**alive**	20	Triclo pH 5 0.2 mg/L 20* 2	5.800	**1.476 ± 0.062**
	3	**alive**	20	Triclo pH 5 0.2 mg/L 20* 3	6.000	**2.016 ± 0.084**
pH 6	5	**alive**	20	Triclo pH 6 0.3 mg/L 20* 5	5.900	**3.04 ± 0.13**
	6	**alive**	20	Triclo pH 6 0.3 mg/L 20* 6	5.900	**4.32 ± 0.18**

	ZFE Triclosan					
	7	**alive**	20	Triclo pH 6 0.3 mg/L 20* 7	14.900	**1.795 ± 0.065**
pH 8	9	**alive**	20	Triclo pH 8 0.35 mg/L 20* 9	5.900	**4.97± 0.21**
	10	**alive**	20	Triclo pH 8 0.35 mg/L 20* 10	5.400	**6.88 ± 0.30**
	11	**alive**	20	Triclo pH 8 0.35 mg/L 20* 11	5.900	**7.14 ± 0.30**
pH 9	13	**alive**	15	Triclo pH 8 0.6 mg/L 15* 13	4.100	**9.70 ± 0.45**
	14	**alive**	15	Triclo pH 8 0.6 mg/L 15* 14	3.900	**13.60 ± 0.64**
	15	**alive**	16	Triclo pH 8 0.6 mg/L 16* 15	3.800	**12.23 ± 0.58**

Source: Own depiction

In the figure below, the C_{int} are presented for each pH value (5,6, 8 and 9) using bar charts. Different pH values are indicated with different colors.

Figure 6.24: Internal concentration (C_{int} (mg kg^{-1})) of Triclosan in the ZFE samples



Source: Own depiction

As far as the results of ZFE samples of the Triclosan exposure experiments (presented in Table 6.44) is concerned, the C_{int} (mg kg^{-1}) were at the same range for most of the samples, especially at pH 6 and pH 8. Most of the ZFE samples at pH 5 and pH 9 had lower and higher respectively internal concentrations in comparison with the rest of the samples at the other pH values. Regarding the results of the ZFE samples at pH 6, the weight of Eppi number 7 was quite large (14.9 mg) in comparison with the number of embryos (Embryos No= 20) contained in the Eppendorf tube. This probably leads to such a low C_{int} in comparison with the C_{int} of the rest of

the ZFE samples. If the weight was according to the No of embryos (≃ 5.9 mg) the internal concentration would be at the same range ((C_{int}= 4.53 mg kg^{-1}).

6.4 Bioconcentration factor [BCF]

The bioconcentration factor (BCF) of each compound was calculated, in order to evaluate the extent of bioaccumulation. The bioconcentration factors (L kg^{-1}) were estimated separately for all the ZFE samples and they are presented below. The data from the bioconcentration factors of the ZFE samples per each pH value, were used for the construction of Boxplots, as well. Boxplots are a way of summarizing the overall data. In the current study, they were used to illustrate the shape of the distribution, its central value, and its variability. Outliers are presented with the symbol (✳).

6.4.1 Pharmaceuticals

6.4.1.1 NSAIDs

Diclofenac

The bioconcentration factors (L kg^{-1}) of diclofenac in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.45).

Table 6.45: Measured bioconcentration factors (L kg^{-1}) of Diclofenac in ZFE samples

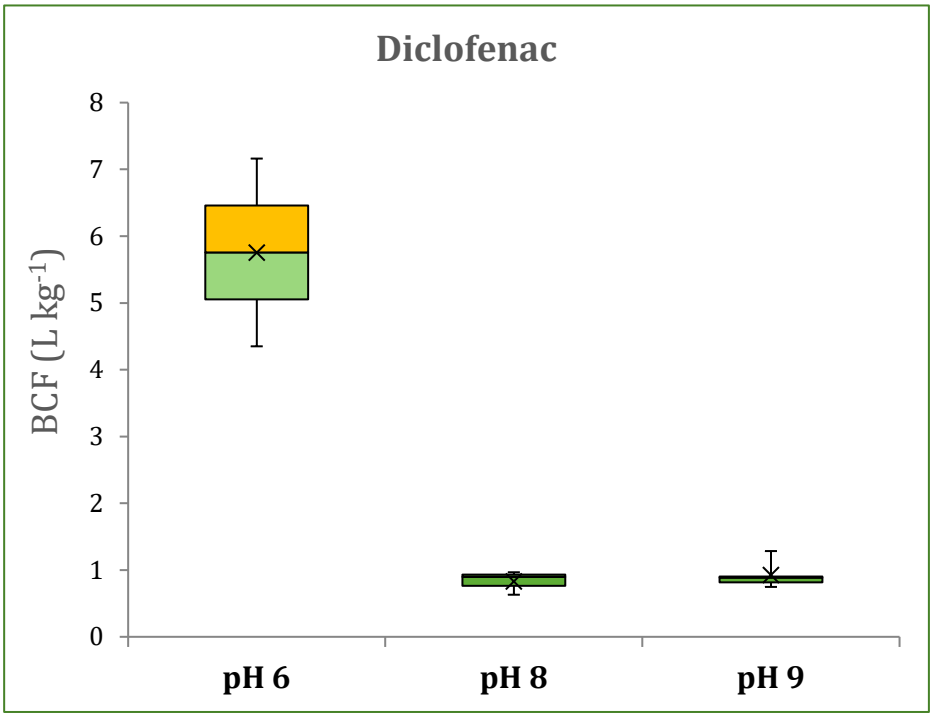
	ZFE Diclofenac					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**alive**	20	Diclo pH 5 0.05 mg/L 20* 1	5.700	-
	2	**alive**	20	Diclo pH 5 0.05 mg/L 20* 2	5.600	-
	3	**alive**	20	Diclo pH 5 0.05 mg/L 20* 3	5.700	-
pH 6	6	**alive**	15	Diclo pH 6 0.4 mg/L 15* 6	4.100	**7.16**
	7	**alive**	15	Diclo pH 6 0.4 mg/L 15* 7	4.300	**4.35**
	8	**alive**	14	Diclo pH 6 0.4 mg/L 14* 8	3.900	-
pH 8	5	**alive**	15	Diclo pH 8 17 mg/L 15* 5	4.800	**0.97**
	6	**alive**	16	Diclo pH 8 17 mg/L 16* 6	4.700	**0.63**
	7	**alive**	15	Diclo pH 8 17 mg/L 15* 7	4.600	**0.90**
pH 9	1	**alive**	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 1	4.500	**0.89**
	2	**alive**	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 2	4.700	**0.82**
	3	**alive**	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 3	4.200	**0.90**
	4	**alive**	16	Diclo pH 9 70 mg/L 16 alive 96 hpf 4	4.500	**0.75**
	5	**dead**	16	Diclo pH 9 70 mg/L 16 dead 96 hpf 5	2.600	**1.28**

Source: Own depiction

The bioconcentrations factors could not be estimated at pH 5 and for one sample from exposure experiment at pH 6, since the internal concentrations of these samples were lower of the limit of detection.

In the figure below, the bioconcentration factors are presented for each pH value (6, 8, 9) using box plots. Different pH values are indicated with different colors.

Figure 6.25: Boxplots with BCF data of ZFE samples per each pH value from Diclofenac exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the trend of the increase in the BCF values per pH value was inversely proportional to the exposure concentration, as anticipated (Figure 6.25). Higher bioaccumulation potential was observed at pH 6 since the bioconcentration factor is almost 5 times higher at this pH value in comparison with the BCF at pH 9 (C_{exp}=70 mg L^{-1}). It's worth mentioning that the exposure concentration at pH 6 (C_{exp}=0.4 mg L^{-1}) is lower than the C_{exp} at pH 9 (C_{exp}=70 mg L^{-1}).

Ibuprofen

The bioconcentration factors (L kg^{-1}) of Ibuprofen in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.46). Different pH values are indicated with different colors.

Table 6.46: Measured bioconcentration factors (L kg^{-1}) of Ibuprofen in ZFE samples

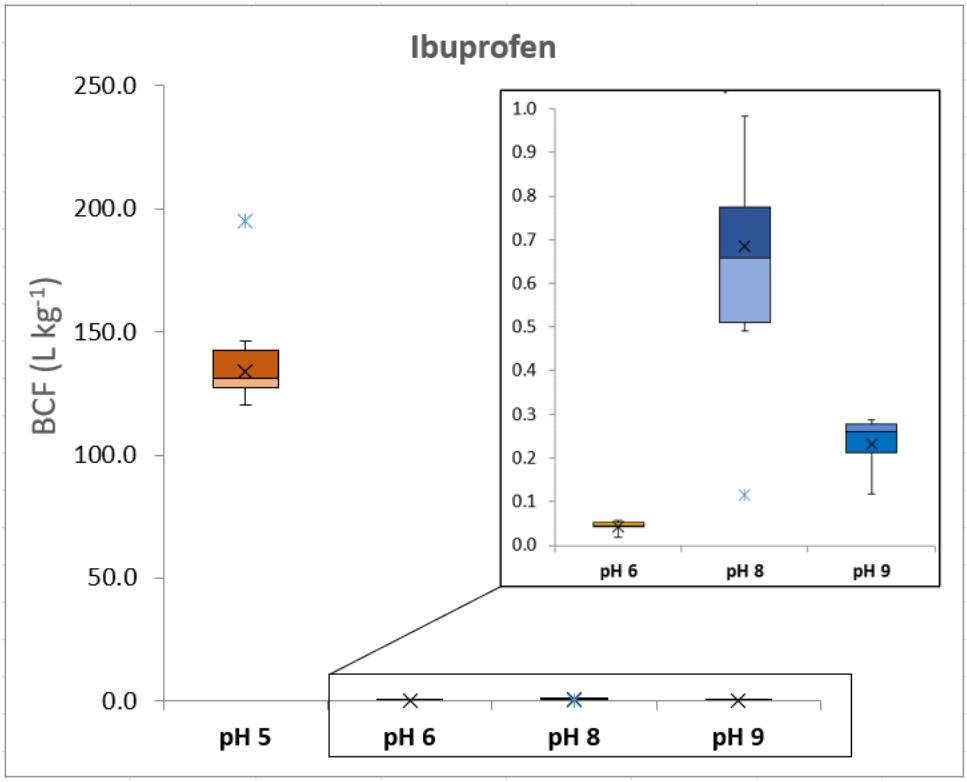
	ZFE Ibuprofen					
	Eppi No.	Status	Embryos No.	Labelling	Weight of sample (mg)	BCF (L kg^{-1})
pH 5	1	dead	17	Ibu pH 5 D17	4.1164	**127.54**
	2	dead	20	Ibu pH 5 D20	4.8428	**146.42**

	ZFE Ibuprofen					
	3	**dead**	19	Ibu pH 5 D19	4.6007	**120.31**
	1	**alive**	15	Ibu pH 5 A15	3.6321	**131.01**
	2	**alive**	15	Ibu pH 5 A15	3.6321	**195.03**
	3	**alive**	18	Ibu pH 5 A18	4.3585	**142.72**
pH 6	1	**dead**	20	Ibu pH 6 D20	4.8428	**0.04**
	2	**dead**	13	Ibu pH 6 D13	3.1478	**0.05**
	1	**alive**	15	Ibu pH 6 A15	3.6321	**0.04**
	2	**alive**	14	Ibu pH 6 A14	3.3900	**0.06**
	3	**alive**	14	Ibu pH 6 A14	3.3900	**0.02**
pH 8	3	**dead**	7	Ibu pH 8 #7 tot 3	1.6950	**0.49**
	4	**dead**	10	Ibu pH 8 #10 tot 4	16.900	**0.12**
	6	**alive**	20	Ibu pH 8 #20 leb.6	6.4000	**0.78**
	7	**alive**	20	Ibu pH 8 #20 leb.7	7.8000	**0.98**
	8	**alive**	15	Ibu pH 8 #15 leb.8	3.7000	**0.51**
	9	**alive**	14	Ibu pH 8 #14 leb.9	2.9000	**0.66**
pH 9	1	**alive**	16	Ibu pH 9 300 mg/L 16 alive 96 hpf 1	0.2750	**0.12**
	2	**alive**	16	Ibu pH 9 300 mg/L 16 alive 96 hpf 2	0.2188	**0.28**
	3	**alive**	20	Ibu pH 9 300 mg/L 20 alive 96 hpf 3	0.2000	**0.29**
	4	**dead**	9	Ibu pH 9 300 mg/L 9 dead 96 hpf 4	0.3111	**0.24**

Source: Own depiction

In the figure below (Figure 6.26), the bioconcentration factors are presented for each pH value (5,6, 8 and 9) using box plots.

Figure 6.26: Boxplots with BCF data of ZFE samples per each pH value from Ibuprofen exposure experiments



Source: Own depiction

Concerning the results from the BCF values, higher bioaccumulation potential was observed at pH 5 since the bioconcentration factor is higher at this pH value in comparison with the BCF values at the other pH values. The exposure concentration at this pH value is lower than the C_{exp} (C_{exp}=8.7 mg L^{-1}) at the other pH values.

Naproxen

The bioconcentration factors (L kg^{-1}) of Naproxen in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.47).

Table 6.47: Measured bioconcentration factors (L kg^{-1}) of Naproxen in ZFE samples

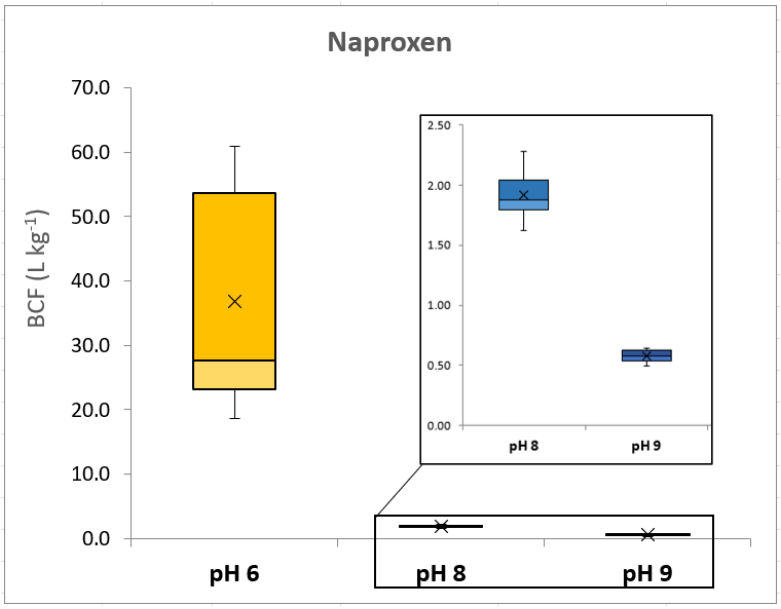
	ZFE Naproxen					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 6	1	**dead**	3	pH 6 Naproxen † 72 hpf no. 3	1.6000	**18.6**
	5	**dead**	6	pH 6 Naproxen † 96 hpf no. 6	3.0000	**27.7**
	7	**alive**	20	pH 6 Naproxen * 96 hpf no. 20	6.6000	**60.9**
	8	**alive**	20	pH 6 Naproxen * 96 hpf no. 20	13.400	**23.2**
	9	**alive**	16	pH 6 Naproxen * 96 hpf no. 16	6.9000	**53.6**

	ZFE Naproxen					
pH 8	2	**dead**	1	pH 8 Naproxen † 72 hpf no. 1	0.9000	**2.1**
	10	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.2000	**2.3**
	11	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.5000	**1.6**
	12	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.1000	**1.9**
	13	**alive**	20	pH 8 Naproxen * 96 hpf no. 20	5.3000	**1.8**
	14	**alive**	15	pH 8 Naproxen * 96 hpf no. 15	3.8000	**1.9**
pH 9	1	**dead**	3	pH 9 Naproxen † 96 hpf no.3	1.6000	**0.6**
	2	**alive**	20	pH 9 Naproxen * 96 hpf no.20	4.6000	**0.6**
	3	**alive**	20	pH 9 Naproxen * 96 hpf no.20	5.3000	**0.6**
	4	**alive**	20	pH 9 Naproxen * 96 hpf no.20	4.6000	**0.6**
	5	**alive**	20	pH 9 Naproxen * 96 hpf no.20	5.3000	**0.5**
	6	**alive**	20	pH 9 Naproxen * 96 hpf no.20	5.6000	**0.5**
	7	**alive**	13	pH 9 Naproxen * 96 hpf no.13	3.3000	**0.5**

Source: Own depiction

In the figure below (Figure 6.27), the bioconcentration factors are presented for each pH value (6, 8 and 9) using box plots. Different pH values are indicated with different colors.

Figure 6.27: Boxplots with BCF data of ZFE samples per each pH value from Naproxen exposure experiments



Source: Own depiction

Regarding the results from the BCF values, the trend of the increase in the BCF values relative to the pH values was inversely proportional to the exposure concentration, as anticipated (Figure 6.27). Higher bioaccumulation potential was observed at pH 6 since the bioconcentration factor is higher at this pH value in comparison with the BCF values at the other two pH values. The exposure concentration at this pH value is lower (almost 100 times) in comparison with the exposure concentration at pH 9.

6.4.1.2 Anaesthetics

Lidocaine

The bioconcentration factors (L kg^{-1}) of Lidocaine in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.48).

Table 6.48: Measured bioconcentration factors (L kg^{-1}) of Lidocaine in ZFE samples

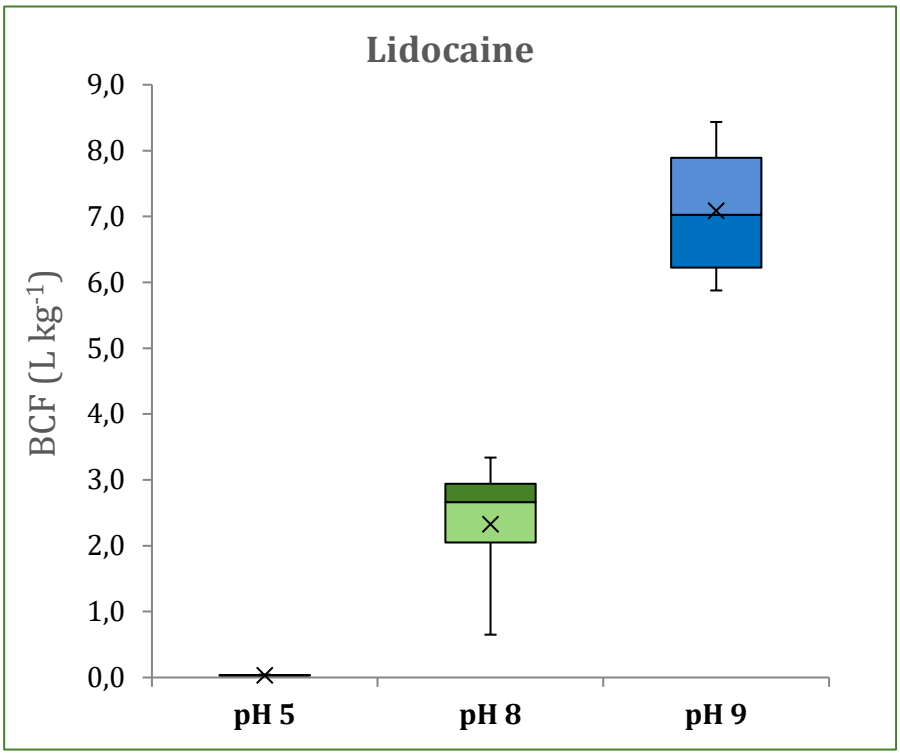
	ZFE Lidocaine					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	2	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 2	4.5000	**0.03**
	3	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 3	5.8000	**0.03**
	4	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 4	4.8000	**0.05**
	5	**alive**	20	Lido pH 5 2000 mg/L 96 h 15 alive 5	5.6000	**0.02**
	6	**alive**	17	Lido pH 5 2000 mg/L 96 h 15 alive 6	6.6000	**0.02**

	ZFE Lidocaine					
pH 8	7	**alive**	15	Lid pH 8 50 mg/L 96 h 15 alive 7	3.0000	**3.3**
	8	**alive**	15	Lid pH 8 50 mg/L 96 h 15 alive 8	11.7000	**0.65**
	9	**alive**	16	Lid pH 8 50 mg/L 96 h 15 alive 9	3.6000	**2.5**
	10	**dead**	15	Lid pH 8 50 mg/L 96 h 15 dead 10	3.3000	**2.8**
pH 9	1	**alive**	15	pH 9 10 mg/L 96 h 15 alive 1	4.9000	**7.7**
	2	**alive**	15	pH 9 10 mg/L 96 h 15 alive 2	4.7000	**8.4**
	3	**alive**	15	pH 9 10 mg/L 96 h 15 alive 3	4.9000	**6.3**
	4	**dead**	15	pH 9 10 mg/L 96 h 15 dead 4	4.1000	**5.9**

Source: Own depiction

In the figure below (Figure 6.28), the bioconcentration factors of Lidocaine are presented for each pH value (5, 8, 9) using box plots. Different pH values are indicated with different colors.

Figure 6.28: Boxplots with BCF data of ZFE samples per each pH value from Lidocaine exposure experiments



Source: Own depiction

Regarding the results from the BCF values, the trend of the increase in the BCF values relative to the pH values was inversely proportional to the exposure concentration, as anticipated (Figure 6.28). Higher bioaccumulation potential was observed at pH 9 since the bioconcentration factor is higher at this pH value in comparison with the BCF values at the other pH values. It's worth

mentioning that the exposure concentration at pH 9 is lower (almost 200 times) in comparison with the exposure concentration at pH 5.

Tetracaine

The bioconcentration factors (L kg^{-1}) of Tetracaine in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.49).

Table 6.49: Measured bioconcentration factors (L kg^{-1}) of Tetracaine in ZFE samples

	ZFE Tetracaine					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**dead**	1	Nr. 1 pH 5 1.0051g+Tc 1Embryo	0.242	-
	5	**dead**	14	Nr. 5 pH 5 +14 Embryos Tc	3.390	**0.07**
	10	**alive**	20	Nr. 10 pH 5 Alive Tc 20 Embryos	4.843	**0.05**
	13	**alive**	21	Nr. 13 pH 5 Alive 21 Embryos Tc	5.085	**0.08**
pH 6	2	**dead**	8	Nr. 2 1.005g+Tc 8 Embryos	1.937	**0.12**
	11	**alive**	3	Nr. 11 pH 6 <3 3 Embryos Tc	0.726	**0.58**
	17	**alive**	20	Nr. 17 pH 6 Alive Tc 1.0044 g 20 Embryos	4.843	**0.28**
	20	**dead**	15	Nr. 20 pH6 + 15 Embryos Tc	3.632	**0.21**
pH 8	3	**dead**	5	Nr. 3 pH 8 1.0075 g + Tc 5 Embryos	1.211	**1.86**
	6	**dead**	10	Nr. 6 pH 8 + 10 Embryos Tc	2.421	**4.91**
	12	**alive**	20	Nr. 12 pH 8 <3 20 Embryos 1.071 g Tc	4.843	**12.0**
	15	**alive**	20	Nr. 15 pH 8 <3 1.0035 20 Embryos Tc	4.843	**5.82**
	18	**alive**	21	Nr. 18 pH 8 <3 21 Embryos 1.0035 g Tc	5.085	**10.62**
pH 9	27	**dead**	2	Tetra 3 mg/L pH9 72hpf t2 27	1.800	-
	32	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 32	5.900	**1.09**
	33	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 33	5.100	**2.56**
	41	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 41	4.900	**3.50**
	43	**dead**	1	Tetra 3 mg/L pH9 48hpf t1 43	1.800	-

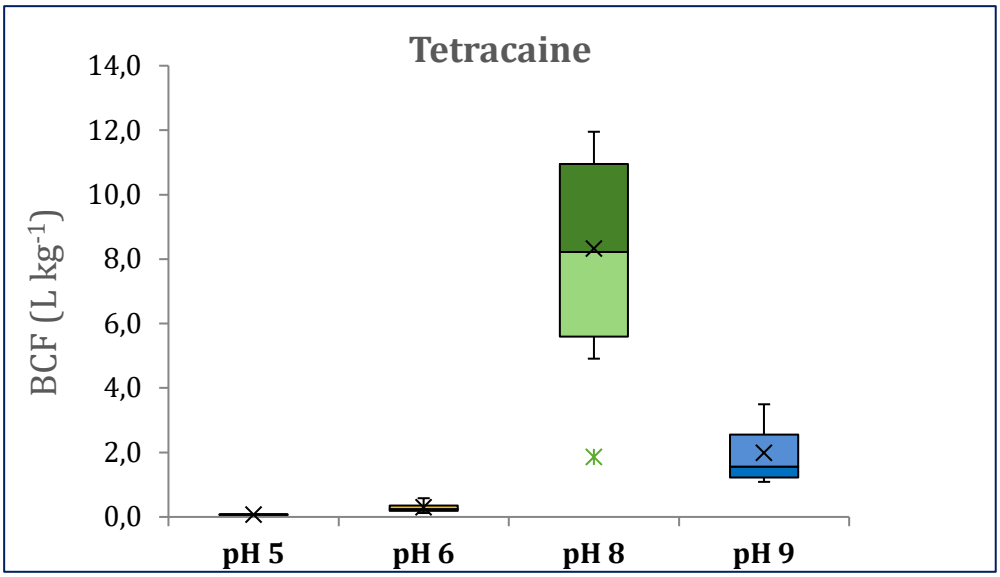
	ZFE Tetracaine					
	45	**alive**	15	Tetra 3 mg/L pH9 96hpf *15 45	6.100	**1.57**
	46	**alive**	20	Tetra 3 mg/L pH9 96hpf *20 46	9.200	**1.23**
	48	**alive**	8	Tetra 3 mg/L pH9 96hpf *8 48	3.400	**1.63**

Source: Own depiction

The bioconcentrations factors are not reported in the table above for some ZFE samples. The internal concentrations of these samples were lower of the limit of detection, therefore the BCF couldn't be calculated.

In figure below, the bioconcentration factors are presented for each pH value (5,6, 8 and 9) using box plots.

Figure 6.29: Boxplots with BCF data of ZFE samples per each pH value from Tetracaine exposure experiments



Source: Own depiction

Regarding the results from the BCF values, the trend of the increase in the BCF values relative to the pH values was inversely proportional to the exposure concentration (Figure 6.29). Higher bioaccumulation potential was observed at pH 8 and pH 9 since the bioconcentration factor is almost 10 times higher at this pH value in comparison with the BCF values at the other two pH values. It's worth mentioning that the exposure concentrations were close enough at pH 8 (C_{exp}= 3.9 mg L^{-1}) and pH 9 (C_{exp}= 3 mg L^{-1}). Therefore, we expected similar BCF values among these pH values.

6.4.1.3 Opioids

Tramadol

The bioconcentration factors (L kg^{-1}) of Tramadol in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.50).

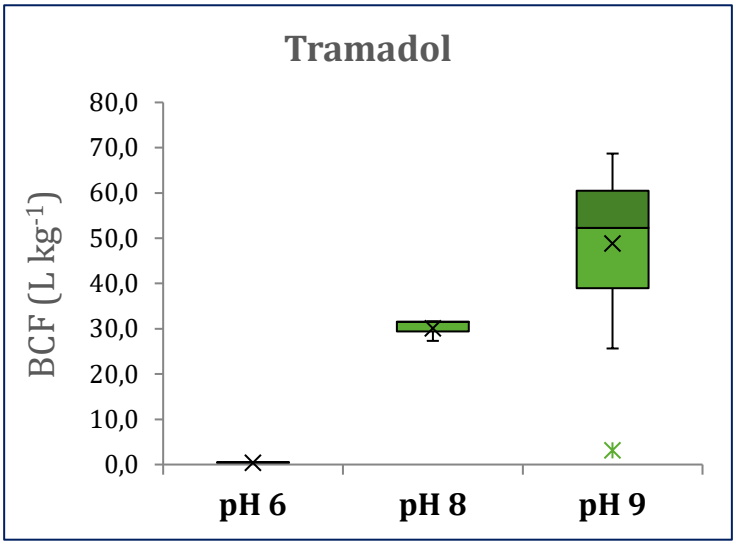
Table 6.50: Measured bioconcentration factors (L kg^{-1}) of Tramadol in ZFE samples

	ZFE Tramadol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 6	1	**alive**	15	Tram pH 6 2750 mg/L 96 h 15 alive 1	3.900	**0.5**
	2	**alive**	15	Tram pH 6 2750 mg/L 96 h 15 alive 2	4.400	**0.4**
	3	**alive**	15	Tram pH 6 2750 mg/L 96 h 15 alive 3	5.100	**0.4**
pH 8	4	**alive**	15	Tram pH 8 40 mg/L 96 h 15 alive 4	4.100	**31.6**
	5	**alive**	15	Tram pH 8 40 mg/L 96 h 15 alive 5	4.300	**31.5**
	6	**alive**	15	Tram pH 8 40 mg/L 96 h 15 alive 6	4.600	**27.3**
pH 9	6	**dead**	2	Tram 15 mg/L pH9 t2 6	9.600	**3.2**
	12	**alive**	15	Tram 15 mg/L pH9 *15 12	5.100	**68.7**
	20	**dead**	6	Tram 15 mg/L 20t pH9 20	2.500	**25.6**
	21	**alive**	15	Tram 15 mg/L pH9 *15 21	4.700	**52.3**

Source: Own depiction

In the figure below (Figure 6.30), the bioconcentration factors of Tramadol are presented for each pH value (6, 8, 9) using box plots. Different pH values are indicated with different colors.

Figure 6.30: Boxplots with BCF data of ZFE samples per each pH value from Tramadol exposure experiments



Source: Own depiction

Regarding the results from the BCF values, the trend of the increase in the BCF values relative to the pH values was inversely proportional to the exposure concentration (Figure 6.30). Higher bioaccumulation potential was observed at pH 9 since the bioconcentration factor is almost 10 times higher at this pH value in comparison with the BCF values at the other two pH values. In addition, the exposure concentration at pH 9 was lower in comparison with the other pH values.

6.4.1.4 Antihistamines

Ketotifen

The bioconcentration factors (L kg^{-1}) of Ketotifen in ZFE were calculated separately for all the ZFE samples from exposure experiments at pH 9 and presented in the Table below (Table 6.51).

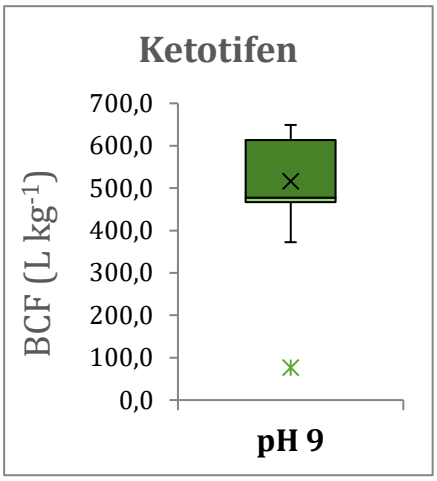
Table 6.51: Measured bioconcentration factors (L kg^{-1}) of Ketotifen in ZFE samples

	ZFE Ketotifen					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 9	9	**alive**	20	Ket 1,5 mg/L pH9 *20 96hpf 9	4.1000	**467**
	15	**alive**	12	Ket 1,5 mg/L pH9 *12 96hpf 15	2.5000	**372**
	17	**alive**	15	Ket 1,5 mg/L pH9 *15 96hpf 17	3.8000	**477**
	19	**alive**	15	Ket 1,5 mg/L pH9 96hpf *15 19	2.9000	**649**
	23	**dead**	7	Ketotifen 1,5 mg/L t7 pH9 96hpf 23	6.6000	**77**
	24	**alive**	15	Ket 1,5 mg/L pH9 96hpf *15 24	2.9000	**614**

Source: Own depiction

In the figure below (Figure 6.31), the bioconcentration factors of Ketotifen are presented for pH 9 using box plots.

Figure 6.31: Boxplot with BCF data of ZFE samples from Ketotifen exposure experiment at pH 9



Source: Own depiction

The Boxplot in Figure 6.31 presents that the measured bioconcentrations factors from exposure experiment to Ketotifen were in the same range for almost all the samples.

6.4.1.5 Beta blockers

Metoprolol

The bioconcentration factors (L kg^{-1}) of Metoprolol in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.52).

Table 6.52: Measured bioconcentration factors (L kg^{-1}) of Metoprolol in ZFE samples

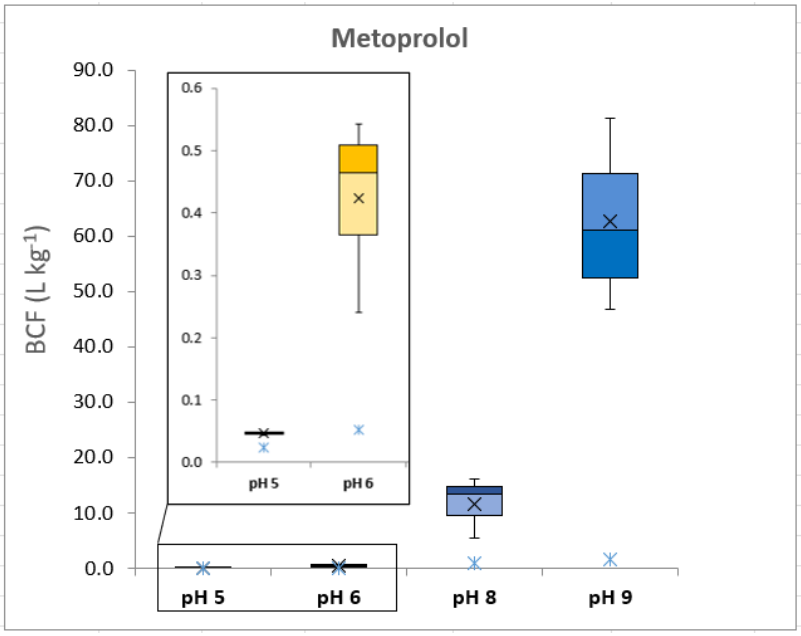
	ZFE Metoprolol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	9	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	5.8000	**0.045**
	10	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	4.3000	**0.049**
	11	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	5.4000	**0.046**
	12	**alive**	20	pH 5 Metoprolol * 96 hpf no. 20	4.7000	**0.048**
	13	**alive**	3	pH 5 Metoprolol * 96 hpf no. 3	1.5000	**0.024**
pH 6	1	**dead**	1	pH 6 Metoprolol † 72 hpf no.1	1.7000	**0.052**
	7	**dead**	20	pH 6 Metoprolol † 96 hpf no.20	6.3000	**0.542**

	ZFE Metoprolol					
	8	**dead**	2	pH 6 Metoprolol † 96 hpf no.20	1.4000	**0.240**
	14	**alive**	20	pH 6 Metoprolol * 96 hpf no. 20	6.0000	**0.509**
	15	**alive**	20	pH 6 Metoprolol * 96 hpf no. 20	5.7000	**0.464**
	16	**alive**	17	pH 6 Metoprolol * 96 hpf no. 17	5.5000	**0.365**
pH 8	2	**dead**	6	pH 8 Metoprolol † 72 hpf no.6	2.2000	**5.5**
	6	**dead**	14	pH 8 Metoprolol † 96 hpf no.14	56.7000	**0.9**
	17	**alive**	20	pH 8 Metoprolol * 96 hpf no. 20	5.3000	**13.5**
	18	**alive**	8	pH 8 Metoprolol * 96 hpf no. 8	2.6000	**16.1**
pH 9	4	**alive**	15	Met 10mg/L *15 pH9 4	4.8000	**68.0**
	11	**alive**	15	Met 10mg/L pH9 *15 11	4.8000	**54**
	13	**dead**	18	Met 10mg/L 18t pH9 13	3.5000	**81**
	16	**dead**	1	Met 10mg/L 16t pH9 16	2.8000	**1.8**
	18	**alive**	7	Met 10mg/L pH9 *7 18	2.3000	**47**

Source: Own depiction

In the figure below (Figure 6.32), the bioconcentration factors of Metoprolol are presented for each pH value (5, 6, 8 and 9) using box plots. Different pH values are indicated with different colors.

Figure 6.32: Boxplots with BCF data of ZFE samples per each pH value from Metoprolol exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the trend of the BCF was inversely proportional to the exposure concentration as anticipated. Higher bioaccumulation potential was observed at pH 9 since the bioconcentration factor is almost 1000 times higher at this pH value in comparison with the BCF at pH 5.

Propranolol

The bioconcentration factors (L kg^{-1}) of Propranolol in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.53). Different pH values are indicated with different colors.

Table 6.53: Measured bioconcentration factors (L kg^{-1}) of Propranolol in ZFE samples

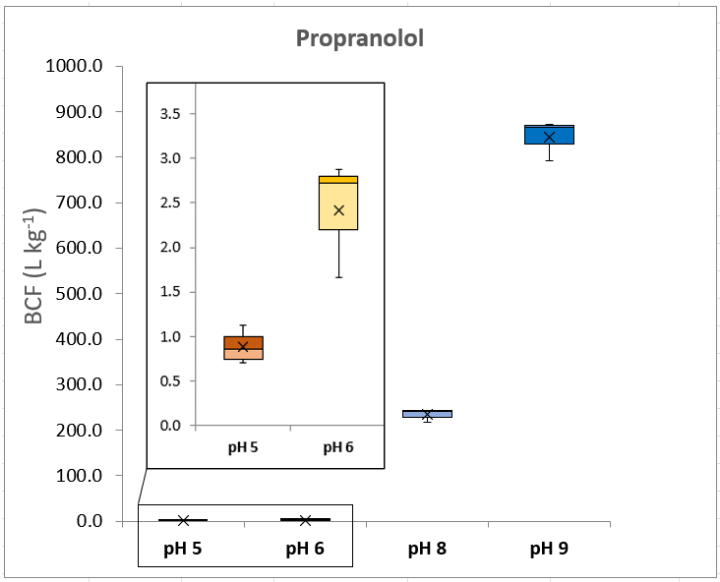
	ZFE Propranolol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**alive**	15	Prop pH 5 1600 mg/L 15 alive 96 hpf 1	4.1000	**0.76**
	2	**alive**	15	Prop pH 5 1600 mg/L 15 alive 96 hpf 2	3.1000	**1.13**
	3	**alive**	13	Prop pH 5 1600 mg/L 13 alive 96 hpf 3	3.4000	**0.95**
	4	**dead**	14	Prop pH 5 1600 mg/L 14 dead 96 hpf 4	3.3000	**0.71**
pH 6	1	**alive**	9	Prop pH 6 440 mg/L 9* 1	2.3000	**2.87**
	2	**alive**	9	Prop pH 6 440 mg/L 9* 2	4.3000	**1.66**
	3	**alive**	8	Prop pH 6 440 mg/L 8* 3	2.1000	**2.73**

	ZFE Propranolol					
pH 8	1	**alive**	20	Prop pH 8 9 mg/L 20* 1	4.3000	**217**
	2	**alive**	20	Prop pH 8 9 mg/L 20* 2	4.1000	**244**
	3	**alive**	20	Prop pH 8 9 mg/L 20* 3	4.8000	**241**
pH 9	4	**alive**	16	Prop pH 9 0.6 mg/L 16* 4	3.4000	**866**
	5	**alive**	16	Prop pH 9 0.6 mg/L 16* 5	3.9000	**793**
	6	**alive**	15	Prop pH 9 0.6 mg/L 15* 6	3.6000	**872**

Source: Own depiction

In the figure below, (Figure 6.33) the bioconcentration factors are presented for each pH value (5,6, 8 and 9) using box plots.

Figure 6.33: Boxplots with BCF data of ZFE samples per each pH value from Propranolol exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the trend of the BCF was inversely proportional to the exposure concentration as anticipated. Higher bioaccumulation potential was observed at pH 9 since the bioconcentration factor is almost 1000 times higher at this pH value in comparison with the BCF at pH 5.

6.4.1.6 Estrogen receptor modulator

Enclomiphene

The bioconcentration factors (L kg^{-1}) of Enclomiphene in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.54).

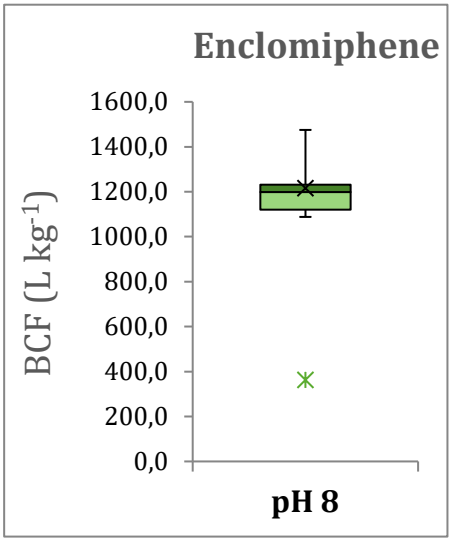
Table 6.54: Measured bioconcentration factors (L kg^{-1}) of Enclomiphene in ZFE samples

	ZFE Enclomiphene					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 8	1	**dead**	4	Enclo pH 8 D4	0.969	**362**
	2	**alive**	15	Enclo pH 8 A15	3.632	**1475**
	3	**alive**	20	Enclo pH 8 A20	4.843	**1097**
	4	**alive**	20	Enclo pH 8 A20	4.843	**1190**
	5	**alive**	20	Enclo pH 8 A20	4.843	**1208**
	6	**alive**	20	Enclo pH 8 A20	4.843	**1088**
	7	**alive**	15	Enclo pH 8 A15	3.632	**1239**
pH 9	1	**dead**	19	Enclo pH 9 D19	4.601	**194.6**
	2	**dead**	1	Enclo pH 9 D1	0.242	**435.0**

Source: Own depiction

In the figure below (Figure 6.34), the bioconcentration factors are presented for enclomiphene from exposure experiment at pH 8 using box plots.

Figure 6.34: Boxplot with BCF data of ZFE samples from enclomiphene exposure experiments at pH 8



Source: Own depiction

Concerning the results from the BCF values, the Boxplot in Figure 6.34 presents that the measured bioconcentrations factors from exposure experiment to Enclomiphene at pH 8 were in the same range for almost all the samples. Regarding the BCF data of ZFE samples from Enclomiphene exposure experiments at pH 9, there were not enough data points for the construction of the respective Boxplot. The exposure concentrations were the same at pH 8 and pH 9 (C_{exp}= 3.1 mg L^{-1}). Therefore, we expected similar BCF values among the two pH values. However, the BCF values at pH 9 was lower (almost 3 times) compared to the corresponding values at pH 8.

6.4.1.7 Anti-depressants

Amitriptyline

The bioconcentration factors (L kg^{-1}) of Amitriptyline in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.55). Different pH values are indicated with different colors.

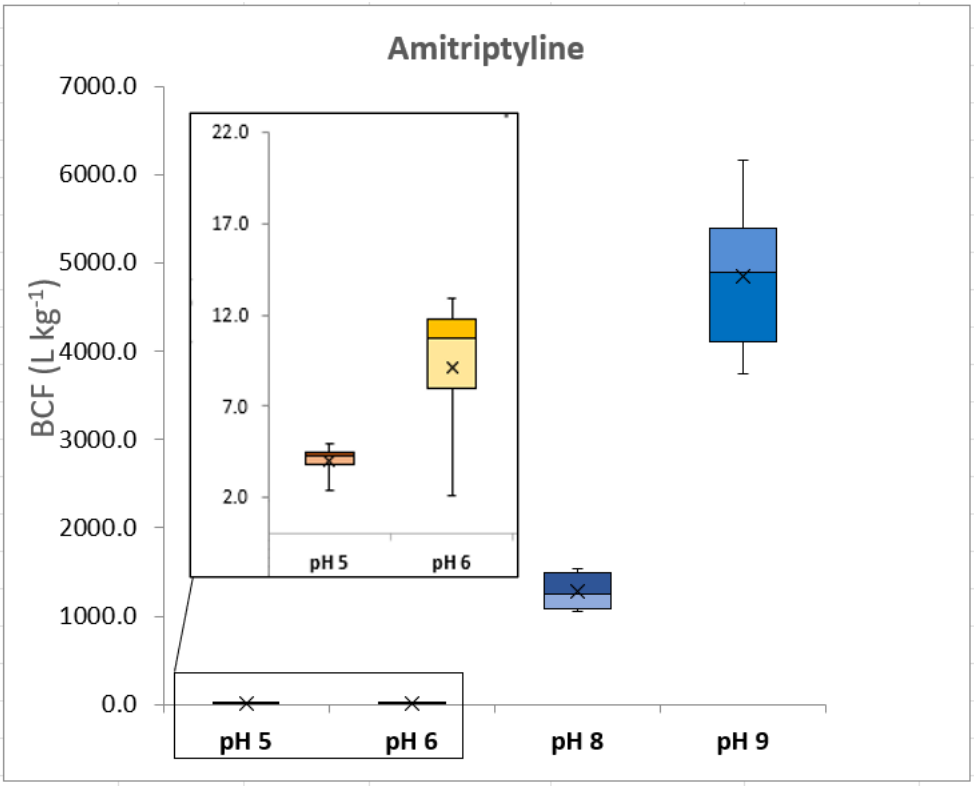
Table 6.55: Measured bioconcentration factors (L kg^{-1}) of Amitriptyline in ZFE samples

	ZFE Amitriptyline					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**alive**	15	Ami pH 5 225 mg/L 15 alive 96 hpf 1	3.600	**4.3**
	2	**alive**	15	Ami pH 5 225 mg/L 15 alive 96 hpf 2	3.300	**5.0**
	3	**alive**	15	Ami pH 5 225 mg/L 15 alive 96 hpf 3	3.300	**4.3**
	4	**dead**	4	Ami pH 5 225 mg/L 4 dead 96 hpf 4	1.400	**2.4**
pH 6	1	**dead**	12	Am pH 6 12x dead 48 h 1	1.600	**12.9**
	2	**dead**	9	Am pH 6 2x dead 96 h 2	16.10	**2.2**
	3	**dead**	2	Am pH 6 2x dead 96 h 3	1.300	**10.0**
	4	**alive**	4	Am pH 6 4x alive 96 h 4	1.400	**11.4**
pH 8	11	**alive**	20	Am pH 8 20x alive 96 h 11	5.900	**1053**
	12	**alive**	20	Am pH 8 20x alive 96 h 12	5.600	**1540**
	13	**alive**	20	Am pH 8 20x alive 96 h 13	5.100	**1544**
	14	**alive**	20	Am pH 8 20x alive 96 h 14	5.200	**1055**
	15	**alive**	20	Am pH 8 20x alive 96 h 15	4.900	**1174**
	16	**alive**	19	Am pH 8 19x alive 96 h 16	4.100	**1341**
pH 9	21	**alive**	20	Am pH 9 20x alive 96 h 21	6.400	**5448**
	22	**dead**	21	Am pH 9 21x dead 96 h 22	6.400	**3746**
	23	**alive**	20	Am pH 9 20x alive 96 h 23	5.500	**5250**
	24	**alive**	20	Am pH 9 20x alive 96 h 24	5.600	**6169**
	25	**alive**	15	Am pH 9 15x alive 96 h 25	5.300	**3964**
	26	**alive**	15	Am pH 9 15x alive 96 h 26	4.900	**4531**

Source: Own depiction

In the figure below, the bioconcentration factors are presented for each pH value (5,6, 8 and 9) using box plots.

Figure 6.35: Boxplots with BCF data of ZFE samples per each pH value from Amitriptyline exposure experiments



Source: Own depiction

Regarding the bioconcentration factors, the trend of the increase in the BCF values per pH value was inversely proportional to the exposure concentration, as anticipated (presented in Figure 6.35). The BCF values were higher at pH 8 and pH 9 since the exposure concentrations (C_{exp}=2.1 mg L^{-1} and C_{exp}= 1.6 mg L^{-1} respectively) at the different pH values were lower that the C_{exp} at the other pH values. Therefore, higher bioaccumulation potential was observed at pH 9 and then at pH 8.

Citalopram

The bioconcentration factors (L kg^{-1}) of Citalopram in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.56).

Table 6.56: Measured bioconcentration factors (L kg^{-1}) of Citalopram in ZFE samples

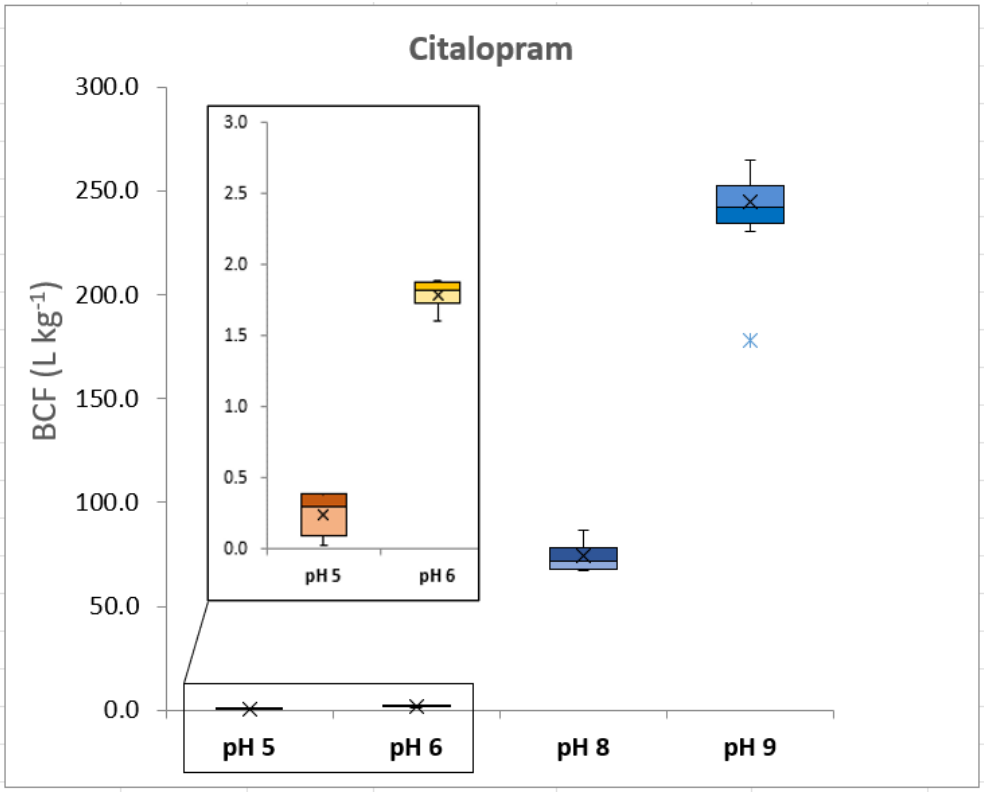
	ZFE Citalopram					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**dead**	1	Cit pH 5 1180 mg/L 1 dead 96 hpf 1	2.200	**0.03**
	2	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 2	12.400	**0.09**
	3	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 3	3.700	**0.29**
	4	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 4	3.300	**0.39**

	ZFE Citalopram					
	5	**alive**	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 5	3.400	**0.39**
pH 6	1	**alive**	20	Cit. pH 6 380 mg/L 20* 1	4.200	**1.9**
	2	**alive**	20	Cit. pH 6 380 mg/L 20* 2	4.400	**1.6**
	3	**alive**	20	Cit. pH 6 380 mg/L 20* 3	4.800	**1.9**
	4	**alive**	15	Cit. pH 6 380 mg/L 15* 4	3.700	**1.8**
pH 8	1	**alive**	20	Cit. pH 8 20 mg/L 20* 1	4.300	**87**
	2	**alive**	20	Cit. pH 8 20 mg/L 20* 2	5.000	**68**
	3	**alive**	20	Cit. pH 8 20 mg/L 20* 3	5.000	**68**
	4	**alive**	15	Cit. pH 8 20 mg/L 15* 4	3.800	**75**
pH 9	2	**alive**	16	Cit 5mg/L pH9 *16 2	4.900	**231**
	3	**alive**	15	Cit 5mg/L pH9 *15 3	5.000	**265**
	5	**alive**	15	Cit 5mg/L pH9 *15 5	5.500	**236**
	8	**dead**	10	Cit 5mg/L pH9 t10 8	3.700	**178**
	14	**alive**	17	Cit 5mg/L pH9 *17 14	5.600	**249**

Source: Own depiction

In the figure below, the bioconcentration factors are presented for each pH value (5,6, 8 and 9) using box plots. Different pH values are indicated with different colors.

Figure 6.36: Boxplots with BCF data of ZFE samples per each pH value from Citalopram exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the trend of the BCF was inversely proportional to the exposure concentration (Figure 6.36). Higher bioaccumulation potential was observed at pH 9 since the bioconcentration factor is almost 1000 times higher at this pH value in comparison with the BCF at pH 5.

Fluoxetine

The bioconcentration factors (L kg^{-1}) of Fluoxetine in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.57).

Table 6.57: Measured bioconcentration factors (L kg^{-1}) of Fluoxetine in ZFE samples

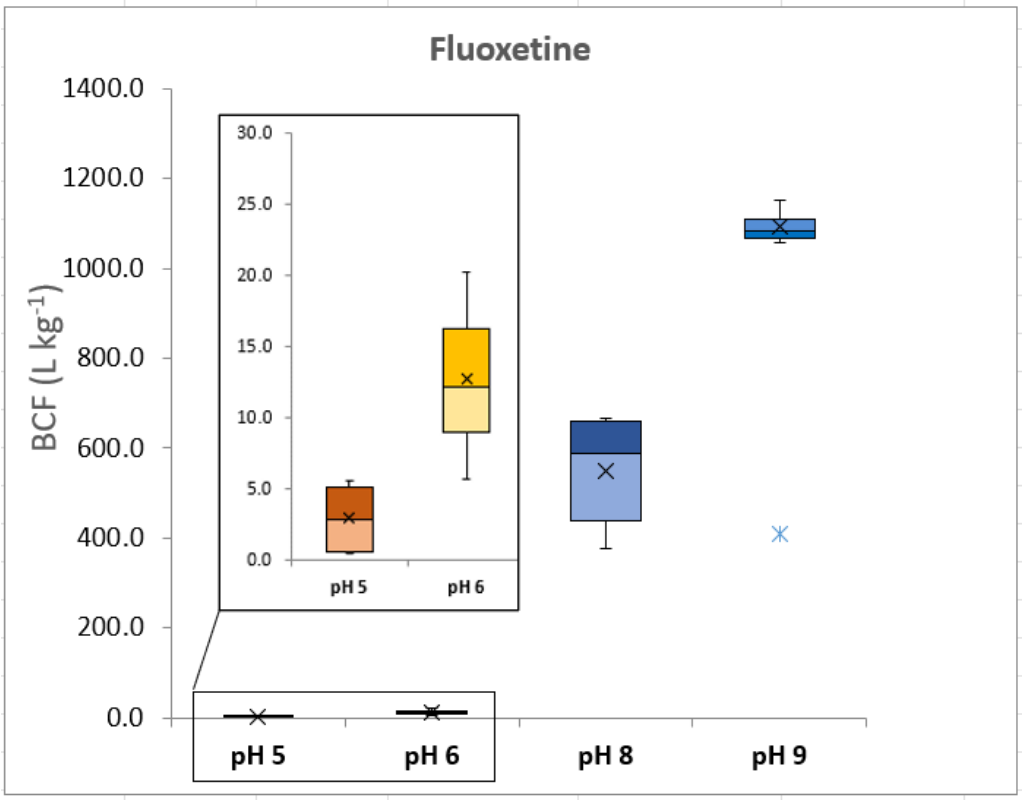
	ZFE Fluoxetine					
	Eppi No.	Status	Embryos No.	Labelling	Weight of sample (mg)	BCF (L kg^{-1})
pH 5	1	dead	1	pH5 Fluo 1	1.700	0.5
	3	dead	2	pH5 Fluo 3	1.500	1
	4	alive	11	pH5 Fluo * 4	2.200	6
	5	alive	13	pH5 Fluo * 5	3.000	5
pH 6	6	dead	5	pH6 Fluo 6	2.600	6
	7	alive	15	pH6 Fluo * 7	4.000	12
	8	alive	18	pH6 Fluo * 8	2.600	20

	ZFE Fluoxetine					
pH 8	15	**alive**	16	pH8 Fluo * 15	4.100	**666**
	16	**alive**	18	pH8 Fluo * 16	4.600	**655**
	17	**alive**	20	pH8 Fluo * 17	5.000	**410**
	18	**alive**	20	pH8 Fluo * 18	4.700	**663**
	19	**alive**	17	pH8 Fluo * 19	6.800	**376**
	20	**alive**	14	pH8 Fluo * 20	5.300	**523**
pH 9	9	**dead**	3	pH9 Fluo 9	1.000	**409**
	11	**dead**	7	pH9 Fluo 11	1.300	**1072**
	12	**alive**	16	pH9 Fluo * 12	3.400	**1153**
	13	**alive**	17	pH9 Fluo * 13	4.300	**1058**
	14	**alive**	13	pH9 Fluo * 14	3.000	**1094**

Source: Own depiction

In the figure below (Fig. 6.37), the bioconcentration factors are presented for each pH value (5,6, 8, 9) using box plots. Different pH values are indicated with different colors.

Figure 6.37: Boxplots with BCF data of ZFE samples per each pH value from Fluoxetine exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the trend of the BCF was inversely proportional to the exposure concentration as anticipated. Higher bioaccumulation potential was observed at

pH 9 since the bioconcentration factor is almost 1000 times higher at this pH value in comparison with the BCF at pH 5.

Sertraline

The bioconcentration factors (L kg^{-1}) of Sertraline in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.58).

Table 6.58: Measured bioconcentration factors (L kg^{-1}) of Sertraline in ZFE samples

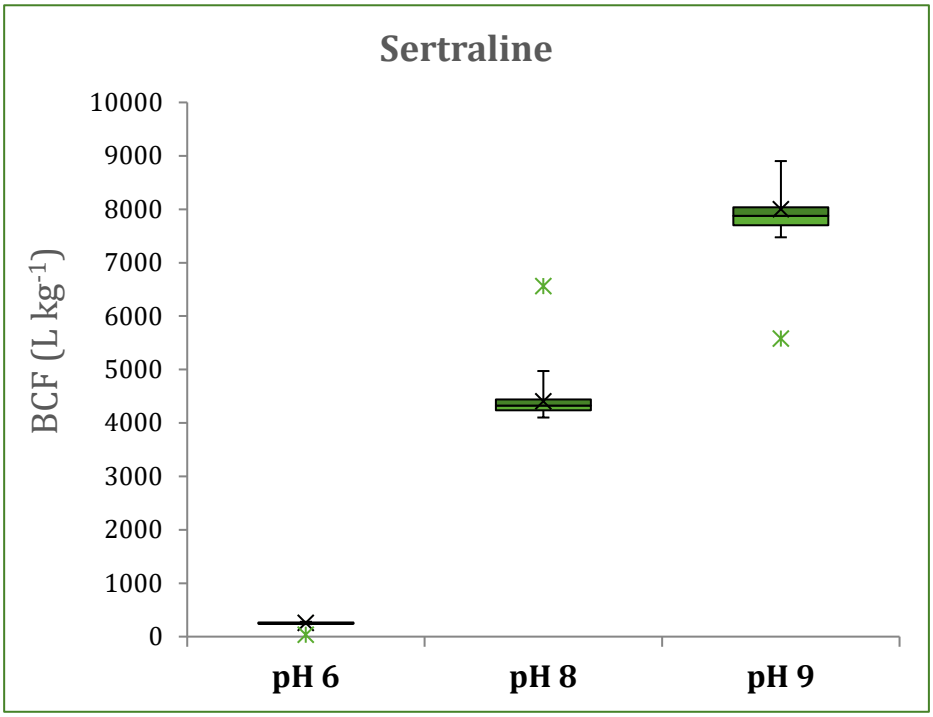
	ZFE Sertraline					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 6	1	**dead**	1	1.PHION pH 6 Sertralin 72h #1	0.400	**31.7**
	5	**alive**	10	5.PHION pH 6 Sertralin 96h lebend #10	2.900	**233.9**
	6	**alive**	16	6.PHION pH 6 lebend Sertralin #16	2.000	**277.7**
	7	**alive**	8	7.PHION pH 6 Sertralin lebend #8 96h	1.700	**260.8**
	8	**alive**	11	8.PHION pH 6 leb.Sertralin 96h #11	2.200	**242.4**
pH 8	15	**alive**	12	15.PHION pH 8 Sertralin 96h #12 lebend	2.700	**4101.5**
	16	**alive**	12	16.PHION pH 8 Sertralin 96h #12 lebend	2.100	**6557.8**
	17	**alive**	12	17.PHION pH 8 Sertralin 96h #12 lebend	2.900	**4220.2**
	18	**alive**	12	18.PHION pH 8 Sertralin 96h #12 lebend	2.700	**4972.2**
	19	**alive**	12	19.PHION pH 8 Sertralin 96h #12 lebend	2.900	**4286.4**
	20	**alive**	12	20.PHION pH 8 Sertralin 96h #12 lebend	2.600	**4465.2**
	21	**alive**	15	21.PHION pH 8 Sertralin 96h #15 lebend	2.900	**4364.0**
pH 9	9	**dead**	8	9. PHION pH 9 tot Sertralin #8 96h	2.000	**5576.0**
	10	**alive**	11	10.PHION pH 9 Sertralin lebend 96h #11	3.000	**8902.3**
	11	**alive**	13	11.PHION pH 9 lebend Sertralin 96h #13	3.500	**7475.2**
	12	**alive**	13	12.PHION pH 9 lebend Sertralin 96h #13	3.700	**8037.0**

	ZFE Sertraline					
	13	**alive**	13	13.PHION pH 9 Sertralin lebend 96h #13	3.300	**7700.6**
	14	**alive**	9	14.PHION pH 9 Sertralin lebend 96h #9	2.600	**7877.0**

Source: Own depiction

In the figure below (Fig. 6.38), the bioconcentration factors are presented for each pH value (6, 8, 9) using box plots. Different pH values are indicated with different colors.

Figure 6.38: Boxplots with BCF data of ZFE samples per each pH value from Sertraline exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the trend of the BCF was inversely proportional to the exposure concentration. Higher bioaccumulation potential was observed at pH 9 since the bioconcentration factor is almost 32 times higher at this pH value in comparison with the BCF values at pH 6.

6.4.2 Pesticides

6.4.2.1 Chlorophenols

2,3-Dichlorophenol (DCP)

The bioconcentration factors (L kg^{-1}) of DPC in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.59).

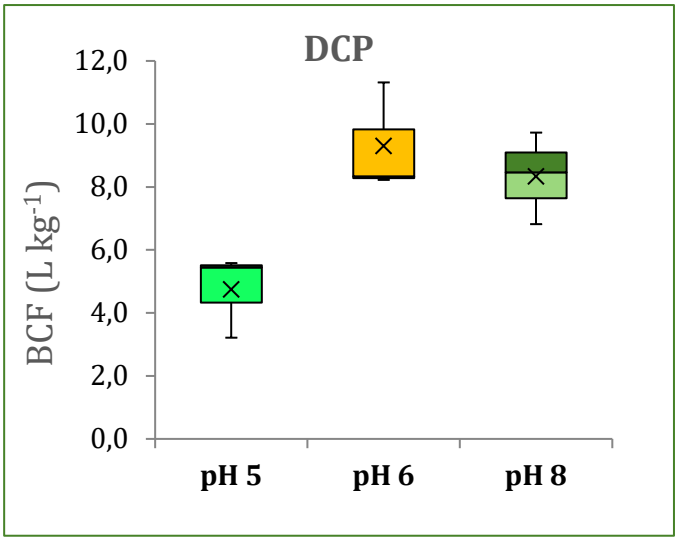
Table 6.59: Measured bioconcentration factors (L kg^{-1}) of DCP in ZFE samples

	ZFE 2,3-Dichlorophenol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**alive**	19	DCP pH5 6 mg/L 19* (1)	17.40	**3.2**
	2	**alive**	20	DCP pH5 6 mg/L 20* (2)	14.50	**5.6**
	3	**alive**	20	DCP pH5 6 mg/L 20* (3)	17.10	**5.4**
pH 6	1	**alive**	20	DCP pH6 5 mg/L 20* (1)	9.70	**11.3**
	2	**alive**	19	DCP pH6 5 mg/L 19* (2)	15.90	**8.3**
	3	**alive**	20	DCP pH6 5 mg/L 20* (3)	9.90	**8.2**
pH 8	1	**alive**	20	DCP pH8 7.5 mg/L 20* (1)	9.90	**6.8**
	2	**alive**	20	DCP pH8 7.5 mg/L 20* (2)	9.30	**9.7**
	3	**alive**	20	DCP pH8 7.5 mg/L 20* (3)	10.10	**8.5**

Source: Own depiction

In the figure below (Figure 6.39), the bioconcentration factors are presented for each pH value (5,6, 8) using box plots. Different pH values are indicated with different colors.

Figure 6.39: Boxplots with BCF data of ZFE samples per each pH value from DCP exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the exposure concentrations were close enough at the different pH values. Therefore, we expected similar BCF values among the three different pH values. Higher bioaccumulation potential was observed at pH 6. The exposure concentration was lower at pH 6 and therefore the BCF values at this pH were higher (inversely proportional to the exposure concentration).

2,4,5-Trichlorophenol (TPC)

The bioconcentration factors (L kg^{-1}) of DPC in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.60).

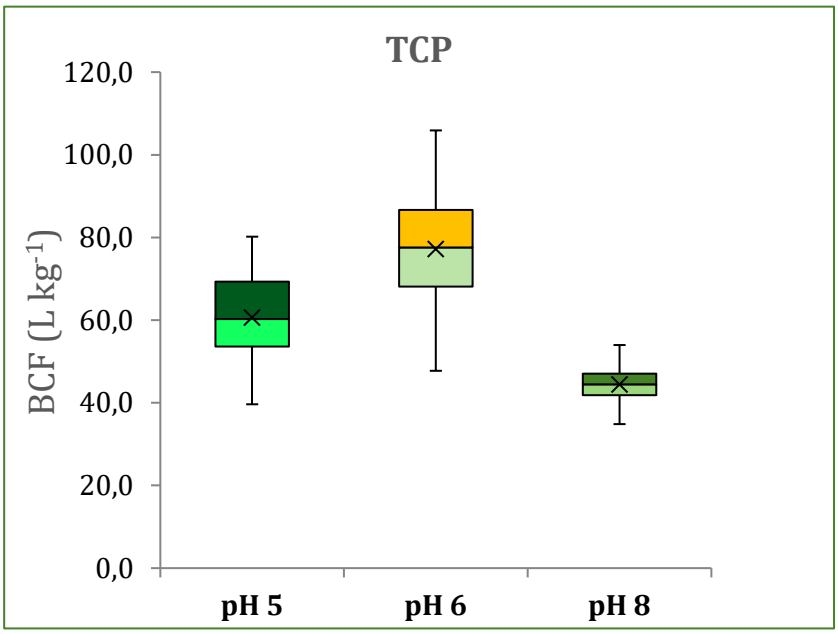
Table 6.60: Measured bioconcentration factors (L kg^{-1}) of TPC in ZFE samples

	ZFE 2,4,5-Trichlorophenol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**alive**	20	1 TCP pH5 0,575 mg/l *20	3.700	**39.6**
	2	**alive**	16	2 TCP pH5 0,575 mg/l *16	3.100	**69.3**
	3	**alive**	20	3 TCP pH5 0,575 mg/l *20	3.800	**60.3**
	4	**alive**	20	4 TCP pH5 0,575 mg/l *20	4.000	**53.6**
	5	**alive**	20	5 TCP pH5 0,575 mg/l *20	4.000	**80.2**
pH 6	1	**alive**	15	1 TCP pH6 0,5 mg/l *15	3.400	**47.74**
	2	**alive**	15	2 TCP pH6 0,5 mg/l *15	3.400	**105.9**
	3	**alive**	15	3 TCP pH6 0,5 mg/l *15	3.100	**80.2**
	4	**alive**	20	4 TCP pH6 0,5 mg/l *20	4.300	**74.9**
pH 8	1	**alive**	20	1 TCP pH8 1 mg/l *20	4.700	**44.2**
	2	**alive**	20	2 TCP pH8 1 mg/l *20	4.500	**44.7**
	3	**alive**	20	3 TCP pH8 1 mg/l *20	4.100	**34.8**
	4	**alive**	20	4 TCP pH8 1 mg/l *20	4.400	**54.0**

Source: Own depiction

In the figure below (Figure 6.40), the bioconcentration factors are presented for each pH value (5,6, 8) using box plots. Different pH values are indicated with different colors.

Figure 6.40: Boxplots with BCF data of ZFE samples per each pH value from TPC exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the exposure concentrations were close enough at the different pH values (especially at pH 5 and 6). Higher bioaccumulation potential was observed at pH 6. The exposure concentration was lower at pH 6 (C_{exp}=0.5 mg L^{-1}) and therefore the BCF values at this pH were higher (inversely proportional to the exposure concentration).

Pentachlorophenol (PCP)

The bioconcentration factors (L kg^{-1}) of DPC in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.61).

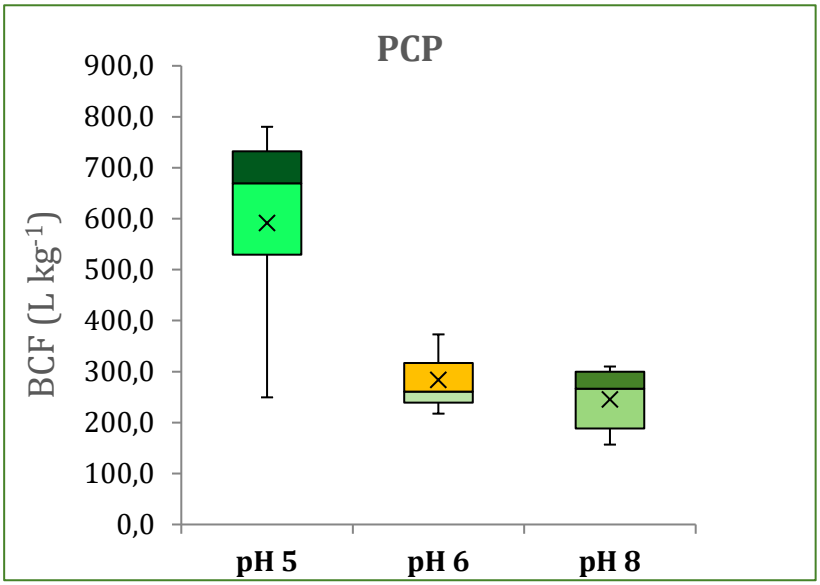
Table 6.61: Measured bioconcentration factors (L kg^{-1}) of PCP in ZFE samples

	ZFE Pentachlorophenol					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	5	**alive**	20	PCP pH 5 0.05 mg/L 20* 5	4.500	**780**
	6	**alive**	20	PCP pH 5 0.05 mg/L 20* 6	8.700	**249**
	7	**alive**	20	PCP pH 5 0.05 mg/L 20* 7	4.700	**716**
	8	**alive**	15	PCP pH 5 0.05 mg/L 15* 8	3.400	**623**
pH 6	1	**alive**	20	PCP pH 6 0.052 mg/L 20* 1	4.700	**217**
	2	**alive**	20	PCP pH 6 0.052 mg/L 20* 2	4.800	**261**
	3	**alive**	20	PCP pH 6 0.052 mg/L 20* 3	4.800	**373**
pH 8	1	**dead**	6	PCP pH 8 0.26 mg/L 6† 72 hpf 1	2.000	**169**
	2	**alive**	15	PCP pH 8 0.26 mg/L 15* 96 hpf 2	6.700	**157**
	3	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 3	4.000	**247**
	4	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 4	4.200	**310**
	5	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 5	4.100	**286**
	6	**alive**	20	PCP pH 8 0.26 mg/L 20* 96 hpf 6	4.100	**304**

Source: Own depiction

In the figure below (Figure 6.41), the bioconcentration factors are presented for each pH value (5,6, 8) using box plots. Different pH values are indicated with different colors.

Figure 6.41: Boxplots with BCF data of ZFE samples per each pH value from PCP exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the exposure concentrations were close enough at the different pH values. Higher bioaccumulation potential was observed at pH 5. The exposure concentration was lower at pH 5 (C_{exp}=0.050 mg L^{-1}) and therefore the BCF values at this pH were higher (inversely proportional to the exposure concentration).

6.4.2.2 Herbicides

Bromoxynil

The bioconcentration factors (L kg^{-1}) of Bromoxynil in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.62).

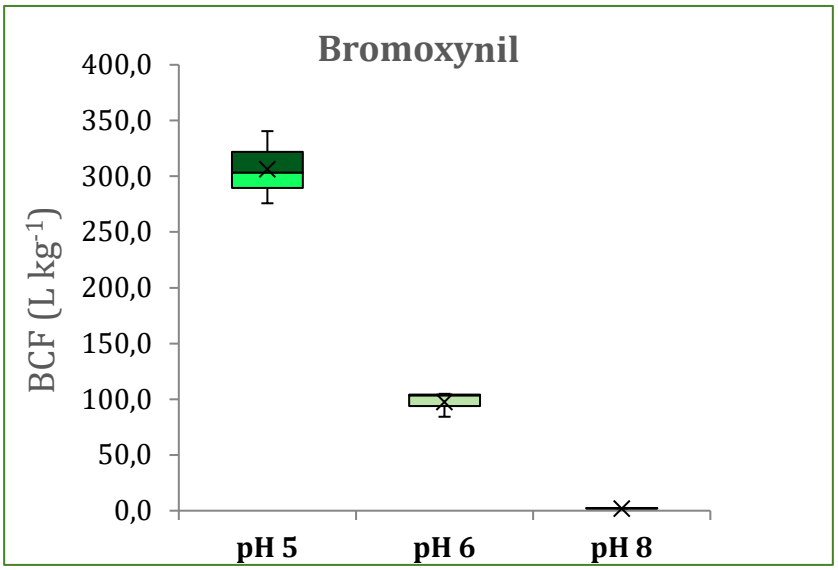
Table 6.62: Measured bioconcentration factors (L kg^{-1}) of Bromoxynil in ZFE samples

	ZFE Bromoxynil					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**alive**	20	Brom. pH5 0,15 mg/L 1 *20	2.700	**340.5**
	2	**alive**	20	Brom. pH5 0,15 mg/L 2 *20	3.000	**303.3**
	3	**alive**	15	Brom. pH5 0,15 mg/L 3 *15	2.000	**275.8**
pH 6	1	**alive**	20	Brom. pH6 0,2 mg/L 1 *20	4.600	**104.83**
	2	**alive**	20	Brom. pH6 0,2 mg/L 2 *20	4.700	**103.47**
	3	**alive**	13	Brom. pH6 0,2 mg/L 3 *13	3.300	**84.27**
pH 8	1	**alive**	20	Brom. pH8 15 mg/L 1 *20	3.200	**2.3**
	2	**alive**	20	Brom. pH8 15 mg/L 2 *20	3.400	**2.4**
	3	**alive**	18	Brom. pH8 15 mg/L 3 *18	2.900	**1.6**
	4	**alive**	20	Brom. pH8 15 mg/L 4 *20	3.100	**2.0**

Source: Own depiction

In the figure below (Figure 6.42), the bioconcentration factors are presented for each pH value (5,6, 8) using box plots. Different pH values are indicated with different colors.

Figure 6.42: Boxplots with BCF data of ZFE samples per each pH value from Bromoxynil exposure experiments



Source: Own depiction

Concerning the results from the BCF values, the trend of the BCF was inversely proportional to the exposure concentration. Higher bioaccumulation potential was observed at pH 5 since the bioconcentration factor is almost 150 times higher at this pH value in comparison with the BCF values at pH 8.

6.4.2.3 Anti-microbial agents

Triclosan

The bioconcentration factors (L kg^{-1}) of Triclosan in ZFE were calculated separately for all the ZFE samples and presented in the Table below (Table 6.63).

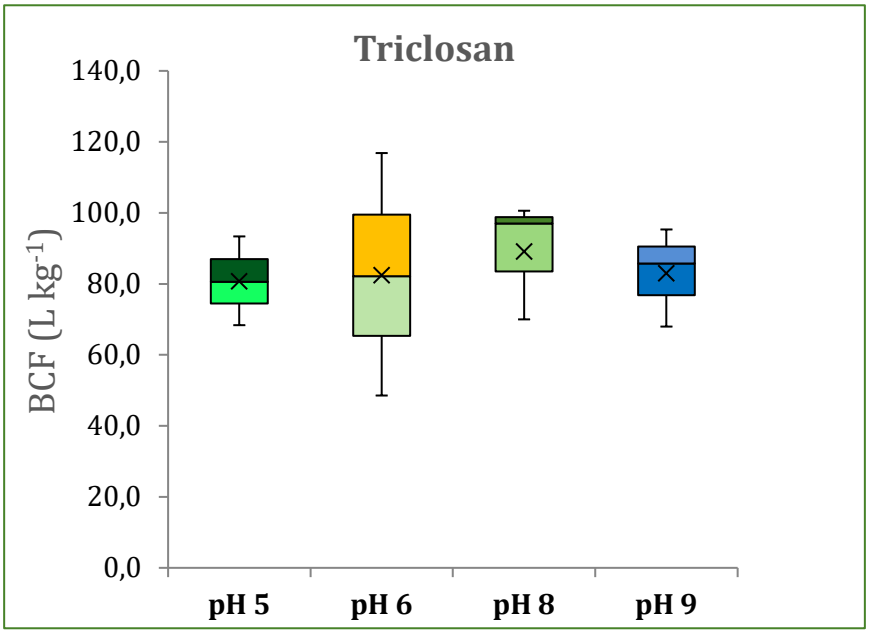
Table 6.63: Measured bioconcentration factors (L kg^{-1}) of Triclosan in ZFE samples

	ZFE Triclosan					
	Eppi No.	**Status**	**Embryos No.**	**Labelling**	**Weight of sample (mg)**	**BCF (L kg^{-1})**
pH 5	1	**alive**	20	Triclo pH 5 0.2 mg/L 20* 1	6.000	**81**
	2	**alive**	20	Triclo pH 5 0.2 mg/L 20* 2	5.800	**68**
	3	**alive**	20	Triclo pH 5 0.2 mg/L 20* 3	6.000	**93**
pH 6	5	**alive**	20	Triclo pH 6 0.3 mg/L 20* 5	5.900	**82**
	6	**alive**	20	Triclo pH 6 0.3 mg/L 20* 6	5.900	**117**
	7	**alive**	20	Triclo pH 6 0.3 mg/L 20* 7	14.900	**49**
pH 8	9	**alive**	20	Triclo pH 8 0.35 mg/L 20* 9	5.900	**70**
	10	**alive**	20	Triclo pH 8 0.35 mg/L 20* 10	5.400	**97**
	11	**alive**	20	Triclo pH 8 0.35 mg/L 20* 11	5.900	**101**
pH 9	13	**alive**	15	Triclo pH 8 0.6 mg/L 15* 13	4.100	**68**
	14	**alive**	15	Triclo pH 8 0.6 mg/L 15* 14	3.900	**95**
	15	**alive**	16	Triclo pH 8 0.6 mg/L 16* 15	3.800	**86**

Source: Own depiction

In the figure below (Figure 6.43), the bioconcentration factors are presented for each pH value (5,6, 8 and 9) using box plots. Different pH values are indicated with different colors.

Figure 6.43: Boxplots with BCF data of ZFE samples per each pH value from Triclosan exposure experiments



Source: Own depiction

The exposure concentrations were close enough at the different pH values from Triclosan exposure experiments. Therefore, similar BCF values among the three different pH values were expected. The Boxplots in Figure above present that the measured bioconcentrations factors were in the same range for almost all the samples and the different pH values.

6.5 Determination of internal fat content of *Lemna minor*

The internal fat content of *Lemna minor* was determined as follows:

The fresh weight of a defined number of *Lemna minor* was determined and then homogenised together with a solvent mixture of cyclohexane and isopropanol using a tissue homogeniser. The pestle of the tissue homogeniser was rinsed with the solvent mixture and water and the residues collected. The plant-solvent-water mixture was vortexed and then centrifuged. The organic phase was pipetted off and collected. Solvent was again added to the aqueous phase and the mixture was vortexed and centrifuged again to separate the remaining organic fraction from the aqueous phase. The entire organic fraction isolated in this way was collected in a crucible and then the solvent was evaporated off and the crucible dried at 75 °C. The amount of the extract obtained was then used as the basis for the extraction. The amount of extract obtained was determined gravimetrically (see results in Table 6.5). Since the dissolving process extracts not only fat but also chlorophyll, the chlorophyll content had to be subtracted finally to determine the real fat content. For chlorophyll content, data from the literature were used. There, the chlorophyll content is estimated at approx. 0.05 %. With an organic content of 1.70 %, this results in a total fat content of 1.65 % for *Lemna minor*.

Table 6.64: Extracted organic content [%] of *Lemna minor*

$G_{Ext.\_i}$ [%]	$G_{Ext.}$ [%]	$S_{GExt.}$ [%]	$VK_{Ext.}$[%]
1.62287	1.70574	0.11269	6.6
1.83406			

$G_{Ext.\_i}$ [%]	$G_{Ext.}$ [%]	$S_{GExt.}$ [%]	$VK_{Ext.}$[%]
1.66028			

Source: Own depiction

7 WP 6: Chemical analyses of tests with *Daphnia* and *Lemna* conducted by the UBA

7.1 Determination of dry and fresh weight of *Daphnia magna* and *Lemna minor*

7.1.1 Daphnia magna

To determine the biomass of *Daphnia major*, four consecutively numbered aluminium trays were dried at 75 °C in a desiccator. They were then cooled to room temperature and weighed promptly (accuracy: 0.1 mg). The determined weight corresponded to the dry weight of the aluminium trays "weight empty [g]" and was recorded. The aluminium trays remained in the desiccator until further usage.

To determine the fresh weight and dry mass of *Daphnia major*, four replicates each were used. The selected organisms (juveniles from the stock colony from the previous weekend) were fed once on Friday with green algae suspension and separated from the stock colony on Monday. Each replicate consisted of 50 individuals, 24 to 72 hours of age. Each replicate was rinsed on a sieve with ultrapure water to remove adhering medium. Subsequently, the organisms were placed on cellulose and dabbed with cellulose so that as little liquid as possible remained on the organisms. Then the replicates (50 individuals each) were placed on the previously dried and weighed aluminium trays (accuracy: 0.1 mg). The total mass of each aluminium tray (dry weight of the aluminium trays + *Daphnia major* fresh weight [FW]) was recorded as. The calculation of the fresh weight was done by subtracting the known dry weights of the aluminium trays from those of the aluminium trays with the *Daphnia* major FW (Table 7.1).

To determine the dry weight [DW], *Daphnia major* was left within the aluminium trays (with known weight) and dried at 75 °C in a desiccator for 48 hours. They were then cooled down to room temperature and weighed promptly (accuracy: 0.1 mg). The total mass of each aluminium tray (dry weight of the aluminium trays + *Daphnia major* dry weight [DW]) was recorded as "weight with *Daphnia* DW [g]". The calculation of the dry weight was done by subtracting the known dry weights of the aluminium trays from those of the aluminium trays with the *Daphnia major* DW.

Table 7.1: Calculation of *Daphnia* fresh [FW] and dry [DW] weight in mg and internal water content in %

Replicate	FW [mg]	DW [mg]	Water content [%]
1	3.87	1.52	60.72
2	6.63	1.68	74.66
3	3.33	1.45	56.46
4	7.47	1.32	82.33
Mean	5.33	1.49	68.54
SD	2.03	0.15	12.04
VarK [%]	31.18	10.05	17.56

Source: Own depiction

7.1.2 Lemna minor

To determine the biomass of *Lemna minor*, four consecutively numbered aluminium trays were dried at 60 °C for 48 hours in a desiccator. They were then cooled to room temperature and weighed promptly (accuracy: 0.1 mg). The determined weight corresponded to the dry weight of the aluminium trays "weight empty [g]" and was recorded. The aluminium trays remained in the desiccator until further usage.

To determine the fresh weight and dry mass of *Lemna minor*, four replicates each were used. The selected organisms corresponded approximately to the size of representative *Lemna minor* from the stock. Each replicate consisted of six colonies with three fronds each. Each replicate was rinsed on a sieve with ultrapure water to remove adhering medium. Subsequently, the organisms were placed on cellulose and dabbed with cellulose so that as little liquid as possible remained on the organisms. Then the replicates (six colonies with three fronds each) were placed on the previously dried and weighed aluminium trays (accuracy: 0.1 mg). The total mass of each aluminium tray (dry weight of the aluminium trays + *Lemna minor* fresh weight [FW]) was recorded as "weight with *Lemna* FW [g]". The calculation of the fresh weight was done by subtracting the known dry weights of the aluminium trays from those of the aluminium trays with the *Lemna minor* FW (Table 7.2).

To determine the dry weight [DW], *Lemna minor* was left within the aluminium trays (with known weight) and dried at 60 °C in a desiccator for 48 hours. They were then cooled down to room temperature and weighed promptly (accuracy: 0.1 mg). The total mass of each aluminium tray (dry weight of the aluminium trays + *Lemna minor* dry weight [DW]) was recorded. The calculation of the dry weight was done by subtracting the known dry weights of the aluminium trays from those of the aluminium trays with the *Lemna minor* DW.

Table 7.2: Calculation of *Lemna* fresh [FW] and dry [DW] weight in mg and internal water content in %

Replicate	FW [mg]	DW [mg]	Water content [%]
1	41.82	3.43	91.80
2	41.45	3.24	92.81
3	43.59	3.16	92.75
4	38.94	2.70	93.07
Mean	41.56	3.13	92.45
SD	1.92	0.31	0.57
VarK [%]	4.62	9.89	0.67

Source: Own depiction

7.2 Material and methods of *Daphnia magna* and *Lemna minor* chemical analyses

7.2.1 Chemical and Reagents

All standards were of high-purity grade (more than 90%) and were purchased from Sigma-Aldrich (Steinheim, Germany). The isotope-labeled internal standards (IS), Citalopram-D4, Propranolol-D7 and Ibuprofen-D3, were purchased from Analytical Standard Solutions (A2S) (France). Furthermore, the internal standard Diclofenac-D4 was purchased from HPC Standards

GmbH (Borsdorf, Germany), whereas Amitriptyline-D3 and was purchased from Cerilliant (Round Rock, TX). Stock standard solutions of individual compounds (1000 μg mL−1) were prepared in MeOH and stored at −20 °C in amber glass bottles to prevent photo-degradation. Regarding the materials used during the sample preparation procedure, bulk beads, 1.4 mm (zirconium oxide) from Bertin Technologies, (France) and regenerated cellulose syringe RC filters (pore size 0.2 μm, diameter 15mm) from Phenomenex (Torrance, CA, USA) were used.

All the solvents used for the sample preparation as well as the LC-QTOF-MS analysis were UPLC-MS grade. Methanol (MeOH) was purchased from Merck (Darmstadt, Germany) and distilled water (H2O) was provided by a Milli-Q purification apparatus (Direct-Q UV; Millipore, Bedford, MA, USA). ACN was supplied from Merck (Darmstadt, Germany). The additives of the mobile phases, ammonium formate (≥ 99.0%), ammonium acetate (99%), and formic acid (99%) were all purchased from Fluka (Buchs, Switzerland).

7.2.2 Sample preparation

7.2.2.1 Water samples from exposure experiments

For each exposure water sample at each pH value and state (start or end), three separated centrifuge tubes were available. So, equal parts of the water exposure samples from each tube were mixed and transferred into a new centrifuge tube, constituted the mix samples. The new mixed samples were diluted properly, in order for the final concentration to reach the linear dynamic range of the instrument for each substance. The diluted water samples were filtered with 0.2 RC filter syringes, transferred to glass vials and spiked with isotope-labeled internal standards. The aim of adding internal standards was to account for potential insufficiencies during the sample preparation. The final solutions in the vials should consist of MeOH:H_2O 1:1 v/v. For this reason, the proper amount of MeOH was added to each filtered water sample. The water samples were stored at -80oC until the LC-HRMS analysis.

7.2.2.2 *Daphnia magna* and *Lemna minor* samples from exposure experiments

The *Daphnia magna* and *Lemna minor* samples were delivered in Eppendorf tubes and stored at -80°C until their sample preparation. Sample preparation was applied to the samples and LC-ESI-QTOF-MS was employed for the analysis.

Initial analyses of the *Daphnia magna* and *Lemna minor* samples (1 sample/pH) were performed, to investigate if their concentration levels were included in the linear dynamic range of the instrument. For some samples, mainly from exposure experiments with Propranolol, we observed high concentrations and therefore, the samples were splinted into smaller ones. The empty and fill weight of the individual Eppendorf were determined and the fresh weight was calculated according to the equation .. below. The objective was to ensure quantification accuracy.

$$Weight\ of\ sample = Weight_{Filled\ Eppendorf} - Weight_{Empty\ Eppendorf}\ [...]$$

Before the sample preparation, the *Daphnia magna* and *Lemna minor* samples werespiked with isotopic-labeled internal standards (prepared in ice-cold methanol) of each analyte, to account for potential insufficiencies during the sample preparation. The samples were then pooled with 1.4 mm bulk beads (zirconium oxide) and subsequently, ice-cold mixture of the extraction solvents MeOH: H_2O (1:1 v/v) was added. The final volume of the extraction solvent was 500 μL. Homogenization and extraction of the samples was performed on a “bead beating” Precellys Evolution 24 homogenizer equipped with a Cryolis Evolution cooler. The homogenizer operated

at 8200 rpm for three cycles of 15 s each, with a 60s break between each cycle. The homogenized samples were centrifugated in a precooled (4 °C) centrifuge NEYA 16R (Remi Neya Centrifuges, Italy) for 10 minutes at 4 °°C and 11.000 rpm. The supernatants were collected and then transferred to Eppendorf tubes. Finally, the extracts were filtered through a 0.2 µm RC syringe filter and then transferred to glass vials for immediate LC-HRMS analysis.

The extracts that were out of the linear dynamic range were then diluted. The diluted extracts were analyzed as well, using LC-HRMS.

7.2.3 Instrumental analysis

The analysis of the exposure water samples and *Daphnia magna* and *Lemna minor* extracts was carried out using an LC-ESI-QTOF system. Ultrahigh-performance liquid chromatography (UHPLC) system with an HPG-3400 pump (Dionex Ultimate 3000 RSLC, Thermo Fischer Scientific, Dreieich, Germany) coupled to a QTOF mass spectrometer (Maxis Impact, Bruker Daltonics, Bremen, Germany) was used. LC-HRMS analyses were conducted in both RPLC and HILIC, as well as in both polarities, and are described in detail in section 6.2.3.

7.2.4 Identification procedure

For the identification of the different analytes, reference standard solutions were analyzed utilizing LC-ESI-QTOFMS. Processing of the raw data was carried out with the software tools Data Analysis 5.1 and TASQ CLIENT 2.1.

The applied procedure, as well as the criteria used for the identification of the different substances in water, *Daphnia magna* and *Lemna minor* samples, are described in detail in section 6.2.4.

7.2.5 Quantification procedure

Calibration curves were constructed with reference standard solutions at different concentration levels. The quantification of all the substances was performed by using the afore-mentioned reference standard calibration curves. The quantification procedure is described in more detail in the section 6.2.5 above.

7.3 Acute tests with *Daphnia magna*

The following tests with *Daphnia magna* were carried out according to OECD TG 202 (2004) at pH 6.0 and pH 8.5 by the UBA Ecotoxicology Laboratory Marienfelde. Endpoints considered were mortality after 24 h as well as after 48 h. EC_{10} and EC_{50} as well as 'no observed effect concentration' (NOEC) and 'lowest observed effect concentration' (LOEC) values were determined (for an overview of all substance effect concentrations see Appendix, Table A3). Modified M4 medium was used as medium.

7.3.1 Pharmaceuticals

7.3.1.1 NSAIDs

a. Diclofenac

Diclofenac induced a much higher toxicity at lower pH levels. While the EC_{50} at pH 8.5 was still 333.97 mg/L (24 h) and 151.02 mg/L (48 h), at pH 6 it dropped significantly to 11.18 mg/L (24 h) and 5.77 mg/L (48 h). At pH 6, 64 mg/L diclofenac at 24 h and only 28.8 mg/L at 48 h were sufficient to induce 100 % mortality. At pH 8.5, 261.04 mg/L was already required at both times.

Basically, mortality only increased significantly in the upper third, which led to a very steep rise in the dose-response curve.

At pH 6, there was a large uncertainty in the derived dose-response relationship both after 24 and 48 h due to the lack of data in the middle range of the curve, whereby the derived EC values were not statistically significant and were therefore not considered valid.

Despite all this, a pH-dependent toxicity of diclofenac can clearly be identified, which, according to an acid, induces increasing toxicity with decreasing pH, whereby, in the case of *Daphnia*, the toxicity at pH 6 increases by a factor of 16.9 (24 h) or 6.2 (48 h), respectively, compared to pH 8.5.

The mortality results for both time points are shown in Table 7.3a and the effect concentrations are depicted in Table 7.3b.

Table 7.3a: Results of the *Daphnia* test with diclofenac sodium salt for mortality

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH %		MW I_{b24h} %	MW I_{b48h} %
							start	end		
6.0	c	0	0	± 0	0	± 0	6.0	6.2	0	0
	1	0.48	0.170	± 0.012	0.194	± 0.014	6.1	6.2	5.00	5.00
	2	1.12	-	-	-	-	6.1	6.2	6.70	6.70
	3	2.50	-	-	-	-	6.1	6.2	0	0
	4	5.76	2.28	± 0.16	2.47	± 0.17	6.1	6.2	5.00	15.00
	5	12.78	-	-	-	-	6.1	6.2	70.00	90.00
	6	28.80	-	-	-	-	6.1	6.3	95.00	100
	7	64.00	19.8	± 1.4	20.1	± 1.4	6.1	6.2	100	100
8.5	c	0	0	± 0	0	± 0	8.5	8.1	0	0
	1	1.96	0.935	± 0.065	0.753	± 0.053	8.5	8.1	0	10.00
	2	4.56	-	-	-	-	8.5	8.1	5.00	15.00
	3	10.44	-	-	-	-	8.5	8.1	5.00	15.00
	4	22.19	11.55	± 0.81	10.52	± 0.74	8.5	8.1	20.00	45.00
	5	52.21	-	-	-	-	8.5	8.1	0	33.30
	6	117.47	-	-	55.7	± 3.9	8.5	8.1	13.30	66.70
	7	261.04	116.5	± 8.2	-	-	8.5	8.2	100	100

Results of the *Daphnia* test with diclofenac sodium salt for the endpoint mortality after 24 and 48 h in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Remark Eleni: UBA inform us that the samples accidentally not taken, test 6 instead.
Source: Own depiction

The $C_{measured}$ of the water samples from the different exposure experiments (Treatments 1, 4, 7) at pH 6 and at pH 8.5 of *Daphnia magna* to diclofenac (reported in Table) were lower (almost 2 times) than the theoretical ones. The $C_{measured}$ of the END water samples from the exposure experiment 1,4 and 7 at pH 6.0 were higher compared to the $C_{measured}$ of the respective START water sample. The $_{measured}$ concentrations of the END and the START water samples were at the same range, if the SD of the $C_{measured}$ is taken into consideration.

Table 7.3b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Daphnia* test with diclofenac sodium salt

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
6.0	5.76	5.76	12.80	12.80	2.55	2.21	8.50	6.47
8.5	117.47	10.44	261.04	22.19	20.08	3.59	143.93	39.97

Source: Own depiction

b. Ibuprofen

Ibuprofen induced an increasing mortality with increasing substance concentration at both pH values. A clear pH dependence of mortality was evident, which was even more pronounced than with propranolol. At pH 6.0, 100 % mortality was already induced at 28.00 mg/L ibuprofen (48 h), whereas 560.66 mg/L were required at pH 8.5. I.e. a decrease in pH led to increased toxicity. The mortality results for both time points are shown in Table 7.4a and the effect concentrations are depicted in Table 7.4b.

Table 7.4a: Results of the *Daphnia* test with ibuprofen sodium salt for mortality

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH %		MW I_{b24h} %	MW I_{b48h} %
							start	end		
6.0	c	0	0	± 0	0	± 0	6.1	6.2	0	0
	1	1.12	0.89	± 0.17	0.931	± 0.047	6.1	6.2	0	5.00
	2	2.52	-	-	-	-	6.1	6.2	0	20.00
	3	5.60	-	-	-	-	6.1	6.2	15.00	20.00
	4	12.32	8.7	± 1.1	6.93	± 0.34	6.1	6.2	45.00	95.00
	5	28.00	-	-	-	-	6.1	6.2	100	100
	6	55.99	-	-	-	-	6.1	6.2	100	100
	7	111.98	105.70	± 3.5	94.5	± 2.7	6.1	6.2	100	100
8.5	c	0	0	± 0	0	± 0	8.4	8.1	0	0
	1	5.05	4.15	± 0.557	3.62	± 0.514	8.4	8.1	0	0
	2	11.21	-	-	-	-	8.4	8.1	0	0

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH %		MW I_{b24h} %	MW I_{b48h} %
							start	end		
	3	25.23	-	-	-	-	8.4	8.1	0	5.00
	4	56.07	46.10	± 5.00	45.60	± 3.40	8.4	8.1	0	0
	5	112.13	-	-	-	-	8.4	8.1	0	15.00
	6	252.30	-	-	-	-	8.4	8.1	25.00	90.00
	7	560.66	459.90	± 24.80	456.50	± 14.90	8.4	8.1	90.00	100

Results of the *Daphnia* test with ibuprofen sodium salt for the endpoint mortality after 24 and 48 h in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Source: Own depiction

The $C_{measured}$ of the water samples from the four different exposure experiments at (NK, Treatments 1, 4, 7) pH 6 and at pH 8.5 of *Daphnia magna* to Ibuprofen (reported in Table 7.4a), were at the same range with the theoretical ones. In addition, the $C_{measured}$ of the START water samples were higher compared to the $C_{measured}$ of the END water samples, for most of the exposure experiments in both pH values. Regarding the water samples of the exposure 1 experiments at pH 6, The measured concentrations of the END and the START water samples were at the same range, if the SD of the $C_{measured}$ is taken into consideration. The SD of the $C_{measured}$ from exposure 7 experiment at pH 6 as well as from exposure 4 and 7 experiment at pH 8.5, were higher compared with the SD of the $C_{measured}$ at the other exposure experiments of each pH value. In these samples, the $C_{exposure}$ were higher and for this reason more dilution steps were required. As mentioned above the standard deviation was higher since the analytical error appears to increase when more dilution steps are performed.

Table 7.4b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Daphnia* test with ibuprofen sodium salt

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
6.0	5.60	1.12	12.32	2.52	5.72	2.19	11.18	5.77
8.5	112.13	112.13	252.30	252.30	201.07	71.71	333.97	151.02

Source: Own depiction

7.3.1.2 Beta blockers

a. Propranolol

Propranolol induced increasing mortality with increasing substance concentration at both pH values, with mortality at pH 6.0 (48 h) varying between 10 and 35 % in the mean concentrations from 7.247 to 60.392 mg/L. Furthermore, a clear pH dependence of mortality was evident, with 100 % mortality being induced at pH 8.5 at 18.624 mg/L propranolol (48 h), whereas 120.784 mg/L were required for this at pH 6.0. I.e. a shift of the pH into the basic range led to increased toxicity. The mortality results for both time points are shown in Table 7.5a and the effect concentrations are depicted in Table 7.5b.

Table 7.5a: Results of the *Daphnia* test with propranolol hydrochloride for mortality

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH %		MW I_{b24h} %	MW I_{b48h} %
							start	end		
6.0	c	0	0	± 0	0	± 0	6.1	6.3	0	0
	1	1.933	1.434	± 0.022	1.468	± 0.070	6.1	6.2	0	0
	2	3.624	-	-	-	-	6.1	6.2	0	5.00
	3	7.247	-	-	-	-	6.1	6.2	0	10.00
	4	14.494	11.88	± 0.79	11.21	± 0.54	6.1	6.2	0	35.00
	5	30.196	-	-	-	-	6.1	6.2	0	10.00
	6	60.392	-	-	-	-	6.1	6.2	15.00	35.00
	7	120.78	92.7	± 5.2	92.3	± 3.6	6.1	6.2	45.00	100
8.5	c	0	0	± 0	0	± 0	8.4	8.1	0	0
	1	0.076	0.0795	± 0.0062	0.0786	± 0.0038	8.5	8.0	0	0
	2	0.190	-	-	-	-	8.5	8.1	0	0
	3	0.476	-	-	-	-	8.5	8.1	0	0
	4	1.194	1.19	± 0.11	1.26	± 0.16	8.5	8.1	0	0
	5	2.976	-	-	-	-	8.5	8.1	0	0
	6	7.450	-	-	-	-	8.5	8.1	5.00	10.00
	7	18.624	19.0	± 2.0	19.0	± 1.0	8.5	8.0	90.00	100

Results of the *Daphnia* test with propranolol hydrochloride for the endpoint mortality after 24 and 48 h in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Source: Own depiction

The $C_{measured}$ of the water samples from the four different exposure experiments (NK, Treatments 1, 4, 7) at pH 6 and at pH 8.5 of *Daphnia magna* to Propranolol (reported in Table 7.5a) were at the same range with the theoretical concentration. The $C_{measured}$ of the START and the END water samples of the exposure experiments at pH 6 and at pH 8.5 were at the same range. Furthermore, the $C_{measured}$ of the START water samples were higher compared to the $C_{measured}$ of the END water samples, for all the exposure experiments at both pH values. The SD of the $C_{measured}$ from exposure experiment 7 at pH 6 and pH 8.5 were higher compared to the SD of the $C_{measured}$ at the other exposure experiments. In these samples, the exposure concentrations were higher and for this reason more dilution steps were required.

Table 7.5b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Daphnia* test with propranolol hydrochloride

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
6.0	60.39	7.25	120.78	14.45	56.88	8.64	126.72	47.82
8.5	7.45	7.45	18.62	18.62	8.35	7.45	12.47	8.99

Source: Own depiction

7.3.1.3 Anti-depressants

a. Amitriptyline

Amitriptyline induced increasing mortality with increasing substance concentrations at both pH levels. At pH 8.5, effects were more pronounced at lower concentrations, e.g. a concentration of around 3 mg/L amitriptyline hydrochloride induced 15 % mortality at pH 6.0 and 100 % mortality at pH 8.5 at time point 48 h. This was also reflected in the EC50 levels which were with 47.12 mg/L (pH 6.0) and 2.18 mg/L (pH 8.5) at 24 h 21 times lower and with 13.01 mg/L (pH 6.0) and 1.14 mg/L (pH 8.5) at 48 h 11 times lower at pH 8.5 than at pH 6.0. The mortality results for both time points are shown in Table 7.6a and the effect concentrations are depicted in Table 7.6b.

Table 7.6a: Results of the *Daphnia* test with amitriptyline hydrochloride for mortality

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH %		MW I_{b24h} %	MW I_{b48h} %
							start	end		
6.0	c	0	0	± 0	0	± 0	6.07	6.20	0	5.00
	1	0.51	0.0320	± 0.0022	0.0318	± 0.0022	6.06	6.20	0	5.00
	2	1.27	-	-	-	-	6.06	6.20	5.00	10.00
	3	3.17	-	-	-	-	6.06	6.20	0	15.00
	4	7.95	5.96	± 0.42	5.95	± 0.42	6.06	6.20	10.00	30.00
	5	19.86	-	-	-	-	6.06	6.20	20.00	30.00
	6	49.64	-	-	-	-	6.06	6.20	25.00	100
	7	124.11	83.5	± 5.3	95.1	± 6.7	6.06	6.20	100	100
8.5	c	0	0	± 0	0	± 0	8.44	8.00	0	0
	1	0.21	0.0914	± 0.0064	0.1027	± 0.0072	8.42	8.10	10.00	15.00
	2	0.54	-	-	-	-	8.42	8.10	5.00	5.00
	3	1.34	-	-	-	-	8.42	8.10	25.00	40.00
	4	3.36	2.22	± 0.16	2.26	± 0.16	8.42	8.10	50.00	100

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH %		MW I_{b24h} %	MW I_{b48h} %
							start	end		
	5	8.40	-	-	-	-	8.42	8.10	100	100
	6	21.01	-	-	-	-	8.42	8.10	100	100
	7	52.53	32.3	± 2.3	36.3	± 2.5	8.42	8.10	100	100

Results of the *Daphnia* test with amitriptyline hydrochloride for the endpoint mortality after 24 and 48 h in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Source: Own depiction

The $C_{measured}$ of the water samples from the different exposure experiments (Treatments 4, 7) at pH 6 and at pH 8.5 of *Daphnia magna* to Amitriptyline (reported in Table 7.6a) were at the same range with the theoretical ones. Although, the $C_{measured}$ of the water samples from the exposure experiment 1 at pH 6.0 and at pH 8.5 was lower (almost 15 and 2 times respectively) than the theoretical one. The water samples were diluted only 5 and 2 times respectively (*in the vial prior to analysis*). In addition, the $C_{measured}$ of the END water samples from the exposure experiment 4 and 7 at pH 6.0 and exposure experiment 7 at pH 8.5, were higher compared to the $C_{measured}$ of the respective START water sample. However, the measured concentrations of the END and the START water samples were at the same range, if the SD of the $C_{measured}$ is taken into consideration.

Table 7.6b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Daphnia* test with amitriptyline hydrochloride

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
6.0	7.95	19.86	19.86	49.64	8.84	2.07	47.12	13.01
8.5	0.54	0.54	1.34	1.34	0.54	0.36	2.18	1.14

Source: Own depiction

b. Citalopram

Citalopram induced increasing mortality with increasing substance concentrations at both pH levels. Effects on mortality were more pronounced at higher pH levels, but the differences were less pronounced than in other substances. The EC_{50} levels for 24 and 48 h were only around two and three times lower in pH 8.5 than in pH 6, respectively. Particularly at pH 8.5, the differences in all effect concentrations between 24 and 48 h were relatively low, whereas at pH 6.0, the effect concentrations were around twice as high at 24 h than 48 h. The mortality results for both time points are shown in Table 7.7a and the effect concentrations are depicted in Table 7.7b.

Table 7.7a: Results of the *Daphnia* test with citalopram hydrobromide for mortality

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH		MW I_{b24h} %	MW I_{b48h} %
							start	end		
6.0	c	0	0	± 0	0	± 0	6.00	6.10	0	5.00
	1	3.00	4.44	± 0.31	4.02	± 0.28	6.10	6.20	5.00	5.00
	2	5.99	-	-	-	-	6.10	6.20	0	0
	3	11.99	-	-	-	-	6.10	6.20	5.00	35.00
	4	23.97	36.6	± 2.6	33.9	± 2.4	6.10	6.20	20.00	25.00
	5	47.94	-	-	-	-	6.10	6.20	60.00	100
	6	95.89	-	-	-	-	6.10	6.20	100	100
	7	191.78	252	± 18	292	± 20	6.10	6.20	90.00	100
8.5	c	0	0	± 0	0	± 0	8.50	8.00	0	0
	1	5.00	3.15	± 0.22	2.55	± 0.18	8.50	8.00	0	10.00
	2	10.00	-	-	-	-	8.50	8.00	5.00	5.00
	3	20.01	-	-	-	-	8.50	8.00	10.00	15.00
	4	40.02	20.2	± 1.4	18.2	± 1.3	8.50	8.00	75.00	90.00
	5	80.03	-	-	-	-	8.50	8.10	80.00	100
	6	160.06	-	-	-	-	8.50	8.10	100	100
	7	320.13	175	± 12	171	± 12	8.50	8.00	100	100

Results of the *Daphnia* test with citalopram hydrobromide for the endpoint mortality after 24 and 48 h in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Source: Own depiction

The $C_{measured}$ of the water samples from the different exposure experiments (NK, Treatments 1, 4, 7) at pH 6 and at pH 8.5 of *Daphnia magna* to Citalopram(reported in Table 7.7a) were at the same range with the theoretical ones. In addition, the $C_{measured}$ of the END water sample from the exposure experiment 7 at pH 6.0, was higher compared to the $C_{measured}$ of the respective START water sample. However, they were at the same range, if the SD of the $C_{measured}$ is taken into consideration.

Table 7.7b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Daphnia* test with citalopram hydrobromide

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
6.0	20.01	10.00	40.02	20.01	19.68	34.58	64.65	34.58
8.5	11.99	11.99	23.97	23.97	8.90	9.11	21.04	15.49

Source: Own depiction

7.4 Chronic tests with *Daphnia magna*

For the chronic *Daphnia* tests, the media to be used must be stabilised so that the selected pH levels remain as stable as possible during the tests in order to determine the pH effect on the ecotoxicity of the individual test subjects. For the acute *Daphnia* and lemna tests, the test media were stabilised by appropriate buffers without observing a relevant influence on the test organisms. For the chronic *Daphnia* test, various buffer systems were tested in several trials with regard to their effect on the mortality and reproductive performance of the *Daphnia*.

Within the framework of preliminary tests for chronic *Daphnia* tests, both the standard medium (M4 medium according to OECD TG 211 (2012)) and a series of modified media (only by pH adjustment as well as with buffer systems to stabilise the pH) were tested with regard to pH stability as well as mortality and reproductive performance of the *Daphnia* used.

The results of these preliminary tests are summarised in Table 7.8 below. The results marked in green met the requirements of OECD TG 211 (2012) with regard to valid results. The results marked red were not valid.

Table 7.8: Preliminary tests for modified media investigating pH stability as well as *Daphnia* mortality and reproduction

Medium	pH	Adjustments	pH stability	Test	
			as ΔpH	mortality	reproduction
Elendt M4 medium		Standard medium (OECD TG 211)	- 0.9	10 %	99.3
Elendt M4 medium		Standard medium (OECD TG 211)	- 1.3	20 %	86.1
Elendt M4 medium	6.0	with phosphate buffer (according to acute tests)	0.3	100 %	0.0
Elendt M4 medium	8.5	with glycine buffer (according to acute tests)	- 0.5	0 %	84.6
Elendt M4 medium	6.5	with phosphate buffer	0.2	100 %	0.0
Elendt M4 medium	7.0	without any buffer adjusted to pH 7.0	1.6	100 %	0.0
Elendt M4 medium	7.0	with HEPES buffer	- 0.1	40 %	24.8
Elendt M4 medium	8.5	with borate buffer according to Sörensen and Clark	- 0.2	100 %	0.0
Elendt M4 medium	8.6	with boric acid buffer according to Clark and Lubs	- 0.2	100 %	0.0

Preliminary tests for modified media investigating pH stability as well as *Daphnia* mortality and reproduction; reproduction is given as mean cumulative offspring per surviving parent animal.

Source: Own depiction

Valid tests can be performed with the standard medium according to OECD TG 211 (pH approx. 8.5) and with the modified Elendt M4 medium with a glycine buffer (pH 8.5). The pH shift during the test was ≤ 1.5, the mortality of the parent animals was ≤ 20 % and the mean cumulative offspring per surviving parent animal is ≥ 60.

With the other modified media (pH 6.0, 6.5, 7.0 as well as 8.5 and 8.6) no valid test results according to OECD TG 211 were achieved.

Hence, no suitable medium was found for the acidic or neutral pH range (pH 6.0, 6.5, 7.0) that meets the validity criteria of OECD TG 211. It was assumed that at pH values < 8.5, and also under the influence of the buffer systems, the daphnids did not achieve optimal development to survive exposure for 21 days and simultaneously produce ≥ 60 offspring.

Thus, it was not possible to run chronic *Daphnia* tests at two different pH levels, preferably in an acidic as well as alkaline pH range.

7.5 *Daphnia major* enrichment

7.5.1 Pharmaceuticals

7.5.1.1 NSAIDs

b. Ibuprofen

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Ibuprofen water samples, sampled at the Start and the End of the different exposure experiments of *Daphnia magna* at two different pH values (6, 8.5), were determined (Table 7.9). The total duration of these experiments was 48h. Different pH values are indicated with different colours in the following table.

Table 7.9: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Ibuprofen exposure experiments

Water Samples		pH	Exposure	Sampling point	C measured (ng mL^{-1}) ± SD	C theoretical (ng mL^{-1})
Ibuprofen	***Daphnia magna***	2021-0030-AADm (pH 6.0)	Test 1	0 h	(3.19 ± 0.25) *10^3	5 *10^3
			Test 1.1	48 h	(3.50 ± 0.27) *10^3	
			Test 1.2	48 h	(3.52 ± 0.27) *10^3	
			Test 1.3	48 h	(3.31 ± 0.26) *10^3	
		2021-0030-AADm (pH 8.5)	Test 1	0 h	(85.6 ± 6.7) *10^3	152 *10^3
			Test 1.1	48 h	(90.4 ± 7.1) *10^3	
			Test 1.2	48 h	(93.1 ± 7.3) *10^3	
			Test 1.3	48 h	(97.3 ± 7.6) *10^3	

Source: Own depiction

Regarding the results of the analysis of the water samples reported in Table 7.9, the $C_{measured}$ of the START and END water samples from all the exposure experiments at pH 6.0 and at pH 8.5 were at the same range, if the SD of the $C_{measured}$ is taken into consideration. Moreover, the $C_{measured}$ of the START and END water samples from the exposure experiments at pH 8.5 of *Daphnia magna* to Ibuprofen, were lower than the theoretical one.

Daphnia magna

- Internal Concentration (C_{int})

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Ibuprofen in the extracts of the *Daphnia magna* samples was determined (Table 7.10). Different pH values are indicated with different colors.

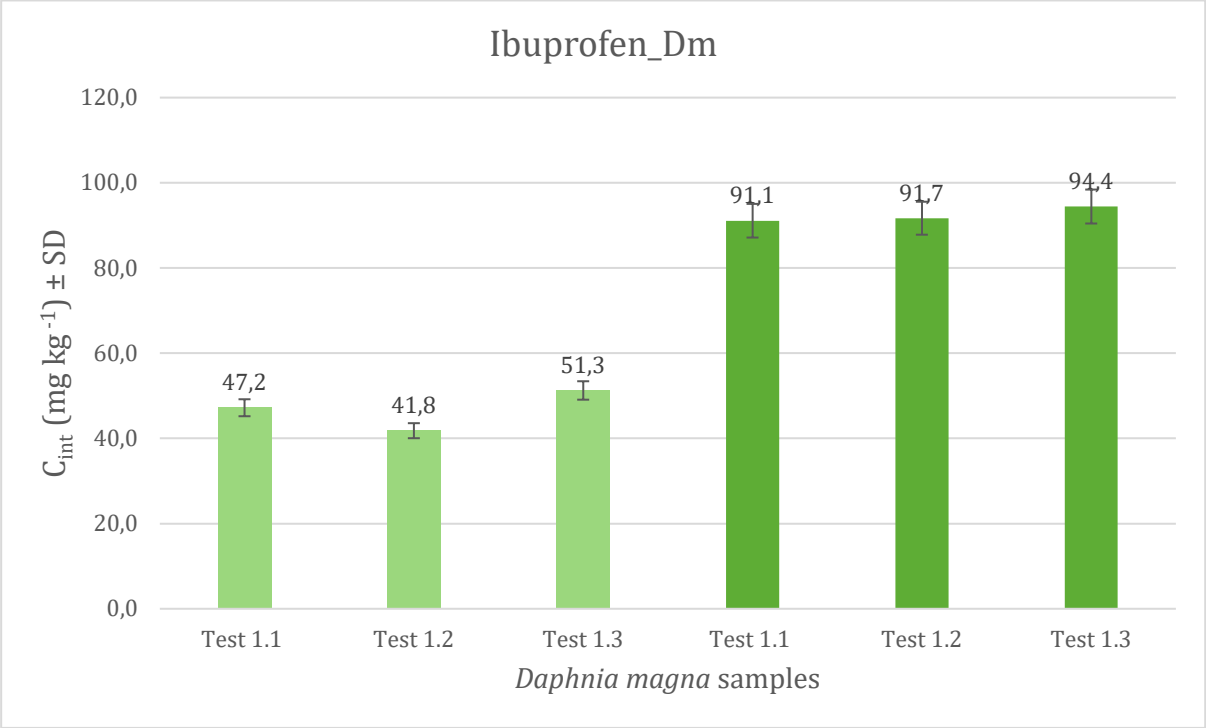
Table 7.10: Measured internal concentrations (mg kg^{-1}) of *Daphnia magna* samples from Ibuprofen exposure experiments

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	C_{int} (mg kg^{-1}) ± SD
Ibuprofen	***Daphnia magna***	2021-0030-AADm (pH 6.0)	Test 1.1	5.0	48 h	5.6	47.2 ± 2.0
			Test 1.2		48 h	12.3	41.8 ± 1.8
			Test 1.3		48 h	10.4	51.3 ± 2.2
		2021-0030-AADm (pH 8.5)	Test 1.1	152.0	48 h	8.4	91.1 ± 4.0
			Test 1.2		48 h	66.0	91.7 ± 3.9
			Test 1.3		48 h	71.2	94.4 ± 4.0

Source: Own depiction

In Figure 7.1 below, the C_{int} are presented for each pH value using bar charts. Different pH values are indicated with different colors.

Figure 7.5: Internal concentrations (C_{int} (mg kg^{-1})) of Ibuprofen in the *Daphnia magna* samples



Source: Own depiction

As far as the results of *Daphnia magna* samples of the Ibuprofen exposure experiments (presented in Table 7.10) is concerned, the C_{int} (mg kg^{-1}) were at the same range at each pH value. The *Daphnia magna* samples at pH 8.5 had higher internal concentrations in comparison with the rest of the samples at the other pH value.

► Bioconcentration Factor (BCF)

The bioconcentration factor (BCF) of each compound was calculated, in order to evaluate the extent of bioaccumulation. The bioconcentration factors (L kg^{-1}) of Ibuprofen were estimated separately for all the samples and they are presented below (Table 7.11).

Table 7.11: Measured bioconcentration factors (L kg^{-1}) of Ibuprofen in *Daphnia magna* samples

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	BCF (L kg^{-1})
Ibuprofen	***Daphnia magna***	2021-0030-AADm (pH 6.0)	Test 1.1	5.0	48 h	5.6	14.0
			Test 1.2		48 h	12.3	12.4
			Test 1.3		48 h	10.4	15.2
		2021-0030-AADm (pH 8.5)	Test 1.1	152.0	48 h	8.4	0.99
			Test 1.2		48 h	66.0	1.00
			Test 1.3		48 h	71.2	1.03

Source: Own depiction

Concerning the results from the BCF values, higher bioaccumulation potential (inversely proportional to the $C_{exposure}$) was observed at pH 6 since the bioconcentration factor is almost 15 times higher at this pH value in comparison with the BCF at pH 8.5

7.5.1.2 Beta blockers

a. Propranolol

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Propranolol water samples, sampled at the Start and the End of the different exposure experiments of *Daphnia magna* at two different pH values (6, 8.5), were determined (Table 7.12). The total duration of these experiments was 48h. Different pH values are indicated with different colors in the following table.

Table 7.12: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from Propranolol exposure experiments

Water Samples		pH	Exposure	Sampling point	C measured (ng mL^{-1}) ± SD	C theoretical (ng mL^{-1})
Propranolol	***Daphnia magna***	2021-0030-AADm (pH 6.0)	Test 1	0 h	(64.3 ±4.5) *10^3	60000
			Test 1.1	48 h	(37.0 ± 2.6) *10^3	
			Test 1.2	48 h	(44.8 ± 3.1) *10^3	
			Test 1.3	48 h	(39.0 ± 2.7) *10^3	
		2021-0030-AADm (pH 8.5)	Test 1	0 h	(8.77 ± 0.61) *10^3	10000
			Test 1.1	48 h	(9.37 ± 0.66) *10^3	
			Test 1.2	48 h	(7.71 ± 0.54) *10^3	
			Test 1.3	48 h	(8.38 ± 0.59) *10^3	

Source: Own depiction

The $C_{measured}$ of the END water samples from the exposure experiments at pH 6.0 of *Daphnia magna* to Propranolol, were lower than the theoretical one. In addition, the $C_{measured}$ of the water samples from the exposure experiments at pH 8.5 of *Daphnia magna* to Propranolol, were at the same range with the theoretical one. The $C_{measured}$ of the START and END water samples from all the exposure experiments at pH 8.5 were at the same range, if the SD of the $C_{measured}$ is taken into consideration.

Daphnia magna

► Internal Concentration (C_{int})

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Propranolol in the extracts of the *Daphnia magna* samples was determined (Table 7.13). Different pH values are indicated with different colors.

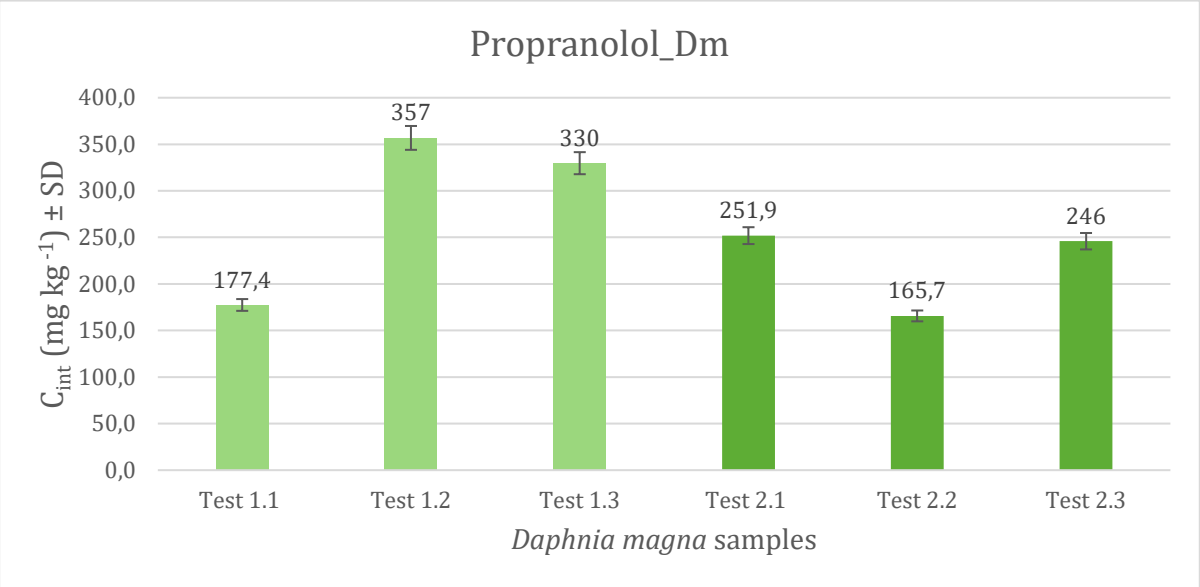
Table 7.13: Measured internal concentrations (mg kg^{-1}) of *Daphnia magna* samples from Propranolol exposure experiments

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	C_{int} (mg kg^{-1}) ± SD
Propranolol	***Daphnia magna***	2021-0030-AADm (pH 6.0)	Test 1.1	60.0	48 h	5.4	177.4 ± 6.3
			Test 1.2		48 h	4.9	357 ± 13
			Test 1.3		48 h	4.8	330 ± 12
		2021-0030-AADm (pH 8.5)	Test 1.1	10.0	48 h	4.8	251.9 ± 9.1
			Test 1.2		48 h	7.2	165.7 ± 5.8
			Test 1.3		48 h	4.9	245.9 ± 9.6

Source: Own depiction

In Figure 7.2 below, the C_{int} are presented for each pH value using bar charts. Different pH values are indicated with different colors.

Figure 7.2: Internal concentrations (C_{int} (mg kg^{-1})) of Propranolol in the *Daphnia magna* samples



Source: Own depiction

As far as the results of *Daphnia magna* samples of the Propranolol exposure experiments (presented in Table 7.13) is concerned, the C_{int} (mg kg^{-1}) were at the same range for most of the samples.

► Bioconcentration Factor (BCF)

The bioconcentration factor (BCF) of each compound was calculated, in order to evaluate the extent of bioaccumulation. The bioconcentration factors (L kg^{-1}) of Propranolol were estimated separately for all the samples and they are presented below (Table 7.14).

Table 7.14: Measured bioconcentration factors (L kg^{-1}) of Propranolol in *Daphnia magna* samples

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	BCF (L kg^{-1})
Propranolol	***Daphnia magna***	2021-0030-AADm (pH 6.0)	Test 1.1	60.0	48 h	5.4	3.83
			Test 1.2		48 h	4.9	7.71
			Test 1.3		48 h	4.8	7.13
		2021-0030-AADm (pH 8.5)	Test 1.1	10.0	48 h	4.8	29.4
			Test 1.2		48 h	7.2	19.4
			Test 1.3		48 h	4.9	28.7

Source: Own depiction

Concerning the results from the BCF values, higher bioaccumulation potential (inversely proportional to the $C_{exposure}$) was observed at pH 8.5 since the bioconcentration factor is almost 4 times higher at this pH value in comparison with the BCF at pH 6.

7.6 Acute tests with *Lemna minor*

7.6.1 Pharmaceuticals

7.6.1.1 NSAIDs

a. Diclofenac

For diclofenac, both pH values tested showed an increasing inhibition of both biomass and offshoot growth with increasing substance concentration. At pH 5.5, inhibition of biomass increase was already induced at 2.247 mg/L diclofenac, while inhibition of offshoot increase was not observed until 25.931 mg/L. At pH 7.5, inhibition of both biomass and offshoot growth occurred at the lowest test concentration of 7.204 mg/L.

In general, the effects on both endpoints increased mainly in the upper third of the tested concentrations, resulting in a very steep dose-response curve.

In summary, diclofenac showed an increase in toxicity with decreasing pH with toxicity increasing from pH 7.5 to pH 5.5 by a factor of 7.6 (inhibition of biomass) or by a factor of 5.3 (inhibition of shoot growth).

The results for the inhibition of biomass and offshoot growth are shown in Table 7.10a, whereas the effect concentrations are depicted in Table 7.10b.

Table 7.10a: Results of the *Lemna* test with diclofenac sodium salt for mean inhibition of biomass increase and mean inhibition of offshoot growth

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH		MW I_{bm} %	MW I_{bAZ} %
							start	end		
5.5	c	0	0	± 0	0	± 0	5.7	6.25	0	0
	1	1.01	0.307	± 0.021	0.317	± 0.022	5.7	6.33	-0.83	-3.34
	2	2.25	-	-	-	-	5.7	6.30	7.51	-3.98
	3	5.04	-	-	-	-	5.7	6.29	13.63	-4.93
	4	11.53	2.67	± 0.19	2.75	± 0.19	5.7	6.18	34.21	-0.16
	5	25.93	-	-	-	-	5.7	5.90	92.07	65.98
	6	57.62	13.45	± 0.94	10.87	± 0.76	5.8	5.90	111.27	98.41
7.5	c	0	0.408	± 0.029	0	± 0	7.3	7.7	0	0
	1	7.20	3.06	± 0.21	3.78	± 0.26	7.2	7.8	2.9	3.3
	2	16.01	-	-	-	-	7.3	7.7	10.7	12.8
	3	35.22	-	-	-	-	7.4	7.7	7.5	5.3
	4	80.04	50.3	± 3.5	56.6	± 4.0	7.4	7.6	18.8	12.8
	5	180.09	-	-	-	-	7.4	7.2	103.2	82.5
	6	400.20	208	± 15	200	± 14	7.3	7.2	108.7	97.7

Results of the *Lemna* test with diclofenac sodium salt for the endpoint mean inhibition of biomass increase (MW I_{bm}) and mean inhibition of offshoot growth (MW I_{bAZ}) in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Source: Own depiction

The $C_{measured}$ of the water samples from the different exposure experiments (Treatments 1, 4, 7) at pH 5.5 of *Lemna minor* to Diclofenac (reported in Table 7.10a) were lower (almost 3-5 times) than the theoretical ones. Also, the $C_{measured}$ of the water samples from the exposure experiment 1 and 6 at pH 7.5 were lower (almost 2 times) than the theoretical one. In addition, the $C_{measured}$ of the END water samples from the exposure experiment 4 at pH 7.5, were higher compared to the $C_{measured}$ of the respective START water sample. However, the $_{measured}$ concentrations of the END and the START water samples were at the same range, if the SD of the $C_{measured}$ is taken into consideration. Furthermore, Diclofenac was detected in the control sample, in low concentration. The sample preparation was implemented 3 separate times in order to ensure the final results. Therefore, the control samples maybe contaminated during the exposure experiments

Table 7.10b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Lemna* test with diclofenac sodium salt

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	biomass	offshoot	biomass	offshoot	biomass	offshoot	biomass	offshoot
5.5	2.25	11.53	5.04	25.91	6.649	17.561	13.543	23.572
7.5	35.22	35.22	80.04	80.04	62.11	75.41	102.83	124.88

Source: Own depiction

b. Ibuprofen

Ibuprofen induced an increasing inhibition of both biomass and offshoot growth with increasing substance concentration at both pH values. the inhibition of both endpoints was much more pronounced at pH 5.5 than at pH 7.5. The results for the inhibition of biomass and offshoot growth are shown in Table 7.11a, whereas the effect concentrations are depicted in Table 7.11b.

Table 7.11a: Results of the *Lemna* test with ibuprofen sodium salt for mean inhibition of biomass increase and mean inhibition of offshoot growth

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH		MW I_{bm} %	MW I_{bAZ} %
							start	end		
5.5	c	0	0	± 0	0	± 0	5.44	6.09	0	0
	1	0.45	0.430	± 0.049	0.424	± 0.046	5.45	6.14	12.1	-2.7
	2	1.36	-	-	-	-	5.46	6.05	22.3	-8.8
	3	4.24	-	-	-	-	5.46	5.90	38.4	3.1
	4	13.03	11.58	± 0.35	13.64	± 1.1	5.47	5.76	53.6	26.3
	5	39.39	-	-	-	-	5.51	5.59	96.4	74.4
	6	121.18	109	± 10	107.5	± 5.5	5.57	5.66	108.5	100.0
7.5	c	0	0	± 0	0	± 0	7.6	7.8	0	0
	1	5.00	3.49	± 0.66	4.717	± 0.058	7.4	7.7	30.8	26.7
	2	12.75	-	-	-	-	7.4	7.7	28.1	22.4
	3	31.37	-	-	-	-	7.2	7.5	35.1	35.4
	4	78.43	59.4	± 6.1	63.4	± 2.9	7.2	7.3	52.6	48.0
	5	196.07	-	-	-	-	7.3	7.2	67.3	58.4
	6	490.18	367	± 18	412	± 17	7.3	7.2	97.3	92.1

Results of the *Lemna* test with ibuprofen sodium salt for the endpoint mean inhibition of biomass increase (MW I_{bm}) and mean inhibition of offshoot growth (MW I_{bAZ}) in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.

Source: Own depiction

The $C_{measured}$ of the water samples from the four different exposure experiments (NK, Exposure 1, 4, 6) at pH 5.5 and at pH 7.5 of *Lemna minor* to Ibuprofen (reported in Table 7.11a), were at the same range with the theoretical ones. The $C_{measured}$ of the START water samples were at the same range with the $C_{measured}$ of the END water samples, for most of the exposure experiments at pH 5.5. However, the $C_{measured}$ of the END water samples from the exposure 4 experiments at pH 5.5 and all the exposure experiments (Exposure 1, 4, 6) at pH 7.5 were higher compared to the $C_{measured}$ of the respective START water samples (presented in Table 7.11a). Nevertheless, the measured concentrations of the END and the START water samples were at the same range for most of the cases, if the SD of the $C_{measured}$ is taken into consideration. Furthermore, the SD of the $C_{measured}$ from exposure 6 experiments at pH 5.5 and exposure 4 and 6 experiments at pH 7.5 were higher compared with the SD of the $C_{measured}$ at the rest of the exposure experiments. In these samples the exposure concentrations were higher and for this reason more stages of dilution were required.

Table 7.11b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Lemna* test with ibuprofen sodium salt

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	biomass	offshoot	biomass	offshoot	biomass	offshoot	biomass	offshoot
5.5	≤ 0.45	4.241	≤ 0.45	13.027	0.7	7.58	6.56	22.29
7.5	< 5.0	< 5.0	≤ 5.0	≤ 5.0	2.05	2.65	48.16	67.32

Source: Own depiction

7.6.1.2 Beta blockers

a. Propranolol

Propranolol induced an increasing inhibition of both biomass and offshoot growth with increasing substance concentration at both pH values, whereby the inhibition of both endpoints was considerably more pronounced at pH 7.5 than at pH 5.5. The results for the inhibition of biomass and offshoot growth are shown in Table 7.12a, whereas the effect concentrations are depicted in Table 7.12b.

Table 7.12a: Results of the *Lemna* test with propranolol hydrochloride for mean inhibition of biomass increase and mean inhibition of offshoot growth

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH		MW I_{bm} %	MW I_{bAZ} %
							start	end		
5.5	c	0	0	± 0	0	± 0	5.9	7.1	0	0
	1	4.98	3.96	± 0.21	3.78	± 0.19	5.9	7.1	-8.0	-14.3
	2	9.95	-	-	-	-	5.9	7.1	-8.6	-10.2
	3	19.91	-	-	-	-	5.9	7.1	-2.7	0
	4	39.81	34.4	± 1.5	32.3	± 1.8	5.9	6.9	4.2	-3.1
	5	79.62	-	-	-	-	5.9	6.5	2.9	12.4
	6	159.25	141.1	± 3.9	114.2	± 3.7	5.9	5.6	75.1	60.9

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH		MW I_{bm} %	MW I_{bAZ} %
							start	end		
7.5	c	0	0	± 0	0	± 0	7.3	7.7	0	0
	1	5.02	3.58	± 0.22	3.53	± 0.17	7.3	7.7	-8.8	-5.1
	2	10.05	-	-	-	-	7.3	7.5	30.2	32.9
	3	20.10	-	-	-	-	7.4	7.4	60.1	58.9
	4	40.19	24.9	± 1.4	10.25	± 0.73	7.4	7.3	50.4	35.6
	5	80.39	-	-	-	-	7.4	7.0	85.2	64.8
	6	160.78	112	± 10	42.1	± 2.6	7.4	6.8	98.2	95.7

Results of the *Lemna* test with propranolol hydrochloride for the endpoint mean inhibition of biomass increase (MW I_{bm}) and mean inhibition of offshoot growth (MW I_{bAZ}) in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Source: Own depiction

The $C_{measured}$ of the water samples from the four different exposure experiments (NK, Exposure 1, 4, 6) at pH 5.5 and at pH 7.5 of *Lemna minor* to Propranolol (reported in Table 7.12a), were at the same range as the theoretical ones. However, as far as the water samples from the exposure 4 experiment at pH 7.5 is concerned, $C_{measured}$ were lower (almost 2 times) compared to the theoretical concentration. In addition, the $C_{measured}$ of the START water samples were higher (or at the same range) compared to the $C_{measured}$ of the END water samples, for all the exposure experiments (Exposure 1, 4, 6) at pH 5.5 and at pH 7.5. The SD of the $C_{measured}$ from exposure 4 and 6 experiments at pH 5.5 and at pH 7.5 were higher compared with the SD of the $C_{measured}$ at the exposure 1 experiments. In these samples, more stages of dilution were required since the exposure concentrations (*initial concentration of the water samples*) were higher.

Table 7.12b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Lemna* test with propranolol hydrochloride

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	biomass	offshoot	biomass	offshoot	biomass	offshoot	biomass	offshoot
5.5	39.81	79.62	79.62	159.25	52.68	75.07	108.72	139.31
7.5	5.02	5.02	10.05	10.05	4.77	3.87	22.93	31.16

Source: Own depiction

7.6.1.3 Anti-depressants

a. Amitriptyline

Amitriptyline induced an increasing inhibition of biomass and offshoot growth with increasing substance concentrations. Effects for the higher pH level was considerably more pronounced, also reflected in the EC_{50} levels that were 16 to 20 times lower at pH 7.5 than at pH 5.5. At pH 7.5, already medium concentrations of ≥ 3.20 mg/L induced very high inhibition rates in both endpoints of more than 80 %. Generally, at both pH levels, the difference between inhibition of biomass and offshoot growth was comparably low. NOEC and LOEC values for inhibition of biomass increase were not determined due to mathematical reasons/inappropriate data. The

results for the inhibition of biomass and offshoot growth are shown in Table 7.13a, whereas the effect concentrations are depicted in Table 7.13b.

Table 7.13a: Results of the *Lemna* test with amitriptyline hydrochloride for mean inhibition of biomass increase and mean inhibition of offshoot growth

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH		MW I_{bm} %	MW I_{bAZ} %
							start	end		
5.5	c	0	0	± 0	0	± 0	5.59	6.06	0	0
	1	0.53	0.276	± 0.019	0.420	± 0.029	5.56	6.12	-6.64	-2.84
	2	1.71	-	-	-	-	5.54	6.15	-1.22	1.50
	3	5.54	-	-	-	-	5.53	6.15	-3.12	3.51
	4	17.54	10.08	± 0.71	10.64	± 0.74	5.52	5.91	34.82	56.93
	5	56.77	-	-	-	-	5.51	5.66	81.44	85.98
	6	184.62	70.3	± 4.9	77.7	± 5.4	5.51	5.68	106.91	99.00
7.5	c	0	0	± 0	0	± 0	7.45	7.70	0	0
	1	0.51	0.268	± 0.019	0.190	± 0.013	7.50	7.49	17.13	15.79
	2	1.28	-	-	-	-	7.51	7.33	50.46	61.97
	3	3.20	-	-	-	-	7.52	7.21	85.42	90.83
	4	8.01	4.26	± 0.30	4.81	± 0.34	7.50	7.21	96.30	97.62
	5	20.01	-	-	-	-	7.51	7.22	96.99	100.00
	6	50.03	27.9	± 2.0	29.9	± 2.1	7.49	7.20	98.15	99.66

Results of the *Lemna* test with amitriptyline hydrochloride for the endpoint mean inhibition of biomass increase (MW I_{bm}) and mean inhibition of offshoot growth (MW I_{bAZ}) in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.
Source: Own depiction

The $C_{measured}$ of the water samples from the different exposure experiments (Treatments 1,4, 6) at pH 5.5 and at pH 7.5 of *Lemna minor* to Amitriptyline (reported in Table 7.13b) were lower (almost 2 times) than the theoretical ones. In addition, the $C_{measured}$ of the END water samples from the exposure experiment 1, 4 and 6 at pH 5.5 and exposure experiment 4 and 6 at pH 7.5, were higher compared to the $C_{measured}$ of the respective START water sample. In some cases, the measured concentrations of the END and the START water samples were at the same range, if the SD of the $C_{measured}$ is taken into consideration.

Table 7.13b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Lemna* test with amitriptyline hydrochloride

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	biomass	offshoot	biomass	offshoot	biomass	offshoot	biomass	offshoot
5.5	5.54	5.54	17.54	17.54	8.54	5.71	25.41	16.77
7.5	n. a.	≤ 0.51	n. a.	≤ 0.51.	0.38	0.40	1.25	1.05

Source: Own depiction

b. Citalopram

Citalopram induced an increasing inhibition of biomass and offshoot growth with increasing substance concentrations at both pH levels. The effects were significantly more pronounced at higher pH levels with EC_{50} levels about four times lower at pH 7.5 than at pH 5.5. At pH 7.5, already the lowest citalopram concentration of 4.42 mg/L affected in both endpoints, so that no NOEC, neither for inhibition of biomass nor offshoot growth, could be determined. At both pH levels, inhibition of offshoot growth was observed at lower concentration than effects on biomass. The results for the inhibition of biomass and offshoot growth are shown in Table 7.14a, whereas the effect concentrations are depicted in Table 7.14b.

Table 7.14a: Results of the *Lemna* test with citalopram hydrobromide for mean inhibition of biomass increase and mean inhibition of offshoot growth

pH	Treatment	$C_{nominal}$ mg/L	C_{start} ± SD mg/L		C_{end} ± SD mg/L		Test pH		MW I_{bm} %	MW I_{bAZ} %
							start	end		
5.5	c	0	0	± 0	0	± 0	5.4	6.1	0	0
	1	4.41	4.01	± 0.28	4.18	± 0.29	5.4	6.1	0.49	4.82
	2	9.46	-	-	-	-	5.4	6.1	-0.49	13.84
	3	22.07	-	-	-	-	5.4	6.2	-0.49	12.91
	4	50.45	44.6	± 3.1	45.8	± 3.2	5.4	5.9	0.24	14.77
	5	113.51	-	-	-	-	5.4	5.7	34.88	43.39
	6	252.24	203	± 14	233	± 16	5.4	6.0	77.32	80.40
7.5	c	0	0	± 0	0	± 0	7.43	7.61	0	0
	1	4.42	4.34	± 0.30	3.52	± 0.25	7.43	7.61	9.90	10.40
	2	9.46	-	-	-	-	7.43	7.49	10.30	14.80
	3	22.08	-	-	-	-	7.43	7.28	26.40	35.30
	4	50.47	52.2	± 3.7	48.7	± 3.4	7.43	7.23	58.60	61.90
	5	113.55	-	-	-	-	7.43	7.22	95.60	92.60
	6	252.33	236	± 17	290	± 20	7.43	7.59	99.30	98.60

Results of the *Lemna* test with citalopram hydrobromide for the endpoint mean inhibition of biomass increase (MW I_{bm}) and mean inhibition of offshoot growth (MW I_{bAZ}) in % per pH with integrated results for the chemical analyses of a low, medium and high concentration of the exposure medium.

Source: Own depiction

The $C_{measured}$ of the water samples from the different exposure experiments (Treatments 1,4, 6) at pH 5.5 and at pH 7.5 of *Lemna minor* to Citalopram (reported in Table 7.14b) were at the same range with the theoretical ones. In addition, the $C_{measured}$ of the END water samples from the exposure experiment 6 at pH 5.5 and at pH 7.5, were higher compared to the $C_{measured}$ of the respective START water sample. The SD of the $C_{measured}$ from exposure 6 experiments at pH 5.5 and at pH 7.5, were higher compared with the SD of the $C_{measured}$ at the rest of the exposure experiments.

Table 7.14b: 24 and 48 h NOEC, LOEC, EC_{10} and EC_{50} values for *Lemna* test with citalopram hydrobromide

pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
	biomass	offshoot	biomass	offshoot	biomass	offshoot	biomass	offshoot
5.5	50.45	4.41	113.51	9.46	66.39	34.63	151.33	123.61
7.5	≤ 4.42	≤ 4.42	≤ 4.42	≤ 4.42	11.99	8.14	37.70	32.87

Source: Own depiction

7.7 Chronic tests with *Lemna minor*

Due to the time limitations and the experiences made during the attempts to establish a buffer regime for chronic tests with *Daphnia magna*, it was refrained to conduct chronic tests with *Lemna minor*, particularly since in case of *Lemna*, a significantly longer exposure time of 21 days compared to 7 days would have been necessary, in which the buffer system had to be stabilised without inducing buffer-related mortality.

7.8 *Lemna minor* enrichment

7.8.1 Pharmaceuticals

7.8.1.1 NSAIDs

Ibuprofen

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Ibuprofen water samples, sampled at the Start and the End of the different exposure experiments of *Lemna minor* at two different pH values (5.5, 7.5), were determined (Table 7.15). The total duration of these experiments was 7d. Different pH values are indicated with different colours in the following table.

Table 7.15: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from the ibuprofen exposure experiments

Water Samples		pH	Exposure	Sampling point	C measured (ng mL^{-1}) ± SD	C theoretical (ng mL^{-1})
Ibuprofen	*Lemna minor*	2021-0031-AALm (pH 5.5)	Test 1	0 h	(11.21 ± 0.87) *10^3	14 *10^3
			Test 1.1	7 d	(10.34 ± 0.81) *10^3	

Water Samples		pH	Exposure	Sampling point	C measured (ng mL^{-1}) ± SD	C theoretical (ng mL^{-1})
			Test 1.2	7 d	(9.51 ± 0.74) *10^3	
			Test 1.3	7 d	(9.40 ± 0.73) *10^3	
		2021-0031-AALm (pH 7.5)	Test 2	0 h	(42.7 ± 3.3) *10^3	60 *10^3
			Test 2.1	7 d	(46.4 ± 3.6) *10^3	
			Test 2.2	7 d	(45.7 ± 3.6) *10^3	
			Test 2.3	7 d	(41.8 ± 3.3) *10^3	

Source: Own depiction

Regarding the results of the analysis of the water samples reported in Table 7.15, the $C_{measured}$ of the START and END water samples from all the exposure experiments at pH 5.5 and at pH 7.5 were at the same range, if the SD of the $C_{measured}$ is taken into consideration.

Lemna minor

- Internal Concentration (C_{int})

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Ibuprofen in the extracts of the *Lemna minor* samples was determined (Table 7.16). Different pH values are indicated with different colors.

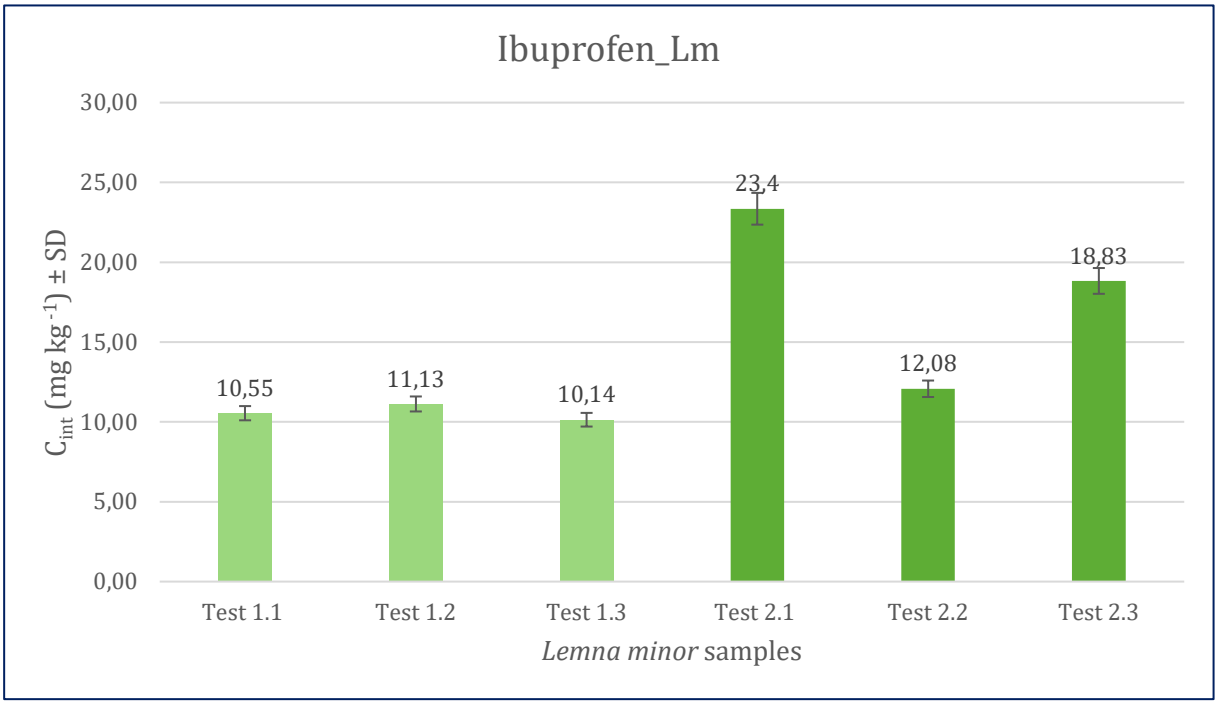
Table 7.16: Measured internal concentrations (mg kg^{-1}) of *Lemna minor* samples from Ibuprofen exposure experiments

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	C_{int} (mg kg^{-1}) ± SD
Ibuprofen	***Lemna minor***	2021-0031-AALm (pH 5.5)	Test 1.1	14.0	7 d	54.5	10.55 ± 0.45
			Test 1.2		7 d	74.5	11.13 ± 0.47
			Test 1.3		7 d	65.3	10.14 ± 0.43
		2021-0031-AALm (pH 7.5)	Test 2.1	60.0	7 d	26.1	23.35 ± 0.99
			Test 2.2		7 d	19.2	12.08 ± 0.52
			Test 2.3		7 d	14.7	18.83 ± 0.81

Source: Own depiction

In Figure 7.3 below, the C_{int} are presented for each pH value using bar charts. Different pH values are indicated with different colors.

Figure 7.3: Internal concentration (C_{int} (mg kg^{-1})) of Ibuprofen in the *Lemna minor* samples



Source: Own depiction

As far as the results of *Lemna minor* samples of the Ibuprofen exposure experiments (presented in Table 7.16) is concerned, the C_{int} (mg kg^{-1}) were at the same range for most of the samples.

► Bioconcentration Factor (BCF)

The bioconcentration factors (L kg^{-1}) of Ibuprofen were estimated separately for all the samples and they are presented below (Table 7.17).

Table 7.17: Measured bioconcentration factors (L kg^{-1}) of Ibuprofen in *Lemna minor* samples

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	BCF (L kg^{-1})
Ibuprofen	***Lemna minor***	2021-0031-AALm (pH 5.5)	Test 1.1	14.0	7 d	54.5	1.04
			Test 1.2		7 d	74.5	1.10
			Test 1.3		7 d	65.3	1.00
		2021-0031-AALm (pH 7.5)	Test 2.1	60.0	7 d	26.1	0.53
			Test 2.2		7 d	19.2	0.27
			Test 2.3		7 d	14.7	0.43

Source: Own depiction

Concerning the results from the BCF values, higher bioaccumulation potential (inversely proportional to the $C_{exposure}$) was observed at pH 6 since the bioconcentration factor is almost 2 times higher at this pH value in comparison with the BCF at pH 7.5.

7.8.1.2 Beta blockers

Propranolol

Water Samples

The concentration levels ($C_{measured}$ ± SD (ng mL^{-1})) of Propranolol water samples, sampled at the Start and the End of the different exposure experiments of *Lemna minor* at two different pH values (5.5, 7.5), were determined (Table 7.18). The total duration of these experiments was 7d. Different pH values are indicated with different colours in the following table.

Table 7.18: Measured concentration ($C_{measured}$) levels (ng mL^{-1}) of water samples from the propranolol exposure experiments

Water Samples		pH	Exposure	Sampling point	C measured (ng mL^{-1}) ± SD	C theoretical (ng mL^{-1})
Propranolol	***Lemna minor***	2021-0024-AALm (pH 5.5)	Test 1	0 h	(74.2 ± 5.2) *10^3	110000
			Test 1.1	7 d	(80.7 ± 5.7) *10^3	
			Test 1.2	7 d	(66.3 ± 4.6) *10^3	
			Test 1.3	7 d	(67.7 ± 4.7) *10^3	
		2021-0024-AALm (pH 7.5)	Test 2	0 h	(13.30 ± 0.93) *10^3	25000
			Test 2.1	7 d	(17.9 ± 1.3) *10^3	
			Test 2.2	7 d	(18.9 ± 1.3) *10^3	
			Test 2.3	7 d	(17.3 ± 1.2) *10^3	

Source: Own depiction

Regarding the results of the analysis of the water samples reported in Table 7.18, the $C_{measured}$ of the START and END water samples from the exposure experiments at pH 5.5 and at pH 7.5 were lower than the theoretical one. The $C_{measured}$ of the START and END water samples from the exposure experiments at pH 5.5 were at the same range, if the SD of the $C_{measured}$ is taken into consideration.

Lemna minor

- Internal concentration (C_{int})

The internal concentration (C_{int} ± SD (mg kg^{-1})) of Propranolol in the extracts of the *Lemna minor* samples was determined (Table 7.19). Different pH values are indicated with different colors.

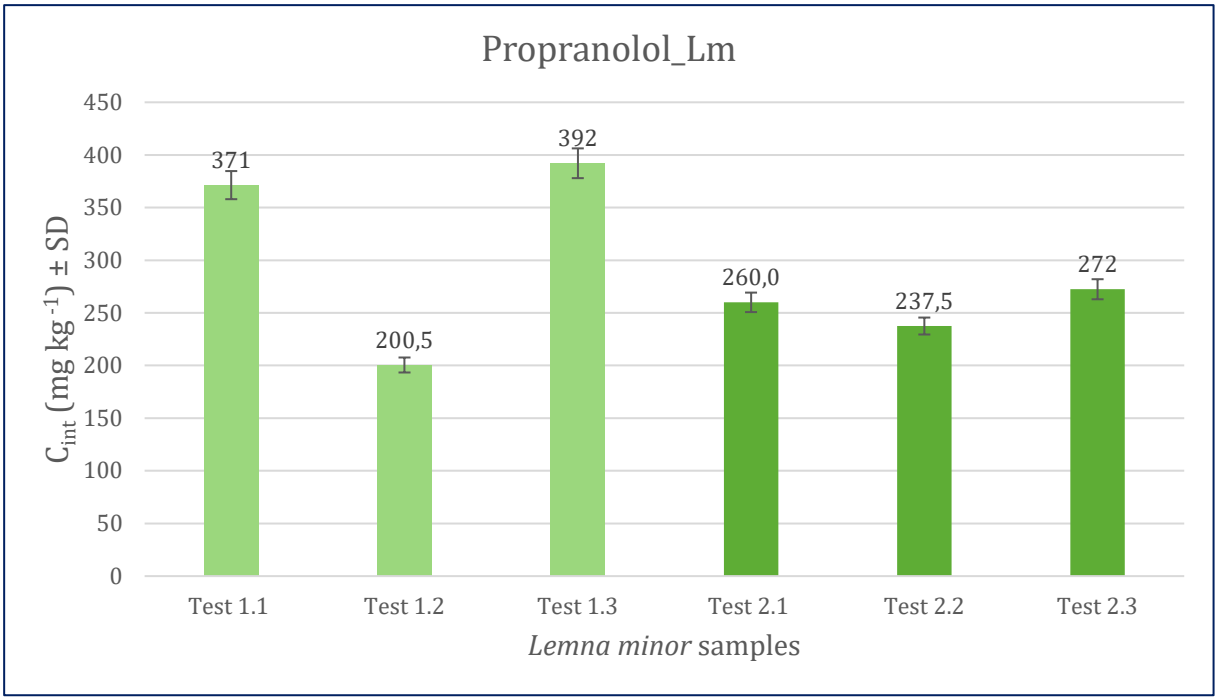
Table 7.19: Measured internal concentrations (mg kg^{-1}) of *Lemna minor* samples from Propranolol exposure experiments

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	C_{int} (mg kg^{-1}) ± SD
Propranolol	***Lemna minor***	2021-0024-AALm (pH 5.5)	Test 1.1	110.0	7 d	4.7	371 ± 13
			Test 1.2		7 d	6.1	200.5 ± 7.1
			Test 1.3		7 d	4.5	392 ± 14
		2021-0024-AALm (pH 7.5)	Test 2.1	25.0	7 d	5.7	260.0 ± 9.3
			Test 2.2		7 d	55.7	237.5 ± 8.0
			Test 2.3		7 d	8.9	272.5 ± 9.5

Source: Own depiction

In Figure 7.4 below, the C_{int} are presented for each pH value using bar charts. Different pH values are indicated with different colors.

Figure 7.4: Internal concentration (C_{int} (mg kg^{-1})) of Propranolol in the *Lemna minor* samples



Source: Own depiction

As far as the results of *Lemna minor* samples of the Propranolol exposure experiments (presented in Table 7.19) is concerned, the C_{int} (mg kg^{-1}) were at the same range for most of the samples.

► Bioconcentration Factor (BCF)

The bioconcentration factors (L kg^{-1}) of Propranolol were estimated separately for all the samples and they are presented below (Table 7.20).

Table 7.20: Measured bioconcentration factors (L kg^{-1}) of Propranolol in *Lemna minor* samples

Tissue Samples		pH	Exposure	$C_{nominal}$ (mg/L)	Sampling point	Weight of sample (mg)	BCF (L kg^{-1})
Propranolol	***Lemna minor***	2021-0024-AALm (pH 5.5)	Test 1.1	110.0	7 d	4.7	5.1
			Test 1.2		7 d	6.1	2.8
			Test 1.3		7 d	4.5	5.4
		2021-0024-AALm (pH 7.5)	Test 2.1	25.0	7 d	5.7	15.4
			Test 2.2		7 d	55.7	14.1
			Test 2.3		7 d	8.9	16.2

Source: Own depiction

Regarding the results from the BCF values (presented above), higher bioaccumulation potential (inversely proportional to the $C_{exposure}$) was observed at pH 7.5 since the bioconcentration factor is almost 3 times higher at this pH value in comparison with the BCF at pH 7.5.

8 WP 7: Integrative analysis of the collected data*

[* The contents of this chapter are envisaged to be published under the title "LogD-based modelling of pH-dependent action of ionizable chemicals reveals the relevance of both neutral and ionic species for fish embryotoxicity and has great potential for practical application in the regulation of chemicals." by Heinz-R. Köhler, Thomas Gräff, Mona Schweizer, Jasmin Blumhardt, Jasmin Burkhardt, Lisa Ehmann, Janine Hebel, Christoph Heid, Lone Kundy, Julia Kuttler, Miroslava Malusova, Friederike-Marie Moroff, Anne-Frida Schlösinger, Pia Schulze-Berge, Eleni I. Panagopoulou, Dimitrios E. Damalas, Nikolaos S. Thomaidis, Rita Triebskorn, Dirk Maletzki, Ute Kühnen and Peter C. von der Ohe]

8.1 Abstract

Depending on the pH, ionizable substances are present in varying proportions in their neutral or charged form. The extent to which these two chemical species contribute to the basal toxicity of ionizable chemicals and whether intracellular ion trapping has a decisive influence in this context is controversially discussed. Against this background, we determined the acute toxicity of more than 20 ionizable substances, each at different pH values, on the embryonic development of the zebrafish, *Danio rerio*, and supplemented this dataset with additional data from the literature. To simulate the toxicity of 12 acids and 12 bases, models were created based on different premises for the uptake and toxic effects of neutal and ionic species, and their abilities to explain the real data set were assessed. Using this approach, we were able to show that both neutral and charged species are taken up into cells according to their logD-based distribution, that both species exert basal toxicity intracellularly in a quantitatively similar manner, and that intracellular ion trapping thus plays no quantitative role in the exerted toxicity. Furthermore, it was possible to attribute differences in toxicity at different pH values for these 24 ionizable substances to the respective differences in logD with high accuracy, enabling this model to be used for predicting potential toxicities in worst-case scenarios, as required in the process of registration of ionizable chemicals and the definition of Environmental Quality Standards (EQS).

8.2 Introduction to the integrative analysis

It has long been known that the quantitative uptake of organic chemicals into cells and, associated with this, their potential to generate baseline toxicity, depends on the lipophilicity of these substances according to their octanol water coefficient (P_{ow}) or lipid water coefficient (P_{lipw}). For substances with variable lipophilicity such as ionisable chemicals, however, this relationship is much more complicated and thus requires a more differentiated view. Due to their sheer number, ionisable compounds are potentially of great importance in the environment. Depending on environmentally relevant pH fluctuations in the medium, these substances switch their speciation and lipophilicity among different states of charge and thus are capable to change their ability to be taken up by cells and to exert toxic effects in them (Rendal et al. 2011a, 2012; Neuwoehner & Escher 2011; Baumer et al. 2017; Bittner et al. 2018, 2019a, 2019b). To mathematically handle the relationship between pH conditions, bioaccumulation of ionisable substances (via bioconcentration factor (BCF) determinations) and the resulting toxicity (mostly EC50 values), various approaches were chosen, each of which referred to selected properties of the chemicals under consideration and - when comparing the toxicity exerted by them on different organisms - the different fat and protein contents of these. Thus, different models exist today that are well capable to explain BCF and/or EC50 for selected topics (Rendal et al. 2011a, 2012; Neuwoehner & Escher 2011; Baumer et al. 2017; Bittner et al. 2018, 2019a, 2019b, Goss et al. 2018) and whose suitability and limitations have been critically

reviewed (Rendal et al. 2011b, Escher et al. 2020). For a reliable modelling of pH-dependent toxicity in this context, it is crucial to know the proportion of charged and uncharged species of chemicals outside and inside the cells of an exposed organism, the quantity of charged and uncharged species that passively diffuse through the outer cell membrane into the cell interior (or are actively taken up by transporters), and whether baseline toxicity within the cell is exclusively caused by a single chemical species (possibly the uncharged species that binds to intracellular membrane structures) or by equally both.

To reduce the complexity of the relationships, here we focus exclusively on monoprotic ionisable chemicals for which no active transmembrane transport is known and for which uptake can therefore be assumed exclusively via passive diffusion, and on a single test system only, the developing zebrafish (*Danio rerio*) in its embryonic stages. Nevertheless, our approach followed the basic mechanistic relationships outlined above and schematically visualised in Figure 8.1, considering the modulating properties of different substance properties (acids vs. bases) and external pH conditions (Figure 8.2). In order to (1) decide which of the uptake and effect pathways of the different specifications are ultimately relevant for the resulting toxicity of a chemical at prevailing pH values and thus (2) establish a model for the reliable prediction of pH-dependent basal toxicity that can be generalised across many substance classes, we have set up several models, each of which taking different numbers of the possible processes and interactions visualised in Figs. 1 and 2 into account, and tested their reliability and predictive power using an extensive data set for 24 ionisable chemicals based on our own experiments and published data (Bittner et al. 2019b, Schweizer et al. 2022). We hypothesise that the model that determines a 'putatively effective' logD such that this value correlates most strongly with experimentally collected data on embryotoxicity includes those parameters and processes that are mechanistically responsible for baseline toxicity.

Therefore, we formulated three sets of models to simulate a most realistic 'putatively effective' logD curve, in which, based on theories formulated in the past, different parameters were included.

Each set consisted of two models based on identical mathematics but either were based on P_{ow} or on P_{lipw}. The reason for this was that, on one hand, P_{ow} is usually used to estimate the bioaccumulation of organic chemicals, whereas P_{lipw} is intended to directly describe the association of organic chemicals with biomembranes.

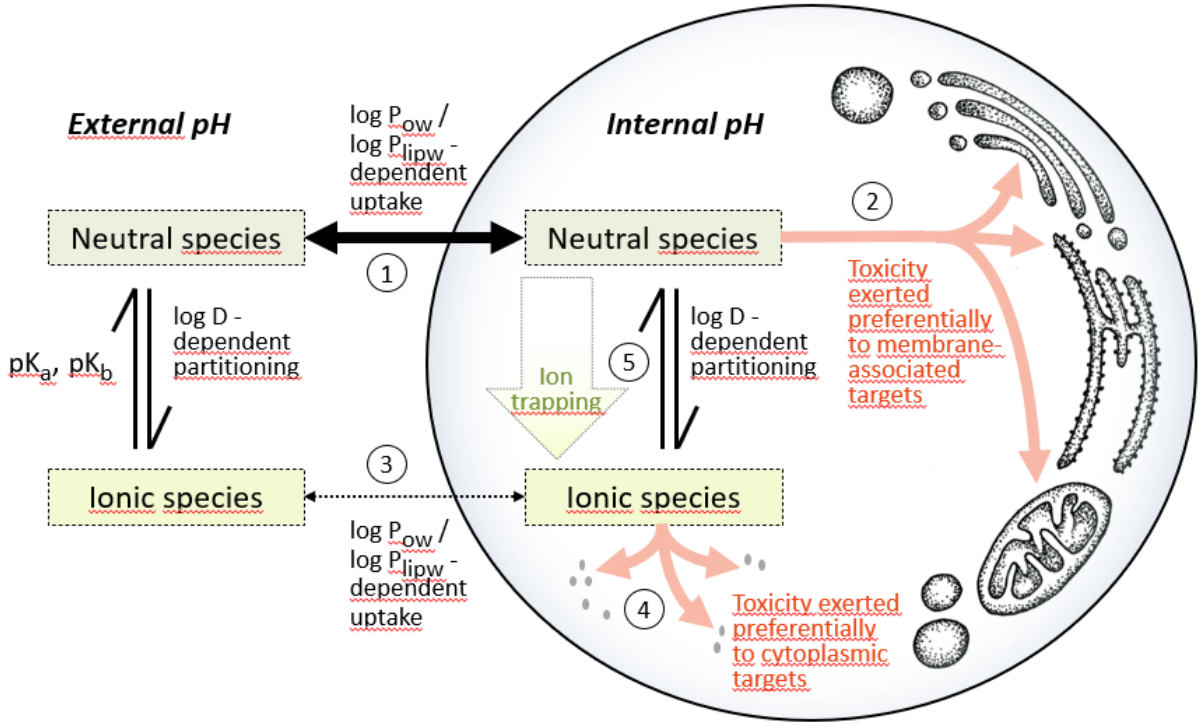
a. The first set of models considered all the processes outlined in Figures 8.1 and 8.2 (processes 1,2,3,4 and 5 in Fig 1) and assumed that both the uncharged and the charged species of a given chemical are passively membrane-permeable, although the uncharged form permeates membranes unequally better. For the pH-dependent ratios and lipophilicities, the logarithmic distribution coefficient, logD, was used as a basis. This model set also implied an automatic adjustment of a log D-dependent equilibrium within the cell according to the intracellular pH and, furthermore, assumed that both the uncharged and the charged species contribute to basic toxicity. We here call these models 'logD-based'.

b. In contrast, the second set of models considered the neutral species present under the given pH conditions only and attributed neither any importance in cellular uptake nor any potential for toxicity to the charged species. Thus, only processes 1 and 2 listed in Figure 8.1 were considered for these models. This type of model was based on the assumption that only the neutral form of an ionisable chemical will partition into the organic phase (ECETOC, 2013) and thus can passively diffuse through membranes. In this model set, there is also no intracellular conversion to an ionic species taken into consideration, and the toxicity is supposed to be exclusively caused by the neutral species. ('neutral-only' models).

c. The third model set also exclusively considered an uptake of neutral species into the cell (process 1 in Fig. 8.1). However, it took into account that, due to the deviation of the intracellular cytoplasmic pH value from that of the external medium, a re-distribution of uncharged and charged species takes place (ion trap, process 5 in Fig. 8.1) and thus the toxic effect of the entirety of the intracellular uncharged molecules (process 2 in Fig. 8.1) is modulated by this ion trapping. In this model, no toxicity is attributed to the charged species and any basal toxic effect is assumed to be exerted exclusively by the intracellular concentration of the neutral species of the chemicals, thus implying that the ion trapping process contributes to the detoxification of part of the ingested amount of substance. This model set will be referred to as 'ion trap-detox' in the following.

All in all, we thus calculated six models (three sets with two models each, based on P_{ow} and P_{lipw}) and assessed their suitability to explain the toxicities (LC_{50} data) found experimentally in the *Danio rerio* embryotoxicity test at different pH values for 24 ionisable substances. We further assumed that those of the theoretically conceivable processes for the uptake and action of chemicals in cells that had been used in the construction of the best-fit models also reflected the real processes responsible for toxicity. However, the central intention of our study was not only to elucidate quantitative aspects of the uptake and toxic effects of the differently charged species of chemicals, but in particular to develop a practicable tool for the reliable prediction of pH-dependent embryotoxicities of those substances, whose effects have been solely determined at a single pH value, e.g. those for which initial registration has been applied for within the framework of the chemical authorisation. Such a model should have great potential to be implemented in the official registration and risk assessment of ionisable chemicals.

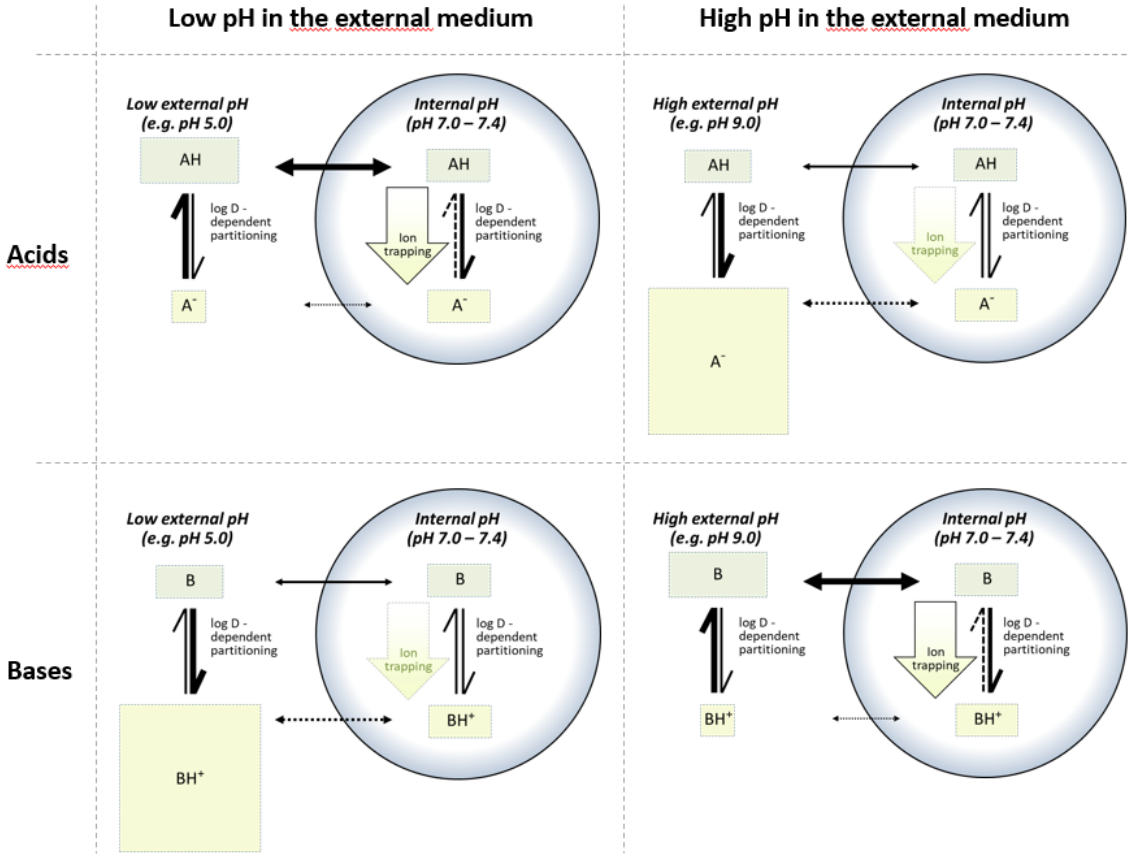
Figure 8.1: Distribution of charged (ionic) and uncharged (neutral) species of ionisable organic chemicals, their possible uptake pathways into the cell and possible targets of toxic action



Distribution of charged (ionic) and uncharged (neutral) species of ionisable organic chemicals, their possible uptake pathways into the cell and possible targets of toxic action. A pH-specific distribution of charged and uncharged species exists both outside and inside the cell. It can be assumed that the diffusion and thus the uptake of the uncharged species through the outer cell membrane (1) is easier and therefore more relevant than that of the charged species (3). The

intracellular conversion of uncharged molecules to charged, poorly membrane-permeable molecules leads to the establishment of an equilibrium (5) corresponding to the cytoplasmic pH and is called ion trapping. In principle, both species of a given ionisable chemical could exert baseline-toxic effects intracellularly, whereby it can be assumed that uncharged species predominantly damage membrane-associated functions and structures (2) and ionic species largely bind to targets dissolved in the cytoplasm (4). The pathways (1) to (5) are considered differently for the different models applied in this study (see text).
Source: Own depiction

Figure 8.2: Quantitative comparison of the proportions of ionic and uncharged species of acids and bases as well as diffusion intensities and direction of reaction equilibria at high and low pH in the external medium of cells



Quantitative comparison of the proportions of ionic and uncharged species of acids and bases as well as diffusion intensities and direction of reaction equilibria at high and low pH in the external medium of cells. Illustration of the dependencies between pH-value in the external medium of cells and the classification of chemicals in the groups of acids and bases considered in this study. Situation at equilibrium. The size of the symbols and the thickness and intensity of the arrows symbolise the quantity ratios and preferred diffusion and reaction directions. Top left: Acids are predominantly present in uncharged form (AH) at low pH of the external medium. The uncharged species diffuses easily into the cell and, since the internal pH in the cytoplasm is higher than in the external medium, is partially converted into the charged species by ion trapping. Diffusion of the charged species across the outer cell membrane hardly occurs. Top right: At high external pH, acids are predominantly charged (A-). Due to the high concentration gradient between the external medium and the cytoplasm, the charged species is somewhat more important for the uptake of the chemical into the cell than at low external pH. The uncharged species (AH) can diffuse through the cell membrane more easily than the charged species; however, due to the low proportion of AH in the external medium, the quantitative uptake of the uncharged acid into the cell is lower than at low pH in the external medium. In this situation, intracellular ion trapping is much less important. Bottom row: The ratios and situation for the charged (BH+) and uncharged (B) species of bases behave inversely to the situation for acids when comparing low (bottom left) and high pH (bottom right) in the external medium.
Source: Own depiction

8.3 Material and methods

8.3.1 Substances and buffer systems

Twenty-seven ionizable substances at up to 4 different pH levels, listed in Tab. 1, have been subject to zebrafish embryo toxicity testing (FET) according to OECD Guideline 236 (OECD 2013). Two of them could not be integrated into modelling because data could be reliably generated for a single pH only and/or could only be unreliably generated by extrapolation (enclomiphene, imidacloprid). In the case of citric acid, the chemical is actively taken up by membrane-bound transporters and therefore could not be incorporated into models based exclusively on passive diffusion as the uptake pathway. Thus, a total of 24 ionisable chemicals, 12 acids and 12 bases, were included in the modelling. The identities and all relevant physicochemical data of the included substances as well as information on the source of the data and the chemicals, the tested pH levels, the concentration range and the occasionally used solvents are given in Tab. 1. Data for five substances (cetirizine, dimethindene, doxylamine, genistein, ketotifen) have been taken from Bittner at al. (2019a) and Bittner at al. (2019b). All other data were collected by ourselves using the methodology described below.

To keep the test systems at constant pHs, four different buffer systems were used: a potassium hydrogen phthalate buffer (pH 5) according to Clark and Lubs (1916), a phosphate buffer based on sodium phosphate monobasic and sodium phosphate dibasic (pH 6), as well as a TRIS buffer (pH 8) (PUFFERAN® ≥ 99.9 % p.a., CAS: 77-86-1, Roth, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and a glycine buffer (pH 9) according to Sørensen (1912). The buffer solutions were prepared with reconstituted water which consisted of 25 mL of 0.23 g/L KCl, 2.59 g/L $NaHCO_3$, 4.93 g/L $MgS_4O \cdot 7\ H_2O$ and 11.76 g/L $CaCl_2 \cdot 2\ H_2O$ each and 900 mL double distilled water. HCl and NaOH were applied to adjust the test media to the correct pH value prior to the test.

8.3.2 Test design and concentrations

In order to determine the concentration of a test substance being lethal to 50 % of the test organisms (LC_{50}) at highest possible accuracy, three test runs were conducted for each substance and pH combination with differing test concentrations (for concentrations see Tab. 1). The spacing of test concentrations for the first run (run 1) was generally set wider to cover mortalities of *Danio rerio* embryos between 0 and 100 % after 96 hpf. Test concentrations were determined on the basis of literature-reported acute lethal concentrations for adult fish (i.e. OECD 203) or based on QSAR predictions (ECOSAR, U.S. EPA 1994). In a second run (i.e. run 2), the concentration range was adjusted to ideally induce mortalities between 20 and 80 % after 96 hpf. A third run (i.e. main run) offered the possibility to cover the concentration range around 50 % mortality more precisely. In runs 1 and 2, usually four concentrations per substance and pH were tested, compared to five concentrations in the main run. Provided that the controls did not differ significantly from each other, data points obtained from all experimental runs were merged into a single dose-response relationship and used collectively for statistical analyses.

8.3.3 Maintenance of zebrafish

Two equally sensitive strains of zebrafish *Danio rerio* are established at the Animal Physiological Ecology laboratory of Tübingen University and equally used for egg production: the ABTL strain obtained from the Institute of Neurobiology (Tübingen University) and the Westaquarium strain that originated from Heidelberg University but had been bred at the Animal Physiological Ecology laboratory for many years. The two strains were kept in separate 90-200 L tanks filled with a 1:1 mixture of filtered tap water (AE-2L water filter coming with an ABL-0240-29 activated carbon filter, 0.3 µm; *Reiser*, Seligenstadt, Germany) and purified water at a constant temperature of 27 ±1 °C. To ensure the water quality, the physicochemical water parameters

were kept in the following ranges: pH 7.4 ±0.2, conductivity 260-350 µS/cm, oxygen saturation 100 ±5 %, total hardness 8-12 °dH (≙ 142.4-213.6 mg/L $CaCO_3$), nitrate 1-5 mg/L, nitrite 0.025-1 mg/L; and the water was changed every two weeks by removing and refilling at least 30-50 % of the total water volume of the tanks. Zebrafish were kept at an artificial 12:12 day/night cycle and fed three times daily with dry flake food (*TetraMin®, Tetra GmbH*, Melle, Germany) and at least twice weekly with a mixture of mosquito larvae and glass worms (*Poseidon Aquakultur Freeze*, Ruppichteroth, Germany) for protein supplement.

8.3.4 Fish embryo test [FET]

To trigger egg production in otherwise unequipped fish tanks, Plexiglas® breeding boxes (20 x 20 x 6 cm) were introduced the evening before the test start. The breeding boxes were covered with a metal mesh to avoid predation on the eggs and artificial sea grass was added on top as optical spawning stimulus. Since zebrafish spawn at sunrise, eggs could be collected the following morning 1-1.5 h after the onset of light. The eggs were rinsed with lukewarm tap water and transferred into glass container filled with artificial water. The fertilisation rate (≥ 80 % according to OECD 236) was checked under a stereo microscope (*Stemi 2000-C, Zeiss*, Oberkochen, Germany).

The embryo test with *D. rerio* was carried out in glass Petri dishes. To avoid a reduction of bioavailability of the test material by adsorption, all Petri dishes were filled with the test solution at least 24 h prior to the test and emptied and refilled directly before test start to assure saturation of the glass walls. Subsequently, the eggs were transferred into pre-exposure Petri dishes (70 mm in diameter) filled with test solution of the respective substance concentrations and incubated at 26 ±1 °C in a heating cabinet for two hours. After incubation, well-developed eggs at the same developmental stage (128 - 256 cell stage according to Kimmel et al. (1995))) were picked and transferred into the test Petri dishes (30 mm in diameter).

For both, the first and second trial, four concentrations per pH were tested, using four Petri dishes with four embryos per treatment each. The main trial consisted of five concentrations per pH and included ten Petri dishes with one embryo each. For each pH, a buffer control was run at the respective pH. The embryos were incubated at 26 ±1 °C in a heating cabinet for 96 hpf with an artificial day/night cycle of 12:12 h.

Embryos were checked every 12-24 h for mortality and dead individuals and empty eggshells were removed to avoid oxygen deprivation due to bacterial degradation processes. The pH was measured during the test in at least one Petri dish per concentration daily exemplarily to assure pH stability (Table A2). Since pH 5 and pH 9 are marking the periphery of the zebrafish's tolerance range (McClure et al. 2006, Spence et al. 2006, Lawrence 2007, Horng et al. 2009), control mortalities occasionally exceeded 10%. Mortality analyses were conducted for 96 hpf and LC50 values determined using non-linear regression analysis (*TableCurve 2D v5.01, SYSTAT Software Inc.*) which were subsequently used for modelling. After 96h all experiments were terminated and the embryos euthanised with MS222 (tricaine methane sulphonate, *PHARMAQ Ltd.*, Fordingbridge, UK).

8.3.5 Chemical analysis

To verify the nominal concentrations determined for the LC_{50} for all chemicals at the different pH values, we carried out further exposures under the same conditions as in the toxicity tests described above, but exclusively with concentrations in close proximity to the LC_{50}s determined in each case by regressions, which enabled us to take sufficiently large volumes of exposure medium for chemical analysis at the beginning and end of the exposure period of 96 h in each case.

All chemicals (analytical standards, internal standards etc.) were purchased in the highest available purity and are listed in detail in the Supplementary Information (S1). Reference standard solutions were used to determine the concentration range that provides a linear signal response for each analyte. Preliminary dilution tests were performed with the exposure media samples to ensure that the concentration of the diluted sample will be included in the linear dynamic range of the instrument.

The exposure media samples were filtered through 0.2 µm RC syringe filters and diluted based on the preliminary test results. Afterwards, equal amounts of MeOH and the diluted samples were transferred into glass vials to reach a final constitution of 1:1 v/v MeOH:H2O. Finally, the samples were spiked with stable isotope-labeled internal standards (IS) in different concentration for each analyte and stored at -80 ˚C until the LC-HRMS analysis. The sample preparation procedure is described in (S2).

Analysis of the exposure media samples was carried out using a UHPLC-QTOF-MS system, equipped with a UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany) and coupled to the QTOF-MS mass analyzer (Maxis Impact, Bruker Daltonics, Bremen, Germany). The LC-HRMS analyses were conducted in reversed phase liquid chromatography (RPLC) in both positive and negative ionization modes. Chromatographic separation was performed using an Acclaim RSLC C18 column (2.1 x 100 mm, 2.2 µm) from Thermo Fischer Scientific (Dreieich, Germany). Detailed information about instrumental analysis is provided in supporting in the Supplementary information file (S3).

A target screening workflow was used to identify the different analytes (discussed in detail in the Supplementary Information (S4). Reference standard solutions were analyzed utilizing LC-ESI-QTOFMS. Processing of the raw data was implemented with the software tools Data Analysis 5.3 and TASQ Client 2.1. (Bruker Daltonics, Bremen,Germany). An in-house database containing analytical information for all the analytes was compiled and used to screen the samples (S4). The detection of the substances was based on specific screening parameters (mass accuracy < 5 mDa, retention time shift ± 0.2 min, isotopic fitting < 200 mSigma (only for confirmation of positive findings), whereas the presence of characteristic qualifier ions was considered mandatory for their successful identification.

Quantification was performed using standard addition calibration curves. The IS were used in the quantification process to achieve reliable and accurate quantitative results. For that reason, relative areas have been used (the area of each substance has been divided by the area of the respective IS (S4). The concentration of each analyte in the samples was determined by using these calibration curves.

8.3.6 Modelling

In order to determine which physicochemical processes during membrane passage and within the cell determine baseline toxicity, three types of models were generated and tested for their suitability to represent experimentally collected toxicity data. For these three model types, two models were generated each, one on the basis of log P_{ow} (n-octanol/water partitioning), and the other on the basis of log P_{lipw}, the partitioning coefficient between artificial liposomes (zwitterionic phosphatidylcholine), which are considered mimicking biological membranes (Escher & Schwarzenbach, 1996) and water. The distribution ratios of the neutral species of ionisable compounds are very similar in the octanol- and the liposome-water system, but the charged species partition was found to be significantly higher in the anisotropic lipid bilayer than into the bulk phase octanol (Escher & Schwarzenbach, 1996). It was therefore assumed that the distribution ratio of K_{lipw} is a more suitable descriptor for the uptake of ionizable compounds

into biological membranes than the corresponding K_{ow} (Escher & Schwarzenbach, 1996; Bittner et al. 2018, 2019a, 2019b; Escher et al. 2020).

In the following, both descriptors are compared for their explanatory value to predict the pH dependent toxicity in the FET for the following different model types. In the following equations, 'logD' is meant to be the putatively effective partition coefficient corresponding to exerted baseline toxicity and the subscript x always stands for either subscript ow (octanol/water) or subscript lipw (liposomes/water):

LogD-based models:

$$logD_X = log10(f_{neutral} \times 10^{log\ X\ \ neutral} + (f_{ionic}) \times 10^{log\ X\ \ ionic}) \quad (1)$$

For the neutral fraction applies:

$$f_{neutral} = \frac{1}{(1+10^{(pH-pK_a)})} \quad (2)$$

For the ionic fraction applies:

$$f_{ionic} = 1 - f_{neutral} \quad (3)$$

Neutral-only models:

For acids:

$$logD_x = logP_x - log[1 + 10^{(pH-pK_a)}] \quad (4)$$

For bases:

$$logD_x = logP_x - log[1 + 10^{(pK_a-pH)}] \quad (5)$$

Ion trap-based models:

$$logD_X = log10(f_{neutral} \times 10^{log\ Px\ \ neutral} + (1 - f_{ionic}) \times 10^{log\ Px\ \ ionic}) \quad (6)$$

assuming pH 7.3 for the cytoplasm of fish embryos:

$$f_{ion\ trap} = (f_{neutral}\ [pH] \times f_{neutral}) \quad (7)$$

The difference between chemical species partitioning between the exterior and the interior of cells is calculated

$$\Delta\ log\ D\ =\ log\ D_{(higher\ pH)}\ -\ log\ D_{(lower\ pH)} \quad (8)$$

The parameter Δ log D strongly depends on the pH of the exposure medium and describes the potential of the test substance to accumulate in the fish embryo. Thereby, log D considers both the neutral and the ionic form of the substance and hence, represents a kind of mixture exposure to both species. However, once chemical species enter the fish embryo cells, they are exposed to the internal pH of the cytoplasm (set to 7.3 according to Madshus (1988) who gives a regular pH 7.0 - 7.4 for this parameter). Depending on the external pH, this results into a higher or lower share of H^+ ions that effect the internal fractions. For example, weak acids entering fish embryos at pH 6 tend to have a lower share of neutral species inside the embryo as compared to what the exposure medium would suggest. The reason is that part of the neutral species that entered the embryo will be ionized due to intensified dissociation (lower mean log D in the cytoplasm). Since this type of model assumes that the ionised species does not develop any toxicity, the result of ion trapping would be a lower toxic effect than suggested from the log D in the external medium.

8.3.7 Statistics

Based on 96 hpf cumulative mortality data, non-linear regressions were run with *TableCurve 2D* (v5.01, *SYSTAT Software Inc.*). From those non-linear regression curves the LC_{50} values were calculated for each substance and pH. The hatching rate was assessed with a Cox regression followed by a sequential Bonferroni-Holm correction for multiple testing (initial p=0.05) and the heart rate was analysed with a linear regression method using *JMP® 14.2.0 (SAS Institute Inc.)*.

The quality of the models to correspond with the mortality data was assessed by root-mean-square error (RMSE) calculation for the 14 chemicals that were tested at ≥4 pH levels. Significance of mean RMSEs was checked by Welch´s ANOVA followed by Benjamini-Hochberg correction for multiple comparisons (initial p=0.05).

To assess the relationship between log D and toxicity, the Δ log LC_{50} values at 96 hpf were calculated for all possible binary combinations of pH for a given substance according to equation 9 for each substance and correlated with the respective Δ log D:

$$\Delta\ log\ LC_{50} = log\ LC_{50\ (higher\ pH)} - log\ LC_{50\ (lower\ pH)} \quad (9)$$

A linear regression was run with the combined data set of all substances using *JMP® 14.2.0 (SAS Institute Inc.).*

8.4 Results of the integrative analysis

8.4.1 Chemical analysis

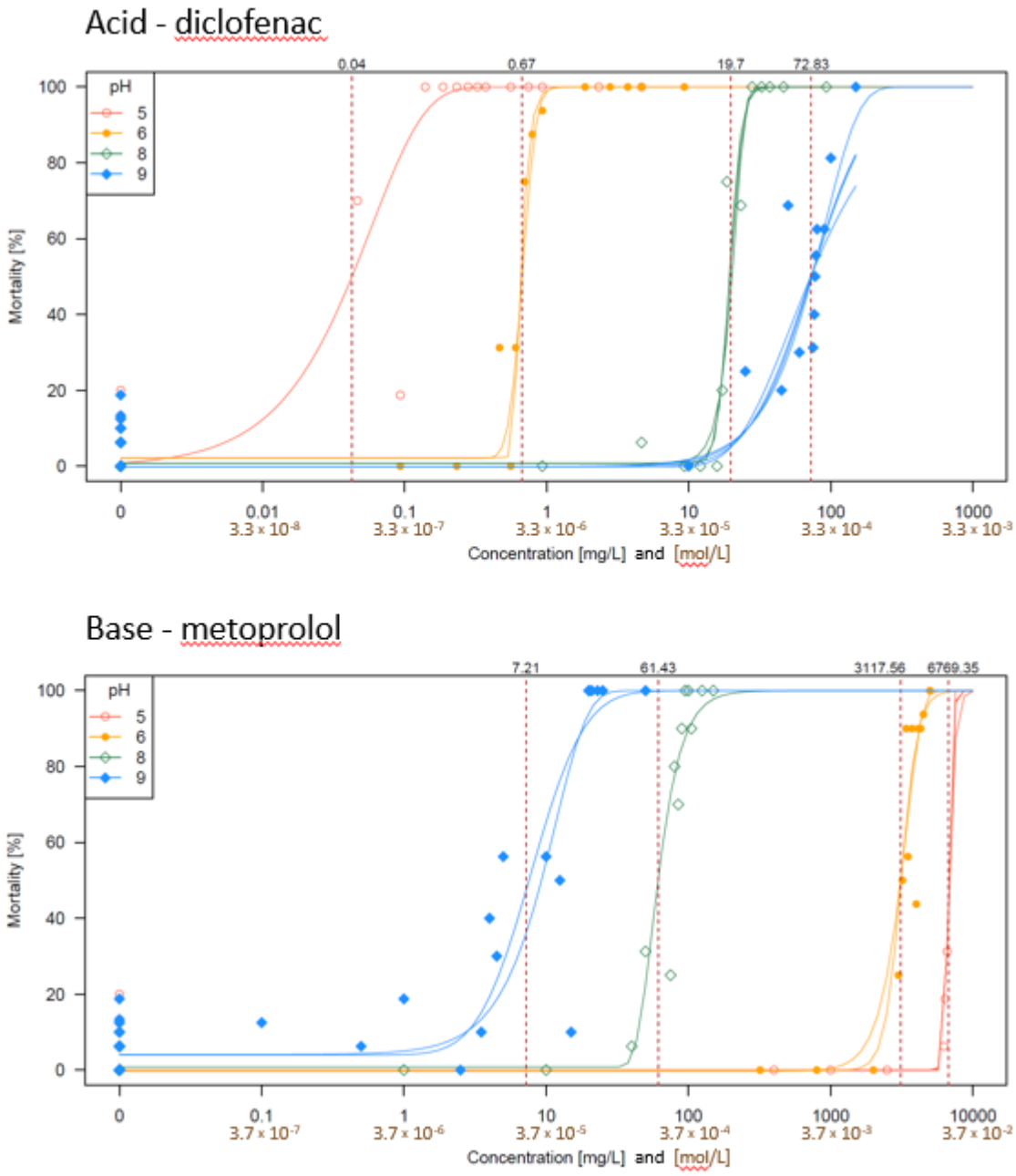
Considered across all chemicals, in the majority (93 out of 121) of cases chemical analytics revealed a nominal vs. measured concentration rate of 30% or higher, in 63 out of 121 cases even a rate of 50% or higher (Table 2). Across most of the chemicals, the measured concentration of the water samples from the start (0 h) and the end (96 h) of the exposure experiments, were at the same range if the standard deviation was taken into consideration. For this reason, all modelling was based on the nominal concentrations of the chemicals.

8.4.2 Embryotoxicity in the fish embryo test (FET)

Based on the toxicity assays carried out for each substance at different pH values at at least 13 concentrations, robust concentration-response relationships and LC_{50} values for 23 substances could be determined with high accuracy. Figure 8.3 exemplarily displays the observed mortalities for an acid, diclofenac, and a base, metoprolol, for pH conditions from 5 to 9. In accordance with the expectations, all acids, including diclofenac, exerted its embryotoxicity at gradually lower concentrations when pH gradually decreases. On the contrary, bases such as metoprolol, exhibited lower LC_{50} values with increasing pH. The concentration response curves for all substances are displayed in the Supplementary Information.

Additional LC_{50} values for 5 of these chemicals (diclofenac, naproxen, ketotifen, metoprolol, propranolol) and for a further 4 substances were determined using data published by Bittner et al. (2018), Bittner et al. (2019a) and Bittner et al. (2019b). However, data for two substances could not be used for modelling because they were uncertain as they could only be generated by extrapolation or just for a single pH only due to low solubility (enclomiphene, imidacloprid). Another chemical (citric acid) known to be actively taken up via membrane-bound transporters was tested for toxicity at three different pH values but showed little pH-dependent difference in toxicity due to this uptake pathway (data not shown). These investigations only served to show that the approach used in our study cannot be transferred to substances actively regulated by cells. Thus, data for 24 ionisable substances, 12 acids and 12 bases, were included in the modelling.

Figure 8.3: Determination of LC_{50} for two exemplarily selected ionizable chemicals, an acid (diclofenac) and a base (metoprolol) at different pH by nonlinear regression analysis

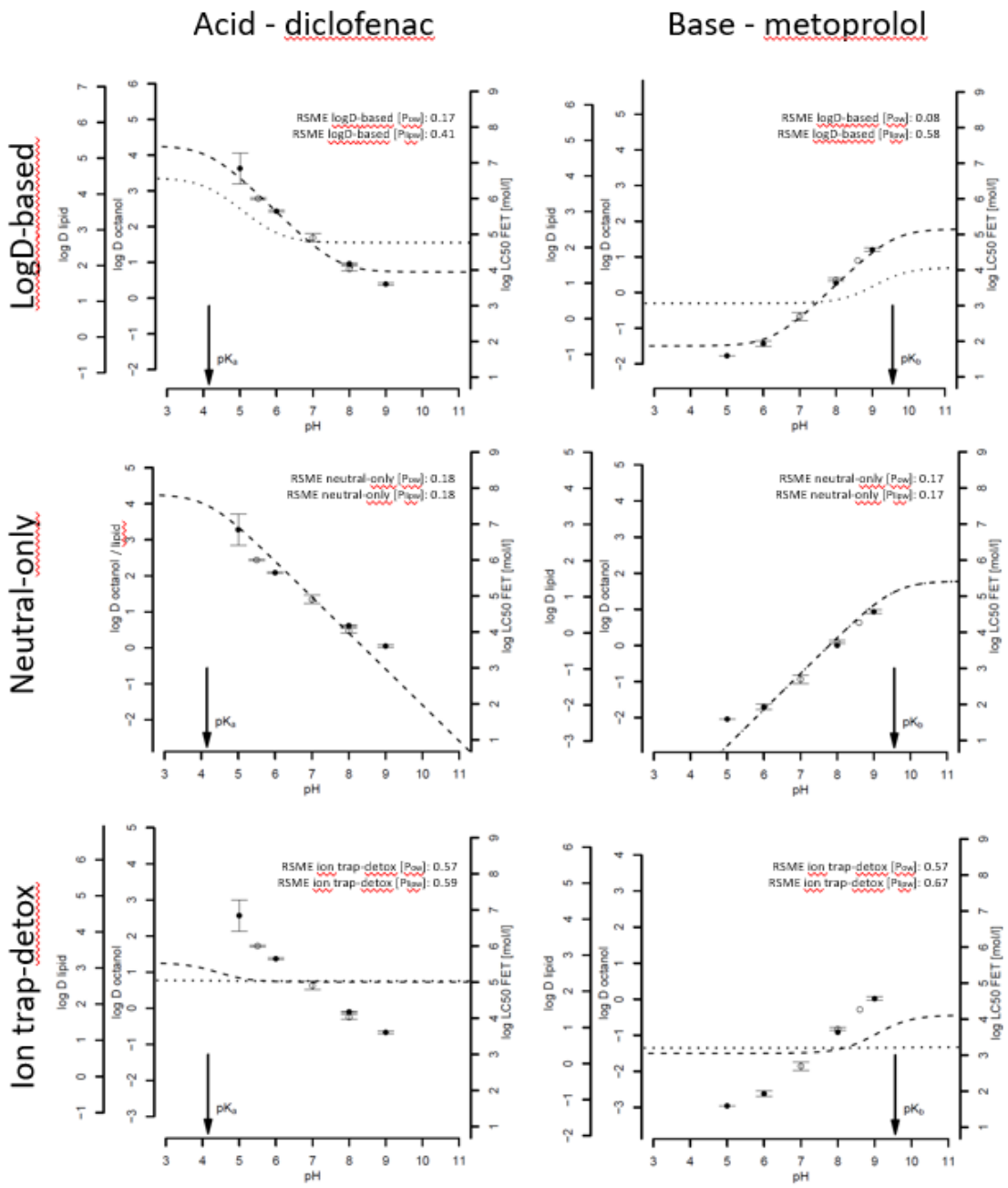


Determination of LC_{50} for two exemplarily selected ionizable chemicals, an acid (diclofenac) and a base (metoprolol) at different pH by nonlinear regression analysis. Mortality in the FET vs. nominal concentrations in mg/L (black) and mol/L (brown) in the aqueous medium. Data obtained for pH5 (red, open circles), pH6 (yellow, dots), pH8 (green, open diamonds) and pH9 (blue, filled diamonds). LC_{50} data are symbolized by red vertical dashed lines and given at the top of the graphs. Whenever two or more equally well-fitting regression lines could be used for LC_{50} determination, preference was given to the lowest LC_{50} value. Graphs and data for all substances tested in this study can be obtained from Supplementary Information.
Source: Own depiction

8.4.3 Modelling

The pH dependence of the embryotoxicity exerted by the 24 substances could be reproduced in very different quality by the six different models. Some models, which took into account different proportions of the possible uptake pathways and theoretically possible toxic effects of charged and uncharged chemical species, reproduced the real courses of these pH dependencies very well, others, however, only insufficiently. Although the potential for reliable modelling of real data was indeed substance-specific and thus some models were suitable for selected chemicals and less suitable for others, it became clear that three models performed best and mirrored the effect data almost equally well. The mean root-mean-square errors (RMSE) ± SD calculated for the respective best fits for those 14 substances with at least 4 tested pH values – for less data RMSEs are hardly meaningful – were almost identical: 0.14 ± 0.06 for the logD-based (P_{ow}) model and 0.14 ± 0.05 for both neutral-only (P_{ow} or P_{lipw}) models. The other three models reflected the real effect (LC50) data significantly less accurately than each of the former ones, and their RMSEs were (in ascending order): 0.30 ± 0.23 for the logD-based (P_{lipw}) model, 0.36 ± 0.21 for the ion trap-detox (P_{ow}) model, and 0.40 ± 0.24 for the ion trap-detox (P_{lipw}) model. These latter three models did not show any significant differences among one another. Exemplarily, the potential of the models to reflect the distribution of the real data measured in the experiments is visualised for the compounds, diclofenac and metoprolol, in Figure 8.4. The best fit of all models for all 24 substances is displayed in the Supplementary Information.

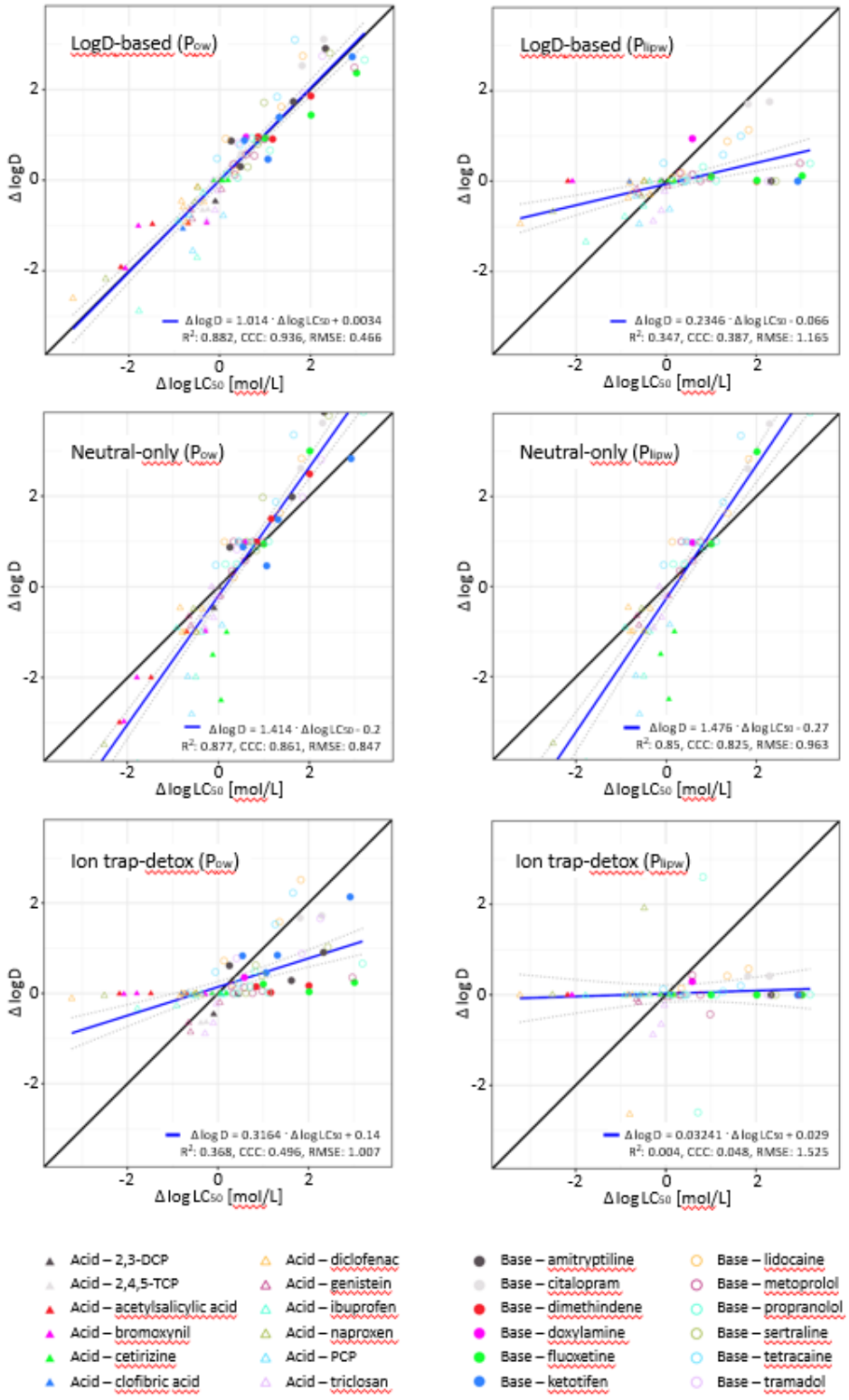
Figure 8.4: Models fitted to experimentally generated mortality data, expressed as log LC_{50}, for two exemplarily selected ionizable chemicals, an acid (diclofenac) and a base (metoprolol) at different pH in the FET



Models fitted to experimentally generated mortality data, expressed as log LC_{50}, for two exemplarily selected ionizable chemicals, an acid (diclofenac) and a base (metoprolol) at different pH in the FET. Black dots: own data, circles: data generated from data of Bittner et al. (2018) and Bittner et al. (2019b). Whiskers: standard deviation. The three rows correspond to the three types of model sets, logD-based (top), neutral-only (middle) and ion trap-detox (bottom). The vertical scaling for the modelled putatively effecting logD data at the left side of each plot was adapted to the experimentally generated data in such a way that the best possible agreement of the modelled data (curves) with the real data was achieved. Dashed lines represent the models based on log P_{ow}, dotted lines those based on log P_{lipw} (in the case of identical curves for both models, only the dashed curve is shown). The vertical arrows point to the pKa of the substances. Root-mean-square errors (RMSE) of the models are given on the top right of each plot. Graphs and data for all substances tested in this study can be obtained from Supplementary Information.
Source: Own depiction

For the three models found to be reliable in the RMSE analysis, there was also a very strong to excellent correlation of the differences in the 'effective' logD values calculated for binary combinations of pH values, the ΔlogD, with the differences in mortality at the same pH values, the ΔLC_{50}. This main finding of the study can be visualised when the LC_{50} measurements are logarithmised and plotted versus the modelled 'effective' distribution coefficient in the external medium (Figure 8.5). It can be clearly seen that, for the log D-based model on the basis of Pow , the now linearised dependence of log LC_{50} and 'effective' log D runs almost identical to the bisector through the origin in a nice uniformity for all substances investigated here, and that all other models lag behind it with regard to the uniformity of the predictions for different substances as well as with regard to the deviations from the bisector. By this correlation, it is thus possible, with the help of the logD-based (Pow) model, to conclude very reliably from an LC_{50} determined for an ionisable chemical at a given pH to the acute toxicity of the same chemical at a different pH. With limitations, this is also possible using one of the two neutral-only models, but with less accuracy. The three remaining models however failed to prove any ability to infer from the toxicity exerted by a chemical at a given pH the baseline toxic effect at another pH.

Figure 8.5: Correlation of ΔlogD with Δlog LC_{50}



Correlation of ΔlogD, the difference in the ability of ionisable chemicals to be passively taken up into cells and putatively cause baseline toxicity at two different pH values predicted by the respective models, with Δlog LC_{50}, the difference in mortality exerted in the FET at these same two pH values. Data for full-factorial binary comparisons among the data collected or modelled for the different pH values for the 24 chemicals listed at the bottom.
Source: Own depiction

The three rows correspond to the three types of model sets, logD-based (top), neutral-only (middle) and ion trap-detox (bottom). Left: $logP_{ow}$-based models, right: $logP_{lipw}$-based models. The black angle bisector in each plot describes the sum of all points for which $\Delta logLC_{50}$ is identical to the modelled value for ΔlogD. Equations for the linear regression curves (blue lines, displayed together with 95% confidence intervals), the coefficients of determination (R^2), the concordance correlation coefficients (CCC) and the root-mean-square errors (RMSE) of the regression curves are given on the bottom right of each plot.

8.5 Discussion

Following our findings and the available literature on the topic, which was critically reviewed in two excellent reviews, an earlier (Rendal et al. 2011b) and a recent one (Escher et al. 2020), the following is a list of necessary discussion points.

- Early studies have shown that the toxicity of an ionizable substance (IOC) increases with increasing fraction of the neural species of this substance (e.g. Rendal et al. 2011b). This finding has led to the suggestion, that the neutral species per se exerts higher toxicity in the cells of an organism. However, it is quite plausible that the increased harmful effect in this case is not caused by the intracellular action of charged and uncharged species but by their differently efficient uptake per time unit into the cell.
- -An exclusive fixation on the fractions, i.e. the relative proportions of neutral and ionic species in the assessment of toxicity is certainly not sufficient, since, according to our results, the respective lipophilicities and the ratio of the two species, symbolised by logD are of decisive importance and the lipophilicities (in absolute numbers) as well as the differences in the lipophilicity of the two species are substance-specific.

8.5.1 Contribution of neutral and charged species to toxicity

Based on our assumption that the model whose 'effective' logD correlates most strongly with experimental data on embryotoxicity takes into account those parameters and processes that are mechanistically responsible for baseline toxicity, the following conclusions emerge from our results on the mean RMSE, when comparing effective logD and LC_{50}, and the correlation between ΔlogD und $\Delta logLC_{50}$: Since the logD-based (P_{ow}) model has proven to be exceptionally suitable for mapping the lethal effects with regard to both analyses (RMSE and ΔlogD vs. $\Delta logLC_{50}$), it must be concluded that both the neutral and the charged species of an ionisable chemical are taken up intracellularly and that both species also contribute to baseline toxicity. Since the neutral-only models, which were equivalent in terms of RMSE and only slightly worse than logD-based (P_{ow}) in terms of ΔlogD vs. $\Delta logLC_{50}$ correlation, ignore uptake and action of the charged species, it has to be assumed that the toxic effect of an ionisable chemical is not exclusively but largely due to the uptake and intracellular action of the neutral species. However, our findings can rule out the possibility that intracellular equilibration between neutral and charged species (the ion trapping mechanism) leads to detoxification, which was the assumption of the rather poorly performing ion trap-detox models – a finding which thus indirectly proves that also the ionic species of ionisable chemicals contribute to the toxicity exerted by ionisable substances.

In our study, all P_{ow}-based models proved to be (slightly) better at describing toxicity than their respective P_{lipw}-based counterparts. As P_{lipw} has been considered more suitable for estimating the distribution of substances between cell membranes and aqueous components such as the outer medium or cytoplasm, especially in recent years (Escher et al. 2020 XX?), our results suggest that this distribution in different cellular compartments cannot be used to infer the resulting toxic effect from chemical distribution patterns in each case without reflection - even in the case of non-specific chemicals whose effects can largely be captured via baseline toxicity.

- In contrast to previous findings, which showed only limited partitioning of ionic species into storage lipids, but strong association with the phospholipids of biological membranes (Escher & Sigg, 2004) and the conclusion that octanol is not suitable as a surrogate for biomembranes for ionisable chemicals (Escher et al. 2020), our results show that toxicity can be modelled more reliably on the basis of P_{ow} than for P_{lipw}. For this reason, it can probably be assumed that the chemical property of biomembranes is predominantly responsible for the estimation of the toxicity exerted by uncharged species and that the toxicity caused by the charged species is unfolded via binding to water-soluble components (which are well represented via P_{ow}) in the cytoplasm, e.g. proteins (Henneberger et al. 2016a, 2016b). The fact that an effect of charged species on membranes is limited according to this interpretation can very well be mechanistically explained by the fact that these species interact with the membrane surface, but (in contrast to the neutral species) intercalate poorly into the lipid part of the bilayer what is considered to trigger narcosis (= baseline toxicity) (McCarty & Mackay 1993).

- Taking into account the assumption, based on our findings, that neutral and charged species differ in their intracellular targets, the prerequisites for a concentration addition mixture toxicity modelling approach to simulate IOC effects (e.g. Altenburger et al. 2000), which assumes the same mode of action of both species, must be critically questioned. The disadvantages of this approach in terms of the lack of mechanistic knowledge and the need for a wide range span of the species fraction, which often cannot be achieved in the ranges of experimentally accessible pH values, have already been recognised by Baumer et al. (2017) and Escher et al. (2020).

- According to Escher et al (2020), we focused on the generation of high quality data at constant pH, which we achieved by using buffered test media. Since, as also criticised by Escher (2020), the simultaneous determination of LC_{50} and BCF did not take place for many substances, it is also not possible for us to calculate internal LC_{50} (ILC50) values for the test substances of the present study and thus to verify the suitability of a full ion-trapping model as proposed by Escher & Hermens (2004) for acids and by Neuwoehner & Escher (2011) for bases. However, since a solely log D-based model, which takes into account the toxic effects of both neutral and charged species, showed surprisingly good agreement with the experimentally collected data even for external pH values of 5 and 9, the ion trap mechanism does not seem to play a decisive role, at least as far as the resulting toxicities of exposure to ionisable substances are concerned.

8.5.2 Implementation in the regulation of chemicals (on the basis of the EQS datasheet for ibuprofen)

Our findings may well contribute to improved toxicity assessment in the process of registration of ionisable chemicals and the definition of Environmental Quality Standards (EQS). The European Food Safety Authority highlights the importance of the pH in toxicity testing in their Aquatic Guidance Document (EFSA 2013): *"Consideration should be given to appropriate pHs (to be) used in the test as solubility may be lower but toxicity may be higher in the unionised form than in the ionised form. Therefore, testing of bioconcentration and toxicity of ionisable substances should preferably be conducted at a pH consistent with the more toxic form of the substance whilst remaining within the range required to maintain the health of the control organisms. (…) A stable pH is important to ensure that the balance between dissociated and non-dissociated forms of the substance is maintained."*

In our study, we were able to show that the toxicity of an ionisable chemical to *D. rerio* embryos (OECD 236) can differ between environmentally relevant pH values by several orders of

magnitude. Other authors noted similar findings (Bittner et al. 2019a, 2019b). To account for such high variation in toxicity, it is critical to consider the presumably most bioaccumulative fraction of a given test chemical in the respective toxicity test guideline.

It is noteworthy, that this is actually also required by the OECD GD 23 on difficult substances, which states that *"The definitive test should be conducted at a pH consistent with the most toxic form (usually the non-dissociated form…) … of the test chemical, whilst remaining within the range required to maintain the health of the control organisms. This may require testing at the pH extremes of the allowable range for test organisms."* (OECD 2019).

The regulatory consequences of all the considerations above are twofold. Firstly, for existing studies and hence endpoints investigated at a pH different from such a 'realistic worst-case pH', the ecotoxicological value should be 'mathematically corrected' to account for those worst-case conditions which would be present in natural conditions, as demanded by OECD GD 23. Secondly, any new study planned or submitted to authorities should consider the "realistic worst-case pH" for the test system used.

We propose that the basis for the mathematical correction should be the delta between the logD value (based on Kow, eq. 1) at the given pH of the test under consideration and the logD value at the realistic worst-case pH of the respective test guideline, both calculated as follows:

$$logD_X = log10(f_{neutral} \times 10^{log\ X\ \ neutral} + (f_{ionic}) \times 10^{log\ X\ \ ionic}) \quad (10)$$

with X referring to the respective K_{ow} constants and where the neutral fraction is calculated as:

$$f_{neutral} = \frac{1}{(1+10^{(pH-pK_s)})} \quad (11)$$

and the ionic fraction as:

$$f_{ionic} = 1 - f_{neutral} \quad (12)$$

The difference in the ratio of uncharged to charged species at the pH used in the toxicity study ('pH st') and at the pH of the worst-case scenario ('pH wc') is simply calculated as the delta between the logD values at the two pHs:

$$\Delta\ log\ D\ (wc - st)\ =\ log\ D\ _{(pH\ wc)}\ -\ log\ D_{(pH\ st)} \quad (13)$$

with pH wc corresponding to the (environmentally realistic) pH with the highest fraction of the neutral species and pH st corresponding to the pH at which the toxicity test has been conducted.

The mathematical correction factor (CF) for toxicity, estimated for the worst-case scenario then can be calculated as

$$CF\ =\ 10^{\Delta\ log\ D\ (wc-st)} \quad (14)$$

Because there is strong evidence for a correlation between log D and baseline toxicity for numerous ionisable chemicals (present study, *Schweizer et al*. 2022), the correction factor (eq. 14) should then be applied to each tested ecotoxicological endpoint in order to derive a realistic worst-case effect of the compound under assessment.

9 Publications

Aleiferi, E.; Panagopoulou, E.I.; Damalas, D.E.; Aalizadeh, R.; Schweizer, M.; Kuttler, J.; Köhler, H.R.; von der Ohe, P.C.; Triebskorn, R.; Thomaidis, N.S. (2020): Assessment of the pH effect on toxicity & biotransformation of the anti-inflammatory drug Diclofenac using the zebrafish (*Danio rerio*) toxicity test. SETAC Europe 30th Annual Meeting (SETAC SciCon 2020), 3-7.05.2020 (poster presentation).

Damalas, D.E.; Aalizadeh, R.; Schweizer, M.; Heid, C.; Köhler, H.R.; von der Ohe, P.C.; Triebskorn, R.; Thomaidis, N.S. (2019): Evaluation of the acute toxicity, uptake and biotransformation potential of citalopram in zebrafish (*Danio rerio*) embryos at three environmentally relevant pHs. SETAC Europe 29th Annual Meeting (SETAC 2019), 26-30.05.2019 (poster presentation).

Damalas, D.E; Panagopoulou, E.I.; Aleiferi, E.; Aalizadeh, R.; Schweizer, M.; Schlösinger, A.; Köhler, H.R.; von der Ohe, P.C.; Triebskorn, R.; Thomaidis, N.S. (2021): Zebrafish toxicity, uptake and biotransformation assessment of tetracaine exposure under different pH values, utilizing embryotoxicity and LC-HRMS. SETAC Europe 31st Annual Meeting (SETAC Europe 2021 Virtual conference), 3-6.05.2021 (poster presentation).

Damalas, D.; Panagopoulou, E.I.; Aleiferi, E.; Tzepkinli, V.; Aalizadeh, R.; Schweizer, M.; Kundy, L.; Köhler, .H.R.; von der Ohe, P.C.; Triebskorn, R.; Thomaidis, N. (2021): Toxicity, uptake, and biotransformation assessment of zebrafish embryos exposed to tetracaine, under different pH values, utilizing embryotoxicity and LC-HRMS. 17th International Conference on Environmental Science and Technology, (CEST-2021), 1-4.09.2021, Athens, Greece (poster presentation).

Köhler H-R, Gräff T, Schweizer M, Blumhardt J, Burkhardt J, Ehmann L, Hebel J, Heid C, Kundy L, Kuttler J, Malusova M, Moroff F-M, Schlösinger A-F, Schulze-Berge P, Panagopoulou EI, Damalas DE, Thomaidis NS, Triebskorn R, Maletzki D, Kühnen U, von der Ohe PC (2023): LogD-based modelling and ΔlogD as a proxy for pH-dependent action of ionizable chemicals reveal the relevance of both neutral and ionic species for fish embryotoxicity and possess great potential for practical application in the regulation of chemicals. Water Research 235, 119864.

Panagopoulou, E.I.; Damalas, D.E.; Aleiferi, E.; Aalizadeh, R.; Schweizer, M.; Kundy, L.; Köhler, H.R.; von der Ohe, P.C.; Triebskorn, R.; Thomaidis, N.S. (2020): Investigation of the influence of pH on the toxicity, uptake and biotransformation of Ibuprofen in zebrafish (*Danio rerio*) embryos. SETAC Europe 30th Annual Meeting (SETAC SciCon 2020), 3-7.05.2020 (poster presentation).

Panagopoulou, E.I.; Tzepkinli, V.; Damalas, D.; Aalizadeh, R.; Schweizer, M.; Kundy, L.; Köhler, H.R.; von der Ohe, P.C.; Triebskorn, R.; Thomaidis, N.(2021): Assessment of the pH-effect on the toxicity, uptake & biotransformation of Fluoxetine in Zebrafish (*Danio rerio*) embryos by LC-ESI-QTOFMS. 17th International Conference on Environmental Science and Technology, (CEST-2021), 1-4.09.2021, Athens, Greece (poster presentation-flash presentation).

Panagopoulou, E.I.; Tzepkinli, V.; Damalas, D.; Aalizadeh, R.; Schweizer, M.; Kundy, L.; Köhler, H.R.; von der Ohe,.P.C.; Triebskorn, R.; Thomaidis, N. (2022): Investigation of the pH-effect on the toxicity, uptake & biotransformation of the Metoprolol in zebrafish (*Danio rerio*) embryos, utilizing LC-ESI-QTOFMS. Society of Environmental Toxicology and Chemistry, SETAC Europe 32th Annual Meeting (SETAC Europe 2022), 15-19.03.2022,Copenhagen, Denmark (poster presentation).

Schweizer M, von der Ohe PC, Gräff T, Kühnen U, Hebel J, Heid C, Kundy L, Kuttler J, Moroff F-M, Schlösinger A-F, Schulze-Berge P, Triebskorn R, Panagopoulou E, Damalas DE, Thomaidis NS, Köhler H-R (2022): Heart rate as an early warning parameter and proxy for subsequent mortality in Danio rerio embryos exposed to ionisable substances. Science of the Total Environment 818: 151744.

Tzepkinli, V.; Panagopoulou, E.I.; Damalas, D.; Aalizadeh, R.; Schweizer, M.; Kundy, L.; Köhler, H.R.; von der Ohe, P.C.; Triebskorn, R.; Thomaidis, N. (2021): Investigation of the influence of environmentally relevant pH values in the toxicity of the b-blocker Metoprolol in zebrafish (*Danio rerio*) embryos through toxicokinetic

approaches (uptake, bioaccumulation, and biotransformation). 17th International Conference on Environmental Science and Technology, (CEST-2021), 1-4.09.2021, Athens, Greece (oral presentation).

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10.1 University of Tübingen

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Jasmin Blumhardt	(master)	bromoxynil, 2,4,5-TCP
Jasmin Burkhardt	(bachelor)	ketotifen, additional pH 9 treatments
Lisa Ehmann	(bachelor)	sertraline
Janine Hebel	(bachelor)	naproxen, metoprolol
Christoph Heid	(state examination)	citalopram, propranolol
Lone Kundy	(master)	ibuprofen, enclomiphene
Julia Kuttler	(state examination)	diclofenac, triclosan
Miroslava Malusova	(master)	ASA, 2,3-DCP
Friederike-Marie Moroff	(bachelor)	lidocaine, tramadol
Anne-Frida Schlösinger	(bachelor)	tetracaine
Pia Schulze-Berge	(bachelor)	clofibric acid, fluoxetine

10.2 University of Athens

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Eleni Aleiferi	(master)	Sertraline, Amitriptyline, Tetracaine, Ibuprofen, Enclomiphene, Triclosan (pH 9), Propranolol & Ibuprofen (Water samples: *Daphnia magna* and *Lemna minor*)
Christina Gatou	(bachelor)	Propranolol (Water samples: *Lemna minor*)
Triantafyllos Souliotis	(bachelor)	Diclofenac (Water samples: *Daphnia magna*)
Vasiliki Tzepkinli	(master)	2,3-Dichlorophenol, 2,4,5-Trichlorophenol, Bromoxynil, Acetylsalicylic acid, Citalopram, Diclofenac,

		Fluoxetine, Ketotifen, Lidocaine, Metoprolol, Naproxen, Pentachlorophenol, Propranolol, Tramadol, Triclosan, Tetracaine (pH 9), Amitriptyline (pH5), Ibuprofen, Citalopram & Diclofenac & Amitriptyline (Water samples: *Daphnia magna* and *Lemna minor*) Propranolol & Ibuprofen (Water samples and organisms: *Daphnia magna* and *Lemna minor*)
Athanasia Drempela	(master)	2,3-Dichlorophenol, 2,4,5-Trichlorophenol, Bromoxynil, Acetylsalicylic acid, Citalopram, Diclofenac, Propranolol

10.3 German Environment Agency

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11 List of references

Aalizadeh, R.; von der Ohe, P. C.; Thomaidis, N. S. (2017): Prediction of acute toxicity of emerging contaminants on the water flea *Daphnia magna* by Ant Colony Optimization-Support Vector Machine QSTR models. Environ Sci Process Impacts 19(3): 438-448.

Aceña, J., Pérez, S., Eichhorn, P., Solé, M., Barceló, D. (2017): Metabolite profiling of carbamazepine and ibuprofen in *Solea senegalensis* bile using high-resolution mass spectrometry, Analytical and Bioanalytical Chemistry, vol. 409: 5441–5450.

Baumer, A.; Bittermann, K.; Klüver, N.; Escher, B.I. (2017): Baseline toxicity and ion-trapping models to describe the pH-dependence of bacterial toxicity of pharmaceuticals. Environmental Science: Processes & Impacts 19(7): 901-916.

Bittermann, K.; Spycher, S.; Endo, S.; Pohler, L.; Huniar, U.; Goss, K.-U.; Klamt, A. (2014): Prediction of phospholipid–water partition coefficients of ionic organic chemicals using the mechanistic model COSMOmic. The Journal of Physical Chemistry B 118(51): 14833-14842.

Bittner, L.; Teixidó, E.; Keddi, I.; Escher, B. I.; Klüver, N. (2019a): pH-dependent uptake and sublethal effects of antihistamines in zebrafish (*Danio rerio*) embryos. Environ. Toxicol. Chem. 38, 1012−1022.

Bittner, L.; Klüver, N.; Henneberger, L., Mühlenbrink, M.; Zarfl, C.; Escher, B.I. (2019b): Combined ion-trapping and mass balance models to describe the pH-dependent uptake and toxicity of acidic and basic pharmaceuticals in zebrafish embryos (*Danio rerio*). Environ. Sci. Technol. 53, 7877−7886.

Bittner, L.; Teixido, E.; Seiwert, B.; Escher, B.I.; Kluver, N. (2018): Influence of pH on the uptake and toxicity of beta-blockers in embryos of zebrafish, *Danio rerio*. Aquat Toxicol 201: 129-137.

Brox, S., Ritter, A.P., Küster, E., Reemtsma, T. (2014): A quantitative HPLC-MS/MS method for studying internal concentrations and toxicokinetics of 34 polar analytes in zebrafish (Danio rerio) embryos, Analytical and Bioanalytical Chemistry, vol. 406: 4831–4840.

Clark, W.M.; Lubs, H.A. (1916): Hydrogen electrode potentials of phthalate, phosphate, and borate buffer mixtures. Journal of Biological Chemistry 25(3): 479-510.

Damalas, D.E., Bletsou, A.A., Agalou, A., Beis, D., Thomaidis, N.S. (2018): Assessment of the Acute Toxicity, Uptake and Biotransformation Potential of Benzotriazoles in Zebrafish (*Danio rerio*) Larvae Combining HILIC- with RPLC-HRMS for High-Throughput Identification, Environmental Science and Technology 52: 6023–6031.

Dasenaki, M.E., Thomaidis, N.S. (2015): Multianalyte method for the determination of pharmaceuticals in wastewater samples using solid-phase extraction and liquid chromatography–tandem mass spectrometry Analytical and Bioanalytical Chemistry 407 (15): 4229-4245

ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals) (2013): Environmental exposure assessment of ionisable organic compounds. Technical Report No. 123, ECETOC AISBL, Brussels, Belgium. 193pp.

Escher, B.I.; Abagyan, R.; Embry, M.; Klüver, N.; Redman, A.D.; Zarfl, C.; Parkerton, T.F. (2020): Recommendations for improving methods and models for aquatic hazard assessment of ionizable organic chemicals. Environ. Toxicol. Chem. 39 (2), 269-286.

Escher, B.I; Hermens, J.L.M. (2004): Internal exposure: Linking bioavailability to effects. Environ. Sci. Technol. 38:455A–462A.

Escher, B.I.; Schwarzenbach, R.P. (1996): Partitioning of substituted phenols in liposome–water, biomembrane–water, and octanol–water systems. Environ. Sci. Technol. 30:260–270.

EU (2010): Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union.

Goss, K. U.; Bittermann, K.; Henneberger, L.; Linden, L. (2018): Equilibrium biopartitioning of organic anions - A case study for humans and fish. Chemosphere 199, 174−181.

Horng, J.-L.; Lin, L.-Y.; Hwang, P.-P. (2009): Functional regulation of H+-ATPase-rich cells in zebrafish embryos acclimated to an acidic environment. American Journal of Physiology-Cell Physiology 296(4): C682-C692. DOI: 10.1152/ajpcell.00576.2008.

Huggett, D. B.; Cook, J. C.; Ericson, J. F.; Williams, R. T. (2003): A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. Human and Ecological Risk Assessment: An International Journal 9(7): 1789-1799.

Lawrence, C. (2007): The husbandry of zebrafish (*Danio rerio*): A review. Aquaculture 269(1): 1-20.

Madshus, I.H. (1988): Regulation of intracellular pH in eukaryotic cells. The Biochemical Journal 250 (1): 1–8. doi:10.1042/bj2500001.

McCarty, L.S.; Mackay, D. (1993): Enhan,cing ecotoxicological modeling and assessment. Environ. Sci. Technol. 27: 1719-1728.

McClure, M.M.; McIntyre, P.B.; McCune, A.R. (2006): Notes on the natural diet and habitat of eight danionin fishes, including the zebrafish *Danio rerio*. Journal of Fish Biology 69(2): 553-570. DOI: 10.1111/j.1095-8649.2006.01125.x.

Neuwoehner, J.; Escher, B.I. (2011): The pH-dependent toxicity of basic pharmaceuticals in the green algae Scenedesmus vacuolatus can be explained with a toxicokinetic ion-trapping model. Aquatic Toxicology 101(1): 266-275.

OECD (2004): Test No. 202: *Daphnia* sp. Acute Immobilisation Test - OECD Guidelines for the Testing of Chemicals, Section 2. Paris, OECD Publishing.

OECD (2006): Test No. 221: Lemna sp. Growth Inhibition Test - OECD Guidelines for the Testing of Chemicals, Section 2. Paris, OECD Publishing.

OECD (2012): Test No. 211: *Daphnia magna* Reproduction Test - OECD Guidelines for the Testing of Chemicals, Section 2. Paris, OECD Publishing.

OECD (2013): Test No. 236: Fish Embryo Acute Toxicity (FET) Test - OECD Guidelines for the Testing of Chemicals, Section 2. Paris, OECD Publishing.

Rendal, C.; Kusk, K.O.; Trapp, S. (2011): The effect of pH on the uptake and toxicity of the bivalent weak base chloroquine tested on Salix viminalis and *Daphnia magna*. Environmental Toxicology and Chemistry 30(2): 354-359.

Rendal, C.; Kusk, K. O.; Trapp, S. (2011a): The effect of pH on the uptake and toxicity of the bivalent weak base chloroquine tested on Salix viminalis and *Daphnia magna*. Environ. Toxicol. Chem. 30, 354−359.

Rendal, C.; Kusk, K. O.; Trapp, S. (2011b): Optimal choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. Environ. Toxicol. Chem. 30, 2395-2406.

Rendal, C.; Trapp, S.; Kusk, K. O. (2012): Critical evaluation and further development of methods for testing ecotoxicity at multiple pH using *Daphnia magna* and Pseudokirchneriella subcapitata. Environ. Toxicol. Chem. 31, 1843−1852.

Russell, W.M.S.; Burch, R.L. (1959): The principles of humane experimental technique.

Schreiber, R.; Gündel, U.; Franz, S.; Küster, A.; Rechenberg, B.; Altenburger, R. (2011): Using the fish plasma model for comparative hazard identification for pharmaceuticals in the environment by extrapolation from human therapeutic data. Regulatory Toxicology and Pharmacology 61(3): 261-275.

Spence, R.; Fatema, M.K.; Reichard, M.; Huq, K.A.; Wahab, M.A.; Ahmed, Z.F.; Smith, C. (2006): The distribution and habitat preferences of the zebrafish in Bangladesh. Journal of Fish Biology 69(5): 1435-1448. DOI: 10.1111/j.1095-8649.2006.01206.x.

Schweizer, M.; Brilisauer, K.; Triebskorn, R.; Forchhammer, K.; Köhler, H.R. (2019): How glyphosate and its associated acidity affect early development in zebrafish (*Danio rerio*). PeerJ 7: e7094.

Schweizer, M.; von der Ohe, P.C.; Gräff, T.; Kühnen, U.; Hebel, J.; Heid, C.; Kundy, L.; Kuttler, J.; Moroff, F.-M.; Schlösinger, A.-F.; Schulze-Berge, P.; Triebskorn, R.; Panagopoulou, E.; Damalas, D.E.; Thomaidis, N.S.; Köhler, H.R. (2022): Heart rate as an early warning parameter and proxy for subsequent mortality in *Danio rerio* embryos exposed to ionisable substances. Science of the Total Environment 818: 151744.

A Appendix: Supplementary information

Table A2: Overview of log D values for all substances (in alphabetical order) used in the toxicity tests at the corresponding pH levels

		Log D			
		pH 5	pH 6	pH 8	pH 9
2,3-DCP	acid	2.88	2.86	2.16	
2,4,5-TCP	acid	3.48	3.42	2.33	
Acetylsalicylic acid	acid	-0.35	-1.30	-2.25	-2.29
Amitriptyline	base	1.33	1.50	3.05	3.98
Bromoxynil	acid	2.82	2.15	1.05	
Citalopram	base	0.28	0.44	1.98	2.91
Citric acid	acid	-3.86	-6.20	-10.54	-11.91
Clofibric acid	acid	1.27	0.32	-0.60	-0.63
Diclofenac	acid	3.21	2.26	0.85	0.74
Enclomiphene	base	2.97	2.97	5.15	5.99
Fluoxetine	base	0.94	1.04	2.38	3.31
Glyphosate	acid	-5.13	-6.39	-7.88	-8.53
Ibuprofen	acid	3.46	2.67	0.85	0.41
Imidacloprid	base				
Ketotifen	base	1.22	2.17	3.29	3.32
Lidocaine	base	0.16	1.09	2.65	2.82
Metoprolol	base	-1.47	-1.34	0.09	1.01
Naproxen	acid	2.11	1.18	-0.36	-0.52
PCP	acid	4.38	3.67	2.68	
Propranolol	base	-0.64	-0.52	0.92	1.83
Sertraline	base	1.93	2.08	3.58	4.48
Tetracaine	base	-0.72	0.41	2.23	2.69
Tramadol	base		-0.59	1.20	2.02
Triclosan	acid	4.98	4.97	4.50	3.70

Source: Own depiction

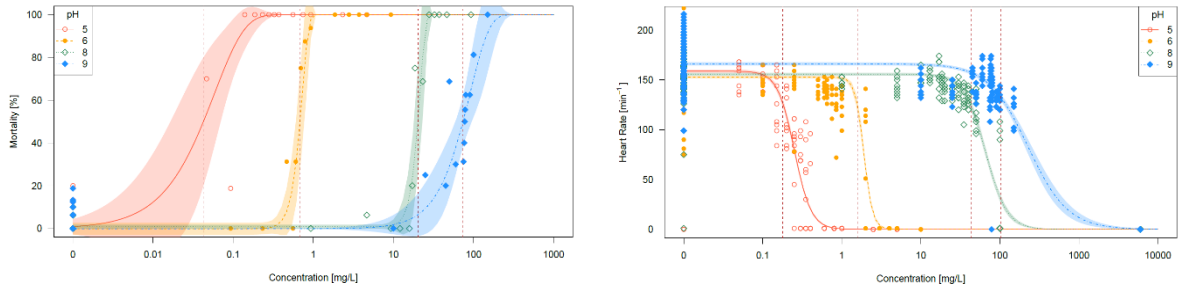
Table A2: Overview of the LC_{50} values determined for all substances tested in the FET

Substance	pH 5		pH 6		pH 8		pH 9	
	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf
2,3-DCP	10.00	6.70	8.97	6.50	17.55	8.36	-	-
2,4,5-TCP	0.60	0.60	0.86	0.83	1.48	1.43	-	-
Acetylsalicylic acid	30.86	19.63	248.42	97.20	3865.00*	2951.60	-	-
Amitriptyline	303.0	245.03	95.18	89.16	3.11	2.10	1.64	1.60
Bromoxynil	0.188	0.161	0.461	0.359	26.99	20.27	-	-
Citalopram	303.08	245.03	426.22	400.00	35.04	19.99	10.66	2.34
Citric acid	-	-	4926.00	3250.00	4559.00	3102.00	-	-
Clofibric acid	67.78	66.83	-	-	-	-	-	469.01*
Diclofenac	0.22	0.07	1.18	0.64	57.40	19.61	172.37*	73.00
Enclomiphene	-	-	-	-	5.58	3.13	-	-
Fluoxetine	490.91*	229.90	99.38	58.33	16.08	2.21	1.93	0.33
Glyphosate	-	239.59	-	-	-	-	-	-
Ibuprofen	13.85	8.71	60.28	45.20	249.39	187.12	-	352.23
Imidacloprid	-	-	-	-	-	-	-	-
Ketotifen	-	-	-	-	-	-	4.65	1.92
Lidocaine	-	3435.20*	-	-	69.32	67.47	41.72	26.99
Metoprolol	-	6739.00*	7008.30*	3183.40	-	77.34	-	-
Naproxen	-	-	9.36	8.27	358.46	327.25	1594.50	1261.60
PCP	0.07	0.07	0.06	0.06	0.27	0.26	-	-
Propranolol	1619.50	1599.40	514.80	436.54	14.67	9.06	4.13	0.67
Sertraline	-	-	14.08	10.55	1.20	1.19	0.59	0.10
Tetracaine	788.50*	170.13	179.56	59.24	6.11	3.94	-	-
Tramadol	-	-	4992.90	3110.50	90.47	46.28	25.00	18.45
Triclosan	0.43	0.30	0.43	0.39	0.47	0.41	0.67	0.66

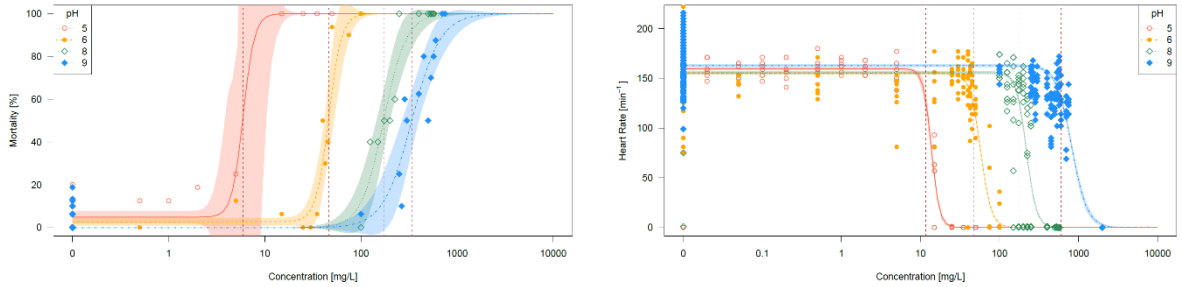
Overview of the LC_{50} values determined for all substances tested (in alphabetical order) in the FET rounded to two decimal places in mg/L at the corresponding pH levels at the time points 72 and 96 hpf; asterisks mark values that were extrapolated since the mortality did not reach 50 %; colour code: red – acids; blue - bases.
Source: Own depiction

Figure A6: Mortality and heart hate in exposed zebrafish embryos

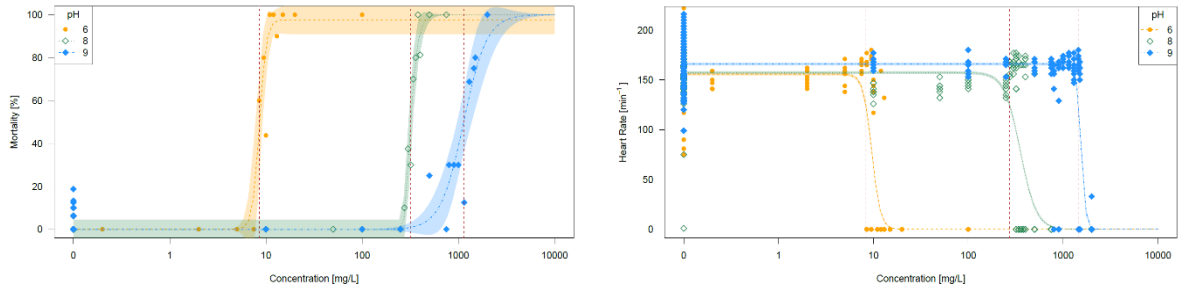
a. Diclofenac



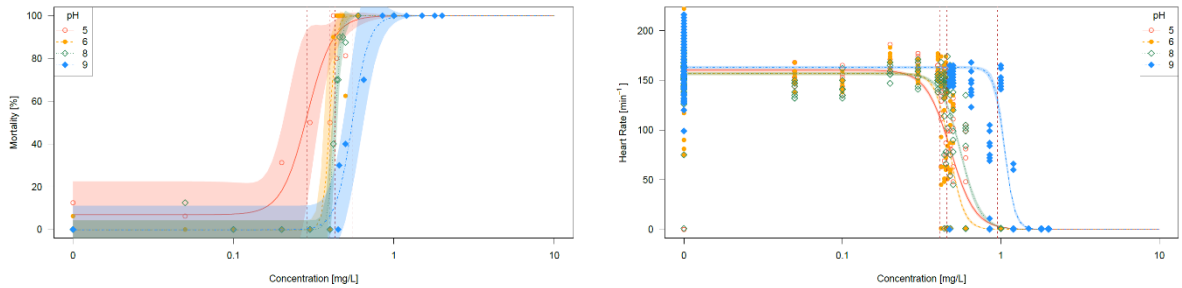
b. Ibuprofen



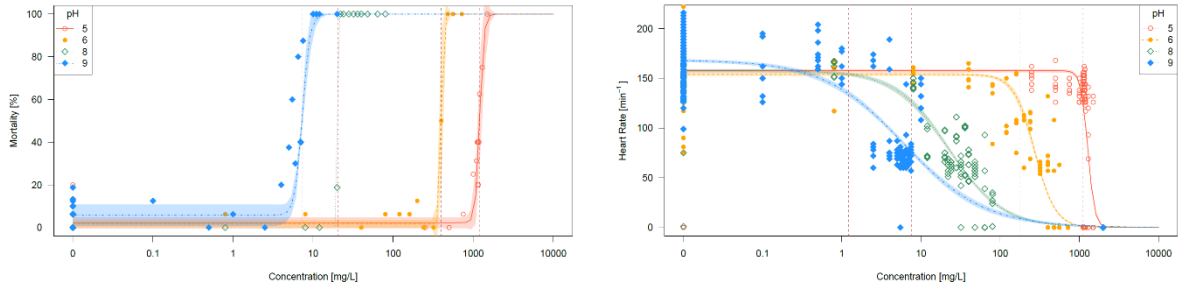
c. Naproxen



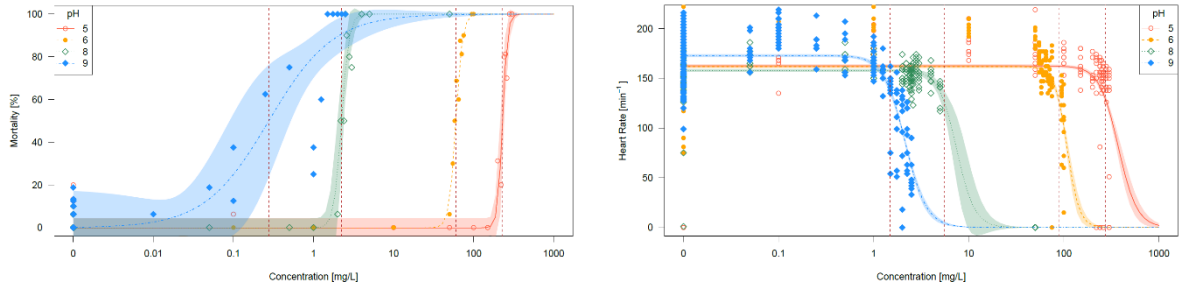
d. Triclosan



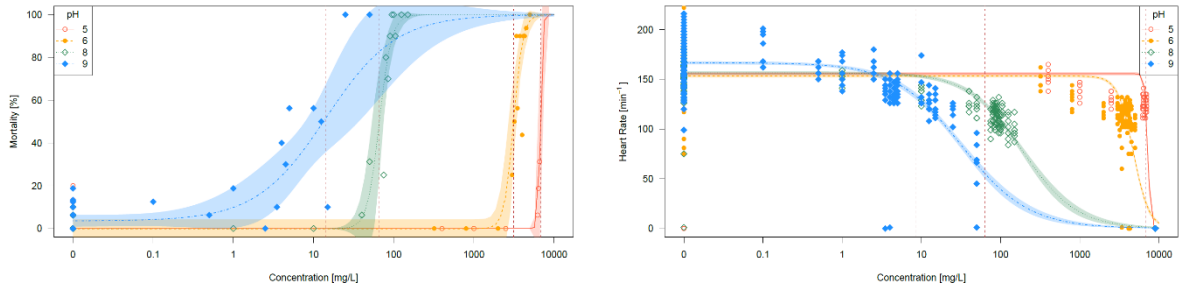
e. Citalopram



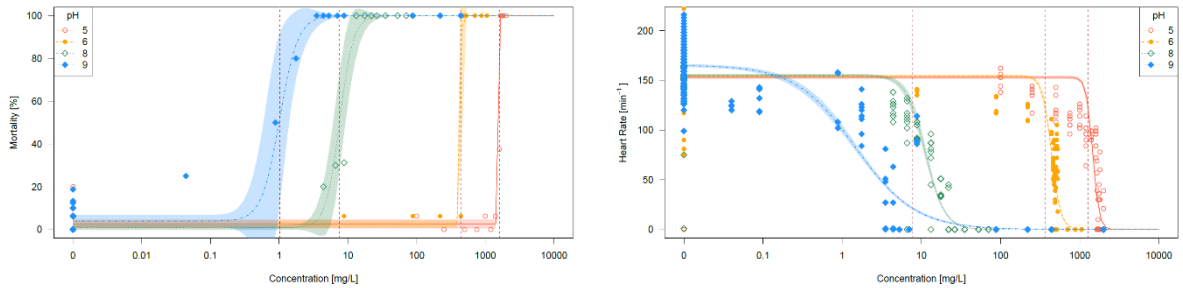
f. Fluoxetine



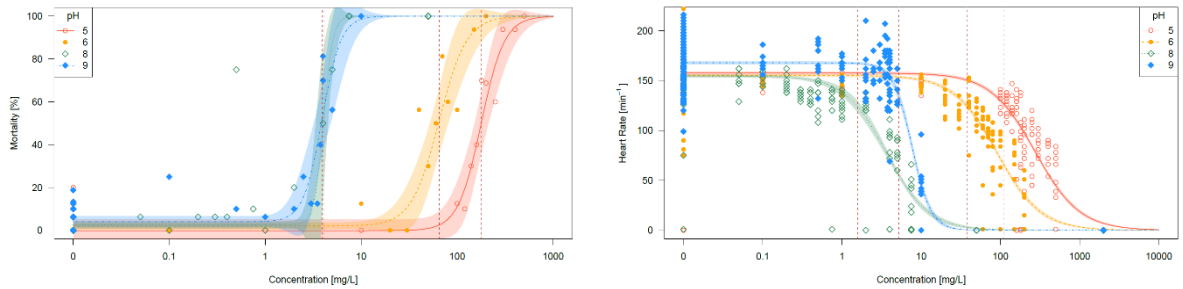
g. Metoprolol



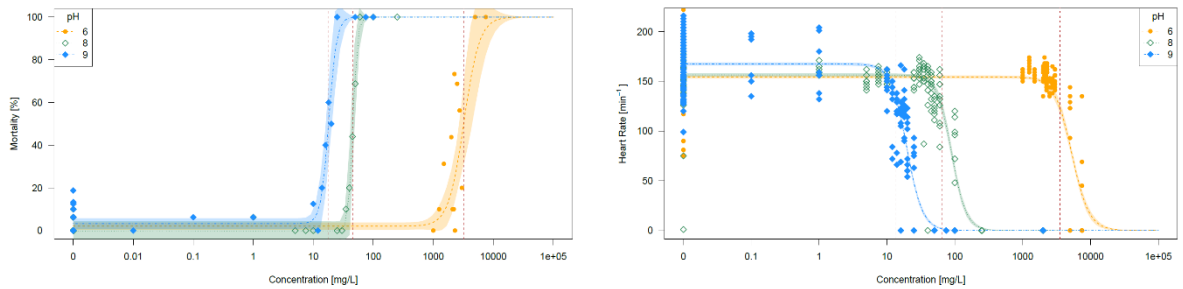
h. Propranolol



i. Tetracaine



j. Tramadol



Non-linear regression analysis for mortality (left) and heart rate (right) in zebrafish embryos depending on pH and substance concentration; vertical dashed lines mark LC_{50} / EC_{20} levels; a. diclofenac; b. ibuprofen; c. naproxen; d. triclosan; e. citalopram; f. fluoxetine; g. propranolol; h. metoprolol; i. tetracaine; j. tramadol.

Source: Own depiction

Table A3: Overview of NOEC, LOEC, EC_{10} and EC_{50} values determined for the substances tested in *Daphnia magna*

Substance	pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
		24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Amitriptyline hydrochloride	6.0								
	8.5								
Diclofenac sodium salt	6.0	5.76	5.76	12.80	12.80	2.55	2.21	8.50	6.47
	8.5	117.47	10.44	261.04	22.19	20.08	3.59	143.93	39.97
Ibuprofen sodium salt	6.0	5.60	1.12	12.32	2.52	5.72	2.19	11.18	5.77
	8.5	112.13	112.13	252.30	252.30	201.07	71.71	333.97	151.02
Propranolol hydrochloride	6.0	60.39	7.25	120.78	14.45	56.88	8.64	126.72	47.82
	8.5	7.45	7.45	18.62	18.62	8.35	7.45	12.47	8.99

Overview of NOEC, LOEC, EC_{10} and EC_{50} values determined for the substances (in alphabetical order) tested in *Daphnia magna* for the endpoint 'mortality' rounded to two decimal places in mg/L at the corresponding pH levels at the time points 24 and 48 h.

Source: Own depiction

Table A4: Overview of NOEC, LOEC, EC_{10} and EC_{50} values determined for the substances tested in *Lemna minor*

Substance	pH	NOEC [mg/L]		LOEC [mg/L]		EC_{10} [mg/L]		EC_{50} [mg/L]	
		biomass	offshoot	biomass	offshoot	biomass	offshoot	biomass	offshoot
Amitriptyline hydrochloride	5.5								
	7.5								
Diclofenac sodium salt	5.5	2.25	11.53	5.04	25.91	6.649	17.561	13.543	23.572
	7.5	35.22	35.22	80.04	80.04	62.11	75.41	102.83	124.88
Ibuprofen sodium salt	5.5	≤ 0.45	4.241	≤ 0.45	13.027	0.7	7.58	6.56	22.29
	7.5	< 5.0	< 5.0	≤ 5.0	≤ 5.0	2.05	2.65	48.16	67.32
Propranolol hydrochloride	5.5	39.81	79.62	79.62	159.25	52.68	75.07	108.72	139.31
	7.5	5.02	5.02	10.05	10.05	4.77	3.87	22.93	31.16

Overview of NOEC, LOEC, EC_{10} and EC_{50} values determined for the substances (in alphabetical order) tested in *Lemna minor* for the endpoint 'mean biomass increase' and 'mean offshoot growth' rounded to two decimal places in mg/L at the corresponding pH levels.

Source: Own depiction

Table A5: Summarised results for the chemical analyses of FET samples including water concentration from test start and end of the exposure experiment

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
2,3-Dichlorophenol	pH 5	START	6.0	WS_DCP_pH5_Start	-	1.278	Y	-	1.278 ± 0.069
2,3-Dichlorophenol	pH 5	END		WS_DCP_pH5_End	-	0.336	Y	-	0.336 ± 0.018
2,3-Dichlorophenol	pH 6	START	5.0	WS_DCP_pH6_Start	-	2.07	Y	-	2.07 ± 0.11
2,3-Dichlorophenol	pH 6	END		WS_DCP_pH6_End	-	0.311	Y	-	0.311 ± 0.017
2,3-Dichlorophenol	pH 8	START	7.5	WS_DCP_pH8_Start	-	2.22	Y	-	2.22 ± 0.12
2,3-Dichlorophenol	pH 8	END		WS_DCP_pH8_End	-	2.60	Y	-	2.60 ± 0.14
2,4,5-Trichlorophenol	pH 5	START	0.575	WS_TCP_pH5_Start	-	0.294	Y	-	0.294 ± 0.015
2,4,5-Trichlorophenol	pH 5	END		WS_TCP_pH5_End	-	< LOD	Y	-	-
2,4,5-Trichlorophenol	pH 6	START	0.50	WS_TCP_pH6_Start	-	0.1742	Y	-	0.1742 ± 0.0091
2,4,5-Trichlorophenol	pH 6	END		WS_TCP_pH6_End	-	< LOD	Y	-	-
2,4,5-Trichlorophenol	pH 8	START	1.00	WS_TCP_pH8_Start	-	0.711	Y	-	0.711 ± 0.037
2,4,5-Trichlorophenol	pH 8	END		WS_TCP_pH8_End	-	0.473	Y	-	0.473 ± 0.025

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured}$ average [mg/L]	$C_{measured}$ [mg/L] ± SD
Acetylsalicylic acid	pH 5	START	18.0	WS_Aca_pH5_Start	-	6.75	Y	-	6.75 ± 0.47
Acetylsalicylic acid	pH 5	END		WS_Aca_pH5_End	-	0.254	Y	-	0.254 ± 0.018
Acetylsalicylic acid	pH 6	START	90.0	WS_Aca_pH6_Start	-	36.5	Y	-	36.5 ± 2.6
Acetylsalicylic acid	pH 6	END		WS_Aca_pH6_End	-	37.8	Y	-	37.8 ± 2.6
Acetylsalicylic acid	pH 8	START	2800	WS_Aca_pH8_Start	-	1.028	Y	-	1.028 ± 0.072
Acetylsalicylic acid	pH 8	END		WS_Aca_pH8_End	-	0.248	Y	-	0.248 ± 0.017
Amitriptyline	pH 5	START	225.0	WS_Ami_pH5_Start	-	110.9	Y	-	110.9 ± 7.8
Amitriptyline	pH 5	END		WS_Ami_pH5_End	-	107.0	Y	-	107.0 ± 7.5
Amitriptyline	pH 6	START	89.16	WS_Ami_pH6_Start	WS_Ami_pH6_Start_1	52.4	Y	58.4	58.4 ± 5.6
Amitriptyline	pH 6	START			WS_Ami_pH6_Start_2	52.5	Y		
Amitriptyline	pH 6	START			WS_Ami_pH6_Start_3	62.3	Y		
Amitriptyline	pH 6	START			WS_Ami_pH6_Start_4	60.1	Y		
Amitriptyline	pH 6	START			WS_Ami_pH6_Start_5	56.6	Y		
Amitriptyline	pH 6	START			WS_Ami_pH6_Start_6	66.5	Y		
Amitriptyline	pH 6	END	89.16	WS_Ami_pH6_End	WS_Ami_pH6_End_1	61.6	Y	60.9	60.9 ± 1.7
Amitriptyline	pH 6	END			WS_Ami_pH6_End_2	58.7	Y		
Amitriptyline	pH 6	END			WS_Ami_pH6_End_3	62.5	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured}$ average [mg/L]	$C_{measured}$ [mg/L] ± SD
Amitriptyline	pH 6	END			WS_Ami_pH6_End_4	62.1	Y		
Amitriptyline	pH 6	END			WS_Ami_pH6_End_5	59.4	Y		
Amitriptyline	pH 6	END			WS_Ami_pH6_End_6	51.7	Outlier		
Amitriptyline	pH 8	START	2.10	WS_Ami_pH8_Start	WS_Ami_pH8_Start_1	0.0692	Y	0.0822	0.0822 ± 0.0071
Amitriptyline	pH 8	START			WS_Ami_pH8_Start_2	0.0808	Y		
Amitriptyline	pH 8	START			WS_Ami_pH8_Start_3	0.0818	Y		
Amitriptyline	pH 8	START			WS_Ami_pH8_Start_4	0.0860	Y		
Amitriptyline	pH 8	START			WS_Ami_pH8_Start_5	0.0898	Y		
Amitriptyline	pH 8	START			WS_Ami_pH8_Start_6	0.0857	Y		
Amitriptyline	pH 8	END	2.10	WS_Ami_pH8_End	WS_Ami_pH8_End_1	0.0660	Y	0.0725	0.0725 ± 0.0060
Amitriptyline	pH 8	END			WS_Ami_pH8_End_2	0.0750	Y		
Amitriptyline	pH 8	END			WS_Ami_pH8_End_3	0.0724	Y		
Amitriptyline	pH 8	END			WS_Ami_pH8_End_4	0.0663	Y		
Amitriptyline	pH 8	END			WS_Ami_pH8_End_5	0.0734	Y		
Amitriptyline	pH 8	END			WS_Ami_pH8_End_6	0.0822	Y		
Amitriptyline	pH 9	START	1.60	WS_Ami_pH9_Start	WS_Ami_pH9_Start_1	0.0236	Y	0.0254	0.0254 ± 0.0020
Amitriptyline	pH 9	START			WS_Ami_pH9_Start_2	0.0234	Y		
Amitriptyline	pH 9	START			WS_Ami_pH9_Start_3	0.0252	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured}$ average [mg/L]	$C_{measured}$ [mg/L] ± SD
Amitriptyline	pH 9	START			WS_Ami_pH9_Start_4	0.0289	Y		
Amitriptyline	pH 9	START			WS_Ami_pH9_Start_5	0.0255	Y		
Amitriptyline	pH 9	START			WS_Ami_pH9_Start_6	0.0256	Y		
Amitriptyline	pH 9	END	1.60	WS_Ami_pH9_End	WS_Ami_pH9_End_1	0.0203	Y	0.01986	0.01986 ± 0.00094
Amitriptyline	pH 9	END			WS_Ami_pH9_End_2	0.0189	Y		
Amitriptyline	pH 9	END			WS_Ami_pH9_End_3	0.0211	Y		
Amitriptyline	pH 9	END			WS_Ami_pH9_End_4	0.0204	Y		
Amitriptyline	pH 9	END			WS_Ami_pH9_End_5	0.0186	Y		
Amitriptyline	pH 9	END			WS_Ami_pH9_End_6	0.0199	Y		
Bromoxynil	pH 5	START	0.15	WS_Bromo_pH5_Start	-	0.1443	Y	-	0.1443 ± 0.0087
Bromoxynil	pH 5	END		WS_Bromo_pH5_End	-	0.0877	Y	-	0.0877 ± 0.0053
Bromoxynil	pH 6	START	0.20	WS_Bromo_pH6_Start	-	0.1360	Y	-	0.1360 ± 0.0082
Bromoxynil	pH 6	END		WS_Bromo_pH6_End	-	0.1264	Y	-	0.1264 ± 0.0076
Bromoxynil	pH 8	START	15.0	WS_Bromo_pH8_Start	-	14.5	Y	-	14.5 ± 1.3
Bromoxynil	pH 8	END		WS_Bromo_pH8_End	-	14.7	Y	-	14.7 ± 1.3
Citalopram	pH 5	START	1180	WS_CTR_pH5_Start	-	1132	Y	-	1132 ± 136

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured}$ average [mg/L]	$C_{measured}$ [mg/L] ± SD
Citalopram	pH 5	END		WS_CTR_pH5_End	-	1196	Y	-	1196 ± 143
Citalopram	pH 6	START	380	WS_CTR_pH6_Start	-	381	Y	-	381 ± 34
Citalopram	pH 6	END		WS_CTR_pH6_End	-	392	Y	-	392 ± 35
Citalopram	pH 8	START	24	WS_CTR_pH8_Start	-	19.9	Y	-	19.9 ± 1.8
Citalopram	pH 8	END		WS_CTR_pH8_End	-	26.4	Y	-	26.4 ± 2.4
Citalopram	pH 9	START	5.0	WS_CTR_pH9_Start	-	4.24	Y	-	4.24 ± 0.51
Citalopram	pH 9	END		WS_CTR_pH9_End	-	3.22	Y	-	3.22 ± 0.39
Diclofenac	pH 5	START	0.05	WS_Diclo_pH5_Start	-	0.0341	Y	-	0.0341 ± 0.0020
Diclofenac	pH 5	END		WS_Diclo_pH5_End	-	0.01566	Y	-	0.01566 ± 0.00094
Diclofenac	pH 6	START	0.4	WS_Diclo_pH6_Start	-	0.302	Y	-	0.302 ± 0.018
Diclofenac	pH 6	END		WS_Diclo_pH6_End	-	0.207	Y	-	0.237 ± 0.014
Diclofenac	pH 8	START	17	WS_Diclo_pH8_Start	-	11.45	Y	-	11.45 ± 0.73
Diclofenac	pH 8	END		WS_Diclo_pH8_End	-	14.5	Y	-	14.5 ± 1.0
Diclofenac	pH 9	START	70.0	WS_Diclo_pH9_Start	-	37.0	Y	-	37.0 ± 2.6
Diclofenac	pH 9	END		WS_Diclo_pH9_End	-	29.5	Y	-	29.5 ± 2.1
Enclomiphene	pH 8	START	3.1	WS_Enclo_pH8_Start	-	< LOQ	-	-	-

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Enclomiphene	pH 8	END		WS_Enclo_pH8_End	-	< LOQ	-	-	-
Enclomiphene	pH 9	START	3.1	WS_Enclo_pH9_Start	-	< LOQ	-	-	-
Enclomiphene	pH 9	END		WS_Enclo_pH9_End	-	< LOQ	-	-	-
Fluoxetine	pH 5	START	220.0	WS_Fluo_pH5_Start	-	152	Y	-	152 ± 11
Fluoxetine	pH 5	END		WS_Fluo_pH5_End	-	215	Y	-	215 ± 15
Fluoxetine	pH 6	START	55.0	WS_Fluo_pH6_Start	-	33.8	Y	-	33.8 ± 2.4
Fluoxetine	pH 6	END		WS_Fluo_pH6_End	-	34.8	Y	-	34.8 ± 2.4
Fluoxetine	pH 8	START	2.0	WS_Fluo_pH8_Start	-	1.106	Y	-	1.106 ± 0.077
Fluoxetine	pH 8	END		WS_Fluo_pH8_End	-	0.758	Y	-	0.758 ± 0.053
Fluoxetine	pH 9	START	0.5	WS_Fluo_pH9_Start	-	0.174	Y	-	0.174 ± 0.012
Fluoxetine	pH 9	END		WS_Fluo_pH9_End	-	0.0828	Y	-	0.0828 ± 0.0058
Ibuprofen	pH 5	START	8.7	WS_Ibu_pH5_Start	WS_Ibu_pH5_Start_1	0.477	Y	0.520	0.520 ± 0.036
Ibuprofen	pH 5	START			WS_Ibu_pH5_Start_2	0.578	Y		
Ibuprofen	pH 5	START			WS_Ibu_pH5_Start_3	0.492	Y		
Ibuprofen	pH 5	START			WS_Ibu_pH5_Start_4	0.534	Y		
Ibuprofen	pH 5	START			WS_Ibu_pH5_Start_5	0.507	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Ibuprofen	pH 5	START			WS_Ibu_pH5_Start_6	0.532	Y		
Ibuprofen	pH 5	END	8.7	WS_Ibu_pH5_End	WS_Ibu_pH5_End_1	0.318	Y	0.328	0.328 ± 0.016
Ibuprofen	pH 5	END			WS_Ibu_pH5_End_2	0.307	Y		
Ibuprofen	pH 5	END			WS_Ibu_pH5_End_3	0.337	Y		
Ibuprofen	pH 5	END			WS_Ibu_pH5_End_4	0.323	Y		
Ibuprofen	pH 5	END			WS_Ibu_pH5_End_5	0.332	Y		
Ibuprofen	pH 5	END			WS_Ibu_pH5_End_6	0.352	Y		
Ibuprofen	pH 6	START	45.2	WS_Ibu_pH6_Start	WS_Ibu_pH6_Start_1	3.54	Y	3.73	3.73 ± 0.28
Ibuprofen	pH 6	START			WS_Ibu_pH6_Start_2	3.88	Y		
Ibuprofen	pH 6	START			WS_Ibu_pH6_Start_3	4.13	Y		
Ibuprofen	pH 6	START			WS_Ibu_pH6_Start_4	3.91	Y		
Ibuprofen	pH 6	START			WS_Ibu_pH6_Start_5	3.51	Y		
Ibuprofen	pH 6	START			WS_Ibu_pH6_Start_6	3.41	Y		
Ibuprofen	pH 6	END	45.2	WS_Ibu_pH6_End	WS_Ibu_pH6_End_1	2.61	Y	2.82	2.82 ± 0.25
Ibuprofen	pH 6	END			WS_Ibu_pH6_End_2	3.24	Y		
Ibuprofen	pH 6	END			WS_Ibu_pH6_End_3	2.94	Y		
Ibuprofen	pH 6	END			WS_Ibu_pH6_End_4	2.87	Y		
Ibuprofen	pH 6	END			WS_Ibu_pH6_End_5	2.58	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured}$ average [mg/L]	$C_{measured}$ [mg/L] ± SD
Ibuprofen	pH 6	END			WS_Ibu_pH6_End_6	2.70	Y		
Ibuprofen	pH 8	START	150.0	WS_Ibu_pH8_Start	WS_Ibu_pH8_Start_1	126	Y	109	109 ± 14
Ibuprofen	pH 8	START			WS_Ibu_pH8_Start_2	96	Y		
Ibuprofen	pH 8	START			WS_Ibu_pH8_Start_3	126	Y		
Ibuprofen	pH 8	START			WS_Ibu_pH8_Start_4	102	Y		
Ibuprofen	pH 8	START			WS_Ibu_pH8_Start_5	107	Y		
Ibuprofen	pH 8	START			WS_Ibu_pH8_Start_6	96	Y		
Ibuprofen	pH 8	END	150.0	WS_Ibu_pH8_End	WS_Ibu_pH8_End_1	132.5	Y	130.5	130.5 ± 5.6
Ibuprofen	pH 8	END			WS_Ibu_pH8_End_2	138.2	Y		
Ibuprofen	pH 8	END			WS_Ibu_pH8_End_3	130.4	Y		
Ibuprofen	pH 8	END			WS_Ibu_pH8_End_4	128.3	Y		
Ibuprofen	pH 8	END			WS_Ibu_pH8_End_5	170.8	Outlier		
Ibuprofen	pH 8	END			WS_Ibu_pH8_End_6	123.1	Y		
Ibuprofen	pH 9	START	300.0	WS_Ibu_pH9_Start	-	178	Y	-	178 ± 14
Ibuprofen	pH 9	END		WS_Ibu_pH9_End	-	127	Y	-	127 ± 10
Ketotifen	pH 9	START	1.5	WS_Ket_pH9_Start	-	0.709	Y	-	0.709 ± 0.016
Ketotifen	pH 9	END		WS_Ket_pH9_End	-	0.587	Y	-	0.587 ± 0.013

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Lidocaine	pH 5	START	2000	WS_Lid_pH5_Start	-	521	Y	-	521 ± 16
Lidocaine	pH 5	END		WS_Lid_pH5_End	-	618	Y	-	618 ± 19
Lidocaine	pH 8	START	50.00	WS_Lid_pH6_Start	-	18.69	Y	-	18.69 ± 0.58
Lidocaine	pH 8	END		WS_Lid_pH6_End	-	17.28	Y	-	17.28 ± 0.54
Lidocaine	pH 9	START	10.0	WS_Lid_pH8_Start	-	4.57	Y	-	4.57 ± 0.14
Lidocaine	pH 9	END		WS_Lid_pH8_End	-	3.97	Y	-	3.97 ± 0.12
Metoprolol	pH 5	START	6600.0	WS_Met_pH5_Start	-	9507	Y	-	9507 ± 665
Metoprolol	pH 5	END		WS_Met_pH5_End	-	8689	Y	-	8689 ± 608
Metoprolol	pH 6	START	3180.0	WS_Met_pH6_Start	-	2297	Y	-	2297 ± 161
Metoprolol	pH 6	END		WS_Met_pH6_End	-	3707	Y	-	3707 ± 259
Metoprolol	pH 8	START	70.0	WS_Met_pH8_Start	-	77.1	Y	-	77.1 ± 5.4
Metoprolol	pH 8	END		WS_Met_pH8_End	-	74.0	Y	-	74.0 ± 5.2
Metoprolol	pH 9	START	10.0	WS_Met_pH9_Start	-	11.60	Y	-	11.60 ± 0.81
Metoprolol	pH 9	END		WS_Met_pH9_End	-	10.39	Y	-	10.39 ± 0.73
Naproxen	pH 6	START	8.0	WS_Napr_pH6_Start	-	5.66	Y	-	5.66 ± 0.57
Naproxen	pH 6	END		WS_Napr_pH6_End	-	5.22	Y	-	5.22 ± 0.52

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Naproxen	pH 8	START	325.0	WS_Napr_pH8_Start	-	147	Y	-	147 ± 15
Naproxen	pH 8	END		WS_Napr_pH8_End	-	163	Y	-	163 ± 16
Naproxen	pH 9	START	900.0	WS_Napr_pH9_Start	-	543	Y	-	543 ± 54
Naproxen	pH 9	END		WS_Napr_pH9_End	-	478	Y	-	478 ± 48
Pentachlorophenol	pH 5	START	0.050	WS_PCP_pH5_Start	-	0.01369	Y	-	0.01369 ± 0.00046
Pentachlorophenol	pH 5	END		WS_PCP_pH5_End	-	< LOD	Y	-	-
Pentachlorophenol	pH 6	START	0.052	WS_PCP_pH6_Start	-	0.02797	Y	-	0.02797 ± 0.00095
Pentachlorophenol	pH 6	END		WS_PCP_pH6_End	-	< LOD	Y	-	-
Pentachlorophenol	pH 8	START	0.26	WS_PCP_pH8_Start	-	0.1459	Y	-	0.1459 ± 0.0049
Pentachlorophenol	pH 8	END		WS_PCP_pH8_End	-	0.1186	Y	-	0.1186 ± 0.0040
Propranolol	pH 5	START	1600	WS_Propr_pH5_new_Start	-	921	Y	-	921 ± 74
Propranolol	pH 5	END		WS_Propr_pH5_new_End	-	846	Y	-	846 ± 68
Propranolol	pH 6	START	440	WS_Propr_pH6_new_Start	-	428	Y	-	428 ± 34
Propranolol	pH 6	END		WS_Propr_pH6_new_End	-	472	Y	-	472 ± 38
Propranolol	pH 8	START	9	WS_Propr_pH8_new_Start	-	6.01	Y	-	6.01 ± 0.48
Propranolol	pH 8	END		WS_Propr_pH8_new_End	-	5.42	Y	-	5.42 ± 0.43

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Propranolol	pH 9	START	0.6	WS_Propr_pH9_new_Start	-	0.413	Y	-	0.413 ± 0.033
Propranolol	pH 9	END		WS_Propr_pH9_new_End	-	0.241	Y	-	0.241 ± 0.019
Sertraline	pH 6	START	10.50	WS_Sertr_pH6_Start	WS_Sertr_pH6_Start_1	2.886	Y	2.87	2.87 ± 0.11
Sertraline	pH 6	START			WS_Sertr_pH6_Start_2	2.144	Outlier		
Sertraline	pH 6	START			WS_Sertr_pH6_Start_3	2.896	Y		
Sertraline	pH 6	START			WS_Sertr_pH6_Start_4	2.822	Y		
Sertraline	pH 6	START			WS_Sertr_pH6_Start_5	3.014	Y		
Sertraline	pH 6	START			WS_Sertr_pH6_Start_6	2.716	Y		
Sertraline	pH 6	END	10.50	WS_Sertr_pH6_End	WS_Sertr_pH6_End_1	3.416	Y	3.38	3.38 ± 0.47
Sertraline	pH 6	END			WS_Sertr_pH6_End_2	2.547	Y		
Sertraline	pH 6	END			WS_Sertr_pH6_End_3	3.789	Y		
Sertraline	pH 6	END			WS_Sertr_pH6_End_4	3.304	Y		
Sertraline	pH 6	END			WS_Sertr_pH6_End_5	3.848	Y		
Sertraline	pH 6	END			WS_Sertr_pH6_End_6	3.369	Y		
Sertraline	pH 8	START	1.10	WS_Sertr_pH8_Start	WS_Sertr_pH8_Start_1	0.2768	Y	0.306	0.306 ± 0.015
Sertraline	pH 8	START			WS_Sertr_pH8_Start_2	0.3109	Y		
Sertraline	pH 8	START			WS_Sertr_pH8_Start_3	0.3169	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Sertraline	pH 8	START			WS_Sertr_pH8_Start_4	0.3104	Y		
Sertraline	pH 8	START			WS_Sertr_pH8_Start_5	0.3022	Y		
Sertraline	pH 8	START			WS_Sertr_pH8_Start_6	0.3179	Y		
Sertraline	pH 8	END	1.10	WS_Sertr_pH8_End	WS_Sertr_pH8_End_1	0.0662	Y	0.0693	0.0693 ± 0.0060
Sertraline	pH 8	END			WS_Sertr_pH8_End_2	0.0659	Y		
Sertraline	pH 8	END			WS_Sertr_pH8_End_3	0.0803	Y		
Sertraline	pH 8	END			WS_Sertr_pH8_End_4	0.0710	Y		
Sertraline	pH 8	END			WS_Sertr_pH8_End_5	0.0637	Y		
Sertraline	pH 8	END			WS_Sertr_pH8_End_6	0.0685	Y		
Sertraline	pH 9	START	0.10	WS_Sertr_pH9_Start	WS_Sertr_pH9_Start_1	0.1164	Y	0.1149	0.1149 ± 0.0035
Sertraline	pH 9	START			WS_Sertr_pH9_Start_2	0.1147	Y		
Sertraline	pH 9	START			WS_Sertr_pH9_Start_3	0.1110	Y		
Sertraline	pH 9	START			WS_Sertr_pH9_Start_4	0.1209	Y		
Sertraline	pH 9	START			WS_Sertr_pH9_Start_5	0.1144	Y		
Sertraline	pH 9	START			WS_Sertr_pH9_Start_6	0.1123	Y		
Sertraline	pH 9	END	0.10	WS_Sertr_pH9_End	WS_Sertr_pH9_End_1	0.0814	Y	0.0835	0.0835 ± 0.0048
Sertraline	pH 9	END			WS_Sertr_pH9_End_2	0.0769	Y		
Sertraline	pH 9	END			WS_Sertr_pH9_End_3	0.0810	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Sertraline	pH 9	END			WS_Sertr_pH9_End_4	0.0883	Y		
Sertraline	pH 9	END			WS_Sertr_pH9_End_5	0.0898	Y		
Sertraline	pH 9	END			WS_Sertr_pH9_End_6	0.0834	Y		
Tetracaine	pH 5	START	170.13	WS_Tetr_pH5_Start	WS_Tetr_pH5_Start_1	155	Y	158	158 ± 14
Tetracaine	pH 5	START			WS_Tetr_pH5_Start_2	154	Y		
Tetracaine	pH 5	START			WS_Tetr_pH5_Start_3	166	Y		
Tetracaine	pH 5	START			WS_Tetr_pH5_Start_4	143	Y		
Tetracaine	pH 5	START			WS_Tetr_pH5_Start_5	182	Y		
Tetracaine	pH 5	START			WS_Tetr_pH5_Start_6	148	Y		
Tetracaine	pH 5	END	170.13	WS_Tetr_pH5_End	WS_Tetr_pH5_End_1	160	Y	152	152 ± 14
Tetracaine	pH 5	END			WS_Tetr_pH5_End_2	163	Y		
Tetracaine	pH 5	END			WS_Tetr_pH5_End_3	150	Y		
Tetracaine	pH 5	END			WS_Tetr_pH5_End_4	167	Y		
Tetracaine	pH 5	END			WS_Tetr_pH5_End_5	131	Y		
Tetracaine	pH 5	END			WS_Tetr_pH5_End_6	142	Y		
Tetracaine	pH 6	START	59.20	WS_Tetr_pH6_Start	WS_Tetr_pH6_Start_1	56.3	Y	56.0	56.0 ± 3.2
Tetracaine	pH 6	START			WS_Tetr_pH6_Start_2	57.4	Y		
Tetracaine	pH 6	START			WS_Tetr_pH6_Start_3	61.3	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Tetracaine	pH 6	START			WS_Tetr_pH6_Start_4	52.6	Y		
Tetracaine	pH 6	START			WS_Tetr_pH6_Start_5	52.8	Y		
Tetracaine	pH 6	START			WS_Tetr_pH6_Start_6	55.9	Y		
Tetracaine	pH 6	END	59.20	WS_Tetr_pH6_End	WS_Tetr_pH6_End_1	49.9	Y	49.8	49.8 ± 1.4
Tetracaine	pH 6	END			WS_Tetr_pH6_End_2	48.7	Y		
Tetracaine	pH 6	END			WS_Tetr_pH6_End_3	48.9	Y		
Tetracaine	pH 6	END			WS_Tetr_pH6_End_4	49.4	Y		
Tetracaine	pH 6	END			WS_Tetr_pH6_End_5	52.1	Y		
Tetracaine	pH 6	END			WS_Tetr_pH6_End_6	59.4	Outlier		
Tetracaine	pH 8	START	3.90	WS_Tetr_pH8_Start	WS_Tetr_pH8_Start_1	2.388	Y	2.406	2.406 ± 0.049
Tetracaine	pH 8	START			WS_Tetr_pH8_Start_2	2.380	Y		
Tetracaine	pH 8	START			WS_Tetr_pH8_Start_3	2.473	Y		
Tetracaine	pH 8	START			WS_Tetr_pH8_Start_4	2.438	Y		
Tetracaine	pH 8	START			WS_Tetr_pH8_Start_5	2.350	Y		
Tetracaine	pH 8	START			WS_Tetr_pH8_Start_6	2.746	Outlier		
Tetracaine	pH 8	END	3.90	WS_Tetr_pH8_End	WS_Tetr_pH8_End_1	1.647	Y	1.705	1.705 ± 0.037
Tetracaine	pH 8	END			WS_Tetr_pH8_End_2	1.710	Y		
Tetracaine	pH 8	END			WS_Tetr_pH8_End_3	1.744	Y		

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured\ average}$ [mg/L]	$C_{measured}$ [mg/L] ± SD
Tetracaine	pH 8	END			WS_Tetr_pH8_End_4	1.697	Y		
Tetracaine	pH 8	END			WS_Tetr_pH8_End_5	1.910	Outlier		
Tetracaine	pH 8	END			WS_Tetr_pH8_End_6	1.727	Y		
Tetracaine	pH 9	START	3.0	WS_Tetr_pH9_Start	-	2.186	Y	-	2.186 ± 0.048
Tetracaine	pH 9	END		WS_Tetr_pH9_End	-	0.0938	Y	-	0.0938 ± 0.0021
Tramadol	pH 6	START	2750.0	WS_Tram_pH6_Start	-	1167	Y	-	1167 ± 117
Tramadol	pH 6	END		WS_Tram_pH6_End	-	1812	Y	-	1812 ± 181
Tramadol	pH 8	START	40.0	WS_Tram_pH8_Start	-	14.8	Y	-	14.8 ± 1.5
Tramadol	pH 8	END		WS_Tram_pH8_End	-	19.9	Y	-	19.9 ± 2.0
Tramadol	pH 9	START	15.0	WS_Tram_pH9_Start	-	8.46	Y	-	8.46 ± 0.85
Tramadol	pH 9	END		WS_Tram_pH9_End	-	9.48	Y	-	9.48 ± 0.95
Triclosan	pH 5	START	0.20	WS_Tricl_pH5_new_Start	-	0.0216	Y	-	0.0216 ± 0.0015
Triclosan	pH 5	END		WS_Tricl_pH5_new_End	-	< LOD	Y	-	< LOD
Triclosan	pH 6	START	0.30	WS_Tricl_pH6_new_Start	-	0.0370	Y	-	0.0370 ± 0.0026
Triclosan	pH 6	END		WS_Tricl_pH6_new_End	-	< LOD	Y	-	< LOD
Triclosan	pH 8	START	0.35	WS_Tricl_pH8_new_Start	-	0.0709	Y	-	0.0709 ± 0.0050

Substance	pH	Start / End	$C_{nominal}$ [mg/L]	Sample Code	Measurement Code	$C_{measured}$ [mg/L]	Used	$C_{measured}$ average [mg/L]	$C_{measured}$ [mg/L] ± SD
Triclosan	pH 8	END		WS_Tricl_pH8_new_End	-	< LOD	Y	-	< LOD
Triclosan	pH 9	START	0.60	WS_Tricl_pH9_new_Start	-	0.264	Y	-	0.264 ± 0.018
Triclosan	pH 9	END		WS_Tricl_pH9_new_End	-	0.0213	Y	-	0.0213 ± 0.0015

Source: Own depiction

Table A6: Summarised results for the chemical analyses of FET samples including the internal concentrations of the zebrafish embryos and the respective calculated bioconcentration factors (BCF)

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
2,3-Dichlorophenol	pH 5	6.0	1	alive	19	DCP pH5 6 mg/L 19* (1)	17.4	90	2.593	0.079	2.593 ± 0.079	3.2
2,3-Dichlorophenol	pH 5		2	alive	20	DCP pH5 6 mg/L 20* (2)	14.5	131	4.51	0.14	4.51 ± 0.14	5.6
2,3-Dichlorophenol	pH 5		3	alive	20	DCP pH5 6 mg/L 20* (3)	17.1	150	4.39	0.13	4.39 ± 0.13	5.4
2,3-Dichlorophenol	pH 6	5.0	1	alive	20	DCP pH6 5 mg/L 20* (1)	9.7	262	13.48	0.45	13.48 ± 0.45	11.3

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
2,3-Dichlorophenol	pH 6		2	alive	19	DCP pH6 5 mg/L 19* (2)	15.9	316	9.93	0.31	9.93 ± 0.31	8.3
2,3-Dichlorophenol	pH 6		3	alive	20	DCP pH6 5 mg/L 20* (3)	9.9	194	9.80	0.32	9.80 ± 0.32	8.2
2,3-Dichlorophenol	pH 8	7.5	1	alive	20	DCP pH8 7.5 mg/L 20* (1)	9.9	325	16.44	0.54	16.44 ± 0.54	6.8
2,3-Dichlorophenol	pH 8		2	alive	20	DCP pH8 7.5 mg/L 20* (2)	9.3	436	23.44	0.78	23.44 ± 0.78	9.7
2,3-Dichlorophenol	pH 8		3	alive	20	DCP pH8 7.5 mg/L 20* (3)	10.1	412	20.40	0.67	20.40 ± 0.67	8.5
2,4,5-Trichlorophenol	pH 5	0.575	1	alive	20	1 TCP pH5 0,575 mg/l *20	3.70	86	11.6	1.1	11.6 ± 1.1	39.6
2,4,5-Trichlorophenol	pH 5		2	alive	16	2 TCP pH5 0,575 mg/l *16	3.10	126	20.4	1.9	20.4 ± 1.9	69.3
2,4,5-Trichlorophenol	pH 5		3	alive	20	3 TCP pH5 0,575 mg/l *20	3.80	135	17.7	1.6	17.7 ± 1.6	60.3
2,4,5-Trichlorophenol	pH 5		4	alive	20	4 TCP pH5 0,575 mg/l *20	4.00	126	15.8	1.4	15.8 ± 1.4	53.6

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
2,4,5-Trichlorophenol	pH 5		5	alive	20	5 TCP pH5 0,575 mg/l *20	4.00	189	23.6	2.1	23.6 ± 2.1	80.2
2,4,5-Trichlorophenol	pH 6	0.50	1	alive	15	1 TCP pH6 0,5 mg/l *15	3.40	57	8.32	0.78	8.32 ± 0.78	47.74
2,4,5-Trichlorophenol	pH 6		2	alive	15	2 TCP pH6 0,5 mg/l *15	3.40	125	18.5	1.7	18.5 ± 1.7	105.9
2,4,5-Trichlorophenol	pH 6		3	alive	15	3 TCP pH6 0,5 mg/l *15	3.10	87	14.0	1.3	14.0 ± 1.3	80.2
2,4,5-Trichlorophenol	pH 6		4	alive	20	4 TCP pH6 0,5 mg/l *20	4.30	112	13.1	1.2	13.1 ± 1.2	74.9
2,4,5-Trichlorophenol	pH 8	1.00	1	alive	20	1 TCP pH8 1 mg/l *20	4.70	246	26.1	2.3	26.1 ± 2.3	44.2
2,4,5-Trichlorophenol	pH 8		2	alive	20	2 TCP pH8 1 mg/l *20	4.50	238	26.5	2.4	26.5 ± 2.4	44.7
2,4,5-Trichlorophenol	pH 8		3	alive	20	3 TCP pH8 1 mg/l *20	4.10	169	20.6	1.9	20.6 ± 1.9	34.8
2,4,5-Trichlorophenol	pH 8		4	alive	20	4 TCP pH8 1 mg/l *20	4.40	281	31.9	2.9	31.9 ± 2.9	54.0
Acetylsalicylic acid	pH 5	18.0	1	dead	13	pH 5 1 ASA	5.1	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 5		2	alive	20	pH 5 2 ASA	3.9	<LOD	-	-	<LOD	-

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Acetylsalicylic acid	pH 5		3	alive	20	pH 5 3 ASA	10.7	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 5		4	alive	20	pH 5 4 ASA	21.8	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 5		5	alive	17	pH 5 5 ASA	7.4	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 6	90.0	1	dead	11	pH 6 1 ASA	1.8	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 6		2	alive	18	pH 6 2 ASA	5.4	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 6		3	alive	18	pH 6 3 ASA	4.2	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 8	2800	1	alive	20	pH 8 ASA 2800 mg/L 20* (1)	10.1	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 8		2	alive	20	pH 8 ASA 2800 mg/L 20* (2)	12.6	<LOD	-	-	<LOD	-
Acetylsalicylic acid	pH 8		3	alive	20	pH 8 ASA 2800 mg/L 20* (3)	12.9	<LOD	-	-	<LOD	-
Amitriptyline	pH 5	225.00	1	alive	15	Ami pH 5 225 mg/L 15 alive 96 hpf 1	3.600	3355	466	19	466 ± 19	4.3
Amitriptyline	pH 5		2	alive	15	Ami pH 5 225 mg/L 15 alive 96 hpf 2	3.300	3568	541	23	541 ± 23	5.0

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Amitriptyline	pH 5		3	alive	15	Ami pH 5 225 mg/L 15 alive 96 hpf 3	3.300	3071	465	20	465 ± 20	4.3
Amitriptyline	pH 5		4	dead	4	Ami pH 5 225 mg/L 4 dead 96 hpf 4	1.400	728	260	17	260 ± 17	2.4
Amitriptyline	pH 6	89.16	1	dead	12	Am pH 6 12x dead 48 h 1	1.600	2463	770	47	770 ± 47	12.9
Amitriptyline	pH 6		2	dead	9	Am pH 6 2x dead 96 h 2	16.100	4146	128.7	3.7	128.7 ± 3.7	2.2
Amitriptyline	pH 6		3	dead	2	Am pH 6 2x dead 96 h 3	1.300	1543	593	42	593 ± 42	10.0
Amitriptyline	pH 6		4	alive	4	Am pH 6 4x alive 96 h 4	1.400	1910	682	46	682 ± 46	11.4
Amitriptyline	pH 8	2.10	11	alive	20	Am pH 8 20x alive 96 h 11	5.900	961	81.5	2.8	81.5 ± 2.8	1053
Amitriptyline	pH 8		12	alive	20	Am pH 8 20x alive 96 h 12	5.600	1335	119.2	4.2	119.2 ± 4.2	1540

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Amitriptyline	pH 8		13	alive	20	Am pH 8 20x alive 96 h 13	5.100	1219	119.5	4.4	119.5 ± 4.4	1544
Amitriptyline	pH 8		14	alive	20	Am pH 8 20x alive 96 h 14	5.200	849	81.6	3.0	81.6 ± 3.0	1055
Amitriptyline	pH 8		15	alive	20	Am pH 8 20x alive 96 h 15	4.900	890	90.9	3.4	90.9 ± 3.4	1174
Amitriptyline	pH 8		16	alive	19	Am pH 8 19x alive 96 h 16	4.100	851	103.8	4.1	103.8 ± 4.1	1341
Amitriptyline	pH 9	1.60	21	alive	20	Am pH 9 20x alive 96 h 21	6.400	1577	123.2	4.2	123.2 ± 4.2	5448
Amitriptyline	pH 9		22	dead	21	Am pH 9 21x dead 96 h 22	6.400	1084	84.7	2.9	84.7 ± 2.9	3746
Amitriptyline	pH 9		23	alive	20	Am pH 9 20x alive 96 h 23	5.500	1306	118.7	4.2	118.7 ± 4.2	5250
Amitriptyline	pH 9		24	alive	20	Am pH 9 20x alive 96 h 24	5.600	1563	139.5	4.9	139.5 ± 4.9	6169
Amitriptyline	pH 9		25	alive	15	Am pH 9 15x alive 96 h 25	5.300	950	89.6	3.2	89.6 ± 3.2	3964

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Amitriptyline	pH 9		26	alive	15	Am pH 9 15x alive 96 h 26	4.900	1004	102.5	3.8	102.5 ± 3.8	4531
Bromoxynil	pH 5	0.15	1	alive	20	Brom. pH5 0,15 mg/L 1 *20	2.700	213	39.5	1.9	39.5 ± 1.9	340.5
Bromoxynil	pH 5		2	alive	20	Brom. pH5 0,15 mg/L 2 *20	3.000	211	35.2	1.6	35.2 ± 1.6	303.3
Bromoxynil	pH 5		3	alive	15	Brom. pH5 0,15 mg/L 3 *15	2.000	128	32.0	1.8	32.0 ± 1.8	275.8
Bromoxynil	pH 6	0.20	1	alive	20	Brom. pH6 0,2 mg/L 1 *20	4.600	127	13.76	0.55	13.76 ± 0.55	104.83
Bromoxynil	pH 6		2	alive	20	Brom. pH6 0,2 mg/L 2 *20	4.700	128	13.58	0.54	13.58 ± 0.54	103.47
Bromoxynil	pH 6		3	alive	13	Brom. pH6 0,2 mg/L 3 *13	3.300	73	11.06	0.50	11.06 ± 0.50	84.27
Bromoxynil	pH 8	15.0	1	alive	20	Brom. pH8 15 mg/L 1 *20	3.200	214	33.4	1.5	33.4 ± 1.5	2.3
Bromoxynil	pH 8		2	alive	20	Brom. pH8 15 mg/L 2 *20	3.400	237	34.9	1.6	34.9 ± 1.6	2.4

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Bromoxynil	pH 8		3	alive	18	Brom. pH8 15 mg/L 3 *18	2.900	138	23.8	1.1	23.8 ± 1.1	1.6
Bromoxynil	pH 8		4	alive	20	Brom. pH8 15 mg/L 4 *20	3.100	182	29.4	1.4	29.4 ± 1.4	2.0
Citalopram	pH 5	1180	1	dead	1	Cit pH 5 1180 mg/L 1 dead 96 hpf 1	2.200	137	31.1	1.9	31.1 ± 1.9	0.03
Citalopram	pH 5		2	alive	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 2	12.40	2539	102.4	4.0	102.4 ± 4.0	0.09
Citalopram	pH 5		3	alive	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 3	3.700	2520	341	17	341 ± 17	0.29
Citalopram	pH 5		4	alive	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 4	3.300	2988	453	23	453 ± 23	0.39
Citalopram	pH 5		5	alive	15	Cit pH 5 1180 mg/L 15 alive 96 hpf 5	3.400	3097	455	23	455 ± 23	0.39

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Citalopram	pH 6	380	1	alive	20	Cit. pH 6 380 mg/L 20* 1	4.200	6063	722	35	722 ± 35	1.9
Citalopram	pH 6		2	alive	20	Cit. pH 6 380 mg/L 20* 2	4.40	5437	618	29	618 ± 29	1.6
Citalopram	pH 6		3	alive	20	Cit. pH 6 380 mg/L 20* 3	4.800	7007	730	34	730 ± 34	1.9
Citalopram	pH 6		4	alive	15	Cit. pH 6 380 mg/L 15* 4	3.700	5060	684	34	684± 34	1.8
Citalopram	pH 8	24	1	alive	20	Cit. pH 8 20 mg/L 20* 1	4.300	17250	2006	95	2006 ± 95	87
Citalopram	pH 8		2	alive	20	Cit. pH 8 20 mg/L 20* 2	5.000	15627	1563	71	1563 ± 71	68
Citalopram	pH 8		3	alive	20	Cit. pH 8 20 mg/L 20* 3	5.000	15831	1583	72	1583 ± 72	68
Citalopram	pH 8		4	alive	15	Cit. pH 8 20 mg/L 15* 4	3.800	13252	1744	86	1744 ± 86	75
Citalopram	pH 9	5.0	2	alive	16	Cit 5mg/L pH9 *16 2	4.900	8437	861	39	861 ± 39	231

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Citalopram	pH 9		3	alive	15	Cit 5mg/L pH9 *15 3	5.000	9878	988	45	988 ± 45	265
Citalopram	pH 9		5	alive	15	Cit 5mg/L pH9 *15 5	5.500	9682	880	39	880 ± 39	236
Citalopram	pH 9		8	dead	10	Cit 5mg/L pH9 t10 8	3.700	4913	664	33	664 ± 33	178
Citalopram	pH 9		14	alive	17	Cit 5mg/L pH9 *17 14	5.600	10397	928	41	928 ± 41	249
Diclofenac	pH 5	0.05	1	alive	20	Diclo pH 5 0.05 mg/L 20* 1	5.700	<LOD	-	-	<LOD	-
Diclofenac	pH 5		2	alive	20	Diclo pH 5 0.05 mg/L 20* 2	5.600	<LOD	-	-	<LOD	-
Diclofenac	pH 5		3	alive	20	Diclo pH 5 0.05 mg/L 20* 3	5.700	<LOD	-	-	<LOD	-
Diclofenac	pH 6	0.4	6	alive	15	Diclo pH 6 0.4 mg/L 15* 6	4.100	15	1.823	0.088	1.823 ± 0.088	7.16
Diclofenac	pH 6		7	alive	15	Diclo pH 6 0.4 mg/L 15* 7	4.300	10	1.107	0.053	1.107 ± 0.053	4.35
Diclofenac	pH 6		8	alive	14	Diclo pH 6 0.4 mg/L 14* 8	3.900	<LOD	-	-	<LOD	-

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Diclofenac	pH 8	17	5	alive	15	Diclo pH 8 17 mg/L 15* 5	4.800	120	12.55	0.58	12.55 ± 0.58	0.97
Diclofenac	pH 8		6	alive	16	Diclo pH 8 17 mg/L 16* 6	4.700	77	8.18	0.38	8.18 ± 0.38	0.63
Diclofenac	pH 8		7	alive	15	Diclo pH 8 17 mg/L 15* 7	4.600	107	11.64	0.54	11.64 ± 0.54	0.90
Diclofenac	pH 9	70.0	1	alive	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 1	4.500	265	29.4	1.4	29.4 ± 1.4	0.89
Diclofenac	pH 9		2	alive	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 2	4.700	255	27.1	1.3	27.1 ± 1.3	0.82
Diclofenac	pH 9		3	alive	15	Diclo pH 9 70 mg/L 15 alive 96 hpf 3	4.200	252	30.0	1.4	30.0 ± 1.4	0.90
Diclofenac	pH 9		4	alive	16	Diclo pH 9 70 mg/L 16 alive 96 hpf 4	4.500	224	24.8	1.2	24.8 ± 1.2	0.75
Diclofenac	pH 9		5	dead	16	Diclo pH 9 70 mg/L 16	2.600	222	42.7	2.4	42.7 ± 2.4	1.28

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
						dead 96 hpf 5						
Enclomiphene	pH 8	3.1	1	dead	4	Enclo pH 8 D4	0.9686	2174	1122	103	1122 ±103	362
Enclomiphene	pH 8		2	alive	15	Enclo pH 8 A15	3.6321	33218	4573	216	4573 ± 216	1475
Enclomiphene	pH 8		3	alive	20	Enclo pH 8 A20	4.8428	32943	3401	147	3401 ± 147	1097
Enclomiphene	pH 8		4	alive	20	Enclo pH 8 A20	4.8428	35723	3688	159	3688 ± 159	1190
Enclomiphene	pH 8		5	alive	20	Enclo pH 8 A20	4.8428	36283	3746	162	3746 ± 162	1208
Enclomiphene	pH 8		6	alive	20	Enclo pH 8 A20	4.8428	32668	3373	146	3373 ± 146	1088
Enclomiphene	pH 8		7	alive	15	Enclo pH 8 A15	3.6321	27896	3840	181	3840 ± 181	1239
Enclomiphene	pH 9	3.1	1	dead	19	Enclo pH 9 D19	4.6007	5552	603	26	603 ± 26	194.6
Enclomiphene	pH 9		2	dead	1	Enclo pH 9 D1	0.2421	653	1348	369	1348 ± 369	435.0
Fluoxetine	pH 5	220.00	1	dead	1	pH5 Fluo 1	1.700	341	100.3	6.6	100.3 ± 6.6	0.5
Fluoxetine	pH 5		3	dead	2	pH5 Fluo 3	1.500	430	143	10	143 ± 10	1

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Fluoxetine	pH 5		4	alive	11	pH5 Fluo * 4	2.200	5352	1216	71	1216 ± 71	6
Fluoxetine	pH 5		5	alive	13	pH5 Fluo * 5	3.000	6631	1105	56	1105 ± 56	5
Fluoxetine	pH 6	55.00	6	dead	5	pH6 Fluo 6	2.600	1618	311	17	311 ± 17	6
Fluoxetine	pH 6		7	alive	15	pH6 Fluo * 7	4.000	5373	672	31	672 ± 31	12
Fluoxetine	pH 6		8	alive	18	pH6 Fluo * 8	2.600	5795	1114	60	1114 ± 60	20
Fluoxetine	pH 8	2.00	15	alive	16	pH8 Fluo * 15	4.100	10928	1333	61	1333 ± 61	666
Fluoxetine	pH 8		16	alive	18	pH8 Fluo * 16	4.600	12053	1310	58	1310 ± 58	655
Fluoxetine	pH 8		17	alive	20	pH8 Fluo * 17	5.000	8200	820	35	820 ± 35	410
Fluoxetine	pH 8		18	alive	20	pH8 Fluo * 18	4.700	12471	1327	58	1327 ± 58	663
Fluoxetine	pH 8		19	alive	17	pH8 Fluo * 19	6.800	10230	752	30	752 ± 30	376
Fluoxetine	pH 8		20	alive	14	pH8 Fluo * 20	5.300	11092	1046	44	1046 ± 44	523
Fluoxetine	pH 9	0.50	9	dead	3	pH9 Fluo 9	1.000	409	205	18	205 ± 18	409
Fluoxetine	pH 9		11	dead	7	pH9 Fluo 11	1.300	1394	536	41	536 ± 41	1072

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Fluoxetine	pH 9		12	alive	16	pH9 Fluo * 12	3.400	3919	576	28	576 ± 28	1153
Fluoxetine	pH 9		13	alive	17	pH9 Fluo * 13	4.300	4550	529	24	529 ± 24	1058
Fluoxetine	pH 9		14	alive	13	pH9 Fluo * 14	3.000	3281	547	28	547 ± 28	1094
Ibuprofen	pH 5	8.70	1	dead	17	Ibu pH 5 D17	4.1164	445	54.1	3.1	54.1 ± 3.1	127.54
Ibuprofen	pH 5		2	dead	20	Ibu pH 5 D20	4.8428	601	62.1	3.4	62.1 ± 3.4	146.42
Ibuprofen	pH 5		3	dead	19	Ibu pH 5 D19	4.6007	469	51.0	2.8	51.0 ± 2.8	120.31
Ibuprofen	pH 5		1	alive	15	Ibu pH 5 A15	3.6321	404	55.6	3.2	55.6 ± 3.2	131.01
Ibuprofen	pH 5		2	alive	15	Ibu pH 5 A15	3.6321	601	82.7	4.8	82.7 ± 4.8	195.03
Ibuprofen	pH 5		3	alive	18	Ibu pH 5 A18	4.3585	528	60.5	3.4	60.5 ± 3.4	142.72
Ibuprofen	pH 6	45.20	1	dead	20	Ibu pH 6 D20	4.8428	2236	231	13	231 ± 13	0.04
Ibuprofen	pH 6		2	dead	13	Ibu pH 6 D13	3.1478	1682	267	16	267 ± 16	0.05
Ibuprofen	pH 6		1	alive	15	Ibu pH 6 A15	3.6321	1633	225	13	225 ± 13	0.04

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Ibuprofen	pH 6		2	alive	14	Ibu pH 6 A14	3.3900	2018	298	18	298 ± 18	0.06
Ibuprofen	pH 6		3	alive	14	Ibu pH 6 A14	3.3900	680	100.3	6.0	100.3 ± 6.0	0.02
Ibuprofen	pH 8	150	3	dead	7	Ibu pH 8 #7 tot 3	1.6950	200	58.9	4.5	58.9 ± 4.5	0.49
Ibuprofen	pH 8		4	dead	10	Ibu pH 8 #10 tot 4	16.9000	469	13.88	0.63	13.88 ± 0.63	0.12
Ibuprofen	pH 8		6	alive	20	Ibu pH 8 #20 leb.6	6.4000	1188	92.8	4.8	92.8 ± 4.8	0.78
Ibuprofen	pH 8		7	alive	20	Ibu pH 8 #20 leb.7	7.8000	1838	117.8	5.9	117.8 ± 5.9	0.98
Ibuprofen	pH 8		8	alive	15	Ibu pH 8 #15 leb.8	3.7000	452	61.1	3.6	61.1 ± 3.6	0.51
Ibuprofen	pH 8		9	alive	14	Ibu pH 8 #14 leb.9	2.9000	457	78.8	4.9	78.8 ± 4.9	0.66
Ibuprofen	pH 9	300	1	alive	16	Ibu pH 9 300 mg/L 16 alive 96 hpf 1	3.5000	126	17.97	0.79	14.29 ± 0.79	0.12
Ibuprofen	pH 9		2	alive	16	Ibu pH 9 300 mg/L 16 alive 96 hpf 2	3.5000	295	42.1	2.5	42.1 ± 2.5	0.28

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Ibuprofen	pH 9		3	alive	20	Ibu pH 9 300 mg/L 20 alive 96 hpf 3	4.0000	350	43.7	2.5	43.7 ± 2.5	0.29
Ibuprofen	pH 9		4	dead	9	Ibu pH 9 300 mg/L 9 dead 96 hpf 4	2.8000	209	37.3	2.4	37.3 ± 2.4	0.24
Ketotifen	pH 9	1.50	9	alive	20	Ket 1,5 mg/L pH9 *20 96hpf 9	4.1	2483	303	12	303 ± 12	467
Ketotifen	pH 9		15	alive	12	Ket 1,5 mg/L pH9 *12 96hpf 15	2.5	1207	241	11	241 ± 11	372
Ketotifen	pH 9		17	alive	15	Ket 1,5 mg/L pH9 *15 96hpf 17	3.8	2351	309	12	309 ± 12	477
Ketotifen	pH 9		19	alive	15	Ket 1,5 mg/L pH9 96hpf *15 19	2.9	2439	421	19	421 ± 19	649
Ketotifen	pH 9		23	dead	7	Ketotifen 1,5 mg/L t7 pH9 96hpf 23	6.6	655	49.6	1.6	49.6 ± 1.6	77

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Ketotifen	pH 9		24	alive	15	Ket 1,5 mg/L pH9 96hpf *15 24	2.9	2306	398	18	398 ± 18	614
Lidocaine	pH 5	2000	2	alive	20	Lido pH 5 2000 mg/L 96 h 15 alive 2	4.500	161	17.92	0.79	17.92 ± 0.79	0.03
Lidocaine	pH 5		3	alive	20	LidopH 5 2000 mg/L 96 h 15 alive 3	5.800	221	19.07	0.79	19.07 ± 0.79	0.03
Lidocaine	pH 5		4	alive	20	Lido pH 5 2000 mg/L 96 h 15 alive 4	4.800	247	25.7	1.1	25.7 ± 1.1	0.05
Lidocaine	pH 5		5	alive	20	Lido pH 5 2000 mg/L 96 h 15 alive 5	5.600	143	12.78	0.53	12.78 ± 0.53	0.02
Lidocaine	pH 5		6	alive	17	Lido pH 5 2000 mg/L 96 h 15 alive 6	4.800	92	9.62	0.28	7.00 ± 0.28	0.02
Lidocaine	pH 8	50	7	alive	15	Lid pH 8 50 mg/L 96 h 15 alive 7	3.000	360	60.1	3.1	60.1 ± 3.1	3.3

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Lidocaine	pH 8		8	alive	15	Lid pH 8 50 mg/L 96 h 15 alive 8	11.700	275	11.77	0.43	11.77 ± 0.43	0.65
Lidocaine	pH 8		9	alive	16	Lid pH 8 50 mg/L 96 h 15 alive 9	3.600	326	45.3	2.2	45.3 ± 2.2	2.5
Lidocaine	pH 8		10	dead	15	Lid pH 8 50 mg/L 96 h 15 dead 10	3.300	333	50.5	2.5	50.5 ± 2.5	2.8
Lidocaine	pH 9	10	1	alive	15	pH 9 10 mg/L 96 h 15 alive 1	4.900	323	32.9	1.4	32.9 ± 1.4	7.7
Lidocaine	pH 9		2	alive	15	pH 9 10 mg/L 96 h 15 alive 2	4.700	339	36.0	1.6	36.0 ± 1.6	8.4
Lidocaine	pH 9		3	alive	15	pH 9 10 mg/L 96 h 15 alive 3	4.900	265	27.1	1.2	27.1 ± 1.2	6.3
Lidocaine	pH 9		4	dead	15	pH 9 10 mg/L 96 h 15 dead 4	4.100	206	25.1	1.1	25.1 ± 1.1	5.9
Metoprolol	pH 5	6600.0	9	alive	20	pH 5 Metoprolol * 96 hpf no. 20	5.800	4750	409	18	409 ± 18	0.045
Metoprolol	pH 5		10	alive	20	pH 5 Metoprolol	4.300	3860	449	21	449 ± 21	0.049

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
						* 96 hpf no. 20						
Metoprolol	pH 5		11	alive	20	pH 5 Metoprolol * 96 hpf no. 20	5.400	4557	422	19	422 ± 19	0.046
Metoprolol	pH 5		12	alive	20	pH 5 Metoprolol * 96 hpf no. 20	4.700	4085	435	20	435 ± 20	0.048
Metoprolol	pH 5		13	alive	3	pH 5 Metoprolol * 96 hpf no. 3	1.500	648	216	16	216 ± 16	0.024
Metoprolol	pH 6	3180.0	1	dead	1	pH 6 Metoprolol † 72 hpf no.1	1.700	528	155	11	155 ± 11	0.052
Metoprolol	pH 6		7	dead	20	pH 6 Metoprolol † 96 hpf no.20	6.300	20502	1627	70	1627 ± 70	0.542
Metoprolol	pH 6		8	dead	2	pH 6 Metoprolol † 96 hpf no.20	1.400	2016	720	55	720 ± 55	0.240

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Metoprolol	pH 6		14	alive	20	pH 6 Metoprolol * 96 hpf no. 20	6.000	18329	1527	67	1527 ± 67	0.509
Metoprolol	pH 6		15	alive	20	pH 6 Metoprolol * 96 hpf no. 20	5.700	15891	1394	62	1394 ± 62	0.464
Metoprolol	pH 6		16	alive	17	pH 6 Metoprolol * 96 hpf no. 17	5.500	12064	1097	49	1097 ± 49	0.365
Metoprolol	pH 8	70.0	2	dead	6	pH 8 Metoprolol † 72 hpf no.6	2.200	1844	419	25	419 ± 25	5.5
Metoprolol	pH 8		6	dead	14	pH 8 Metoprolol † 96 hpf no.14	56.700	7459	65.8	2.3	65.8 ± 2.3	0.9
Metoprolol	pH 8		17	alive	20	pH 8 Metoprolol * 96 hpf no. 20	5.300	10812	1020	46	1020 ± 46	13.5
Metoprolol	pH 8		18	alive	8	pH 8 Metoprolol * 96 hpf no. 8	2.600	6332	1218	69	1218 ± 69	16.1

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Metoprolol	pH 9	10.0	4	alive	15	Met 10mg/L *15 pH9 4	4.800	7180	748	34	748 ± 34	68.0
Metoprolol	pH 9		11	alive	15	Met 10mg/L pH9 *15 11	4.800	5737	598	28	598 ± 28	54
Metoprolol	pH 9		13	dead	18	Met 10mg/L 18t pH9 13	3.500	6258	894	45	894 ± 45	81
Metoprolol	pH 9		16	dead	1	Met 10mg/L 16t pH9 16	2.800	108	19.4	1.1	19.4 ± 1.1	1.8
Metoprolol	pH 9		18	alive	7	Met 10mg/L pH9 *7 18	2.300	2364	514	31	514 ± 31	47
Naproxen	pH 6	8.0	1	dead	3	pH 6 Naproxen † 72 hpf no. 3	1.600	324	101.4	7.5	101.4 ± 7.5	18.6
Naproxen	pH 6		5	dead	6	pH 6 Naproxen † 96 hpf no. 6	3.000	905	150.8	8.5	150.8 ± 8.5	27.7
Naproxen	pH 6		7	alive	20	pH 6 Naproxen * 96 hpf no. 20	6.600	4373	331	15	331 ± 15	60.9

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Naproxen	pH 6		8	alive	20	pH 6 Naproxen * 96 hpf no. 20	13.400	3375	125.9	5.2	125.9 ± 5.2	23.2
Naproxen	pH 6		9	alive	16	pH 6 Naproxen * 96 hpf no. 16	6.900	4027	291.8	13	292 ± 13	53.6
Naproxen	pH 8	325	2	dead	1	pH 8 Naproxen † 72 hpf no. 1	0.900	286	159	16	159 ± 16	2.1
Naproxen	pH 8		10	alive	20	pH 8 Naproxen * 96 hpf no. 20	5.200	1806	173.7	8.4	173.7 ± 8.4	2.3
Naproxen	pH 8		11	alive	20	pH 8 Naproxen * 96 hpf no. 20	5.500	1358	123.4	5.9	123.4 ± 5.9	1.6
Naproxen	pH 8		12	alive	20	pH 8 Naproxen * 96 hpf no. 20	5.100	1438	141.0	6.8	141.0 ± 6.8	1.9
Naproxen	pH 8		13	alive	20	pH 8 Naproxen * 96 hpf no. 20	5.300	1428	134.7	6.5	134.7 ± 6.5	1.8

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Naproxen	pH 8		14	alive	15	pH 8 Naproxen * 96 hpf no. 15	3.800	1096	144.2	7.6	144.2 ± 7.6	1.9
Naproxen	pH 9	900.0	1	dead	3	pH 9 Naproxen † 96 hpf no.3	1.600	315	98.4	7.3	98.4 ± 7.3	0.6
Naproxen	pH 9		2	alive	20	pH 9 Naproxen * 96 hpf no.20	4.600	824	89.5	4.5	89.5± 4.5	0.6
Naproxen	pH 9		3	alive	20	pH 9 Naproxen * 96 hpf no.20	5.300	1052	99.2	4.8	99.2 ± 4.8	0.6
Naproxen	pH 9		4	alive	20	pH 9 Naproxen * 96 hpf no.20	4.600	885	96.2	4.8	96.2 ± 4.8	0.6
Naproxen	pH 9		5	alive	20	pH 9 Naproxen * 96 hpf no.20	5.300	889	83.9	4.0	83.9 ± 4.0	0.5
Naproxen	pH 9		6	alive	20	pH 9 Naproxen * 96 hpf no.20	5.600	859	76.7	3.6	76.7 ± 3.6	0.5

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Naproxen	pH 9		7	alive	13	pH 9 Naproxen * 96 hpf no.13	3.300	533	80.7	4.4	80.7 ± 4.4	0.5
Pentachlorophenol	pH 5	0.05	5	alive	20	PCP pH 5 0.05 mg/L 20* 5	4.500	96	10.68	0.50	10.68 ± 0.50	780
Pentachlorophenol	pH 5		6	alive	20	PCP pH 5 0.05 mg/L 20* 6	8.700	59	3.42	0.14	3.42 ± 0.14	249
Pentachlorophenol	pH 5		7	alive	20	PCP pH 5 0.05 mg/L 20* 7	4.700	92	9.81	0.45	9.81 ± 0.45	716
Pentachlorophenol	pH 5		8	alive	15	PCP pH 5 0.05 mg/L 15* 8	3.400	58	8.53	0.44	8.53 ± 0.44	623
Pentachlorophenol	pH 6	0.052	1	alive	20	PCP pH 6 0.052 mg/L 20* 1	4.700	57	6.08	0.28	6.08 ± 0.28	217
Pentachlorophenol	pH 6		2	alive	20	PCP pH 6 0.052 mg/L 20* 2	4.800	70	7.29	0.34	7.29 ± 0.34	261
Pentachlorophenol	pH 6		3	alive	20	PCP pH 6 0.052 mg/L 20* 3	4.800	100	10.43	0.48	10.43 ± 0.48	373

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Pentachlorophenol	pH 8	0.26	1	dead	6	PCP pH 8 0.26 mg/L 6† 72 hpf 1	2.000	89	22.3	1.4	22.3 ± 1.4	169
Pentachlorophenol	pH 8		2	alive	15	PCP pH 8 0.26 mg/L 15* 96 hpf 2	6.700	278	20.73	0.88	20.73 ± 0.88	157
Pentachlorophenol	pH 8		3	alive	20	PCP pH 8 0.26 mg/L 20* 96 hpf 3	4.000	261	32.6	1.6	32.6 ± 1.6	247
Pentachlorophenol	pH 8		4	alive	20	PCP pH 8 0.26 mg/L 20* 96 hpf 4	4.200	344	41.0	2.0	41.0 ± 2.0	310
Pentachlorophenol	pH 8		5	alive	20	PCP pH 8 0.26 mg/L 20* 96 hpf 5	4.100	310	37.8	1.8	37.8 ± 1.8	286
Pentachlorophenol	pH 8		6	alive	20	PCP pH 8 0.26 mg/L 20* 96 hpf 6	4.100	330	40.2	1.9	40.2 ± 1.9	304
Propranolol	pH 5	1600	1	alive	15	Prop pH 5 1600 mg/L 15 alive 96 hpf 1	4.100	5525	674	32	674 ± 32	0.76

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Propranolol	pH 5		2	alive	15	Prop pH 5 1600 mg/L 15 alive 96 hpf 2	3.100	6193	999	53	999 ± 53	1.13
Propranolol	pH 5		3	alive	13	Prop pH 5 1600 mg/L 13 alive 96 hpf 3	3.400	5720	841	43	841 ± 43	0.95
Propranolol	pH 5		4	dead	14	Prop pH 5 1600 mg/L 14 dead 96 hpf 4	3.300	4145	628	32	628 ± 32	0.71
Propranolol	pH 6	440 9	1	alive	9	Prop pH 6 440 mg/L 9* 1	2.300	5946	1293	77	1293 ± 77	2.87
Propranolol	pH 6		2	alive	9	Prop pH 6 440 mg/L 9* 2	4.300	6410	745	35	745 ± 35	1.66
Propranolol	pH 6		3	alive	8	Prop pH 6 440 mg/L 8* 3	2.100	5152	1227	76	1227 ± 76	2.73
Propranolol	pH 8		1	alive	20	Prop pH 8 9 mg/L 20* 1	4.300	10672	1241	59	1241 ± 59	217
Propranolol	pH 8		2	alive	20	Prop pH 8 9 mg/L 20* 2	4.100	11452	1397	67	1397 ± 67	244

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Propranolol	pH 8		3	alive	20	Prop pH 8 9 mg/L 20* 3	4.800	13250	1380	64	1380 ± 64	241
Propranolol	pH 9	0.6	4	alive	16	Prop pH 9 0.6 mg/L 16* 4	3.400	1925	283	14	283 ± 14	866
Propranolol	pH 9		5	alive	16	Prop pH 9 0.6 mg/L 16* 5	3.900	2022	259	13	259 ± 13	793
Propranolol	pH 9		6	alive	15	Prop pH 9 0.6 mg/L 15* 6	3.600	2054	285	14	285 ± 14	872
Sertraline	pH 6	10.5	1	dead	1	1.PHION pH 6 Sertralin 72h #1	0.400	79	99	18	99 ± 18	31.7
Sertraline	pH 6		5	alive	10	5.PHION pH 6 Sertralin 96h lebend #10	2.900	4237	731	37	731 ± 37	233.9
Sertraline	pH 6		6	alive	16	6.PHION pH 6 lebend Sertralin #16	2.000	3469	867	52	867 ± 52	277.7
Sertraline	pH 6		7	alive	8	7.PHION pH 6 Sertralin lebend #8 96h	1.700	2769	814	53	814 ± 53	260.8

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Sertraline	pH 6		8	alive	11	8.PHION pH 6 leb.Sertralin 96h #11	2.200	3330	757	43	757 ± 43	242.4
Sertraline	pH 8	1.10	15	alive	12	15.PHION pH 8 Sertralin 96h #12 lebend	2.700	4154	769	40	769 ± 40	4101.5
Sertraline	pH 8		16	alive	12	16.PHION pH 8 Sertralin 96h #12 lebend	2.100	5166	1230	71	1230 ± 71	6557.8
Sertraline	pH 8		17	alive	12	17.PHION pH 8 Sertralin 96h #12 lebend	2.900	4591	792	40	792 ± 40	4220.2
Sertraline	pH 8		18	alive	12	18.PHION pH 8 Sertralin 96h #12 lebend	2.700	5036	933	48	933 ± 48	4972.2
Sertraline	pH 8		19	alive	12	19.PHION pH 8 Sertralin 96h #12 lebend	2.900	4663	804	40	804 ± 40	4286.4

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Sertraline	pH 8		20	alive	12	20.PHION pH 8 Sertralin 96h #12 lebend	2.600	4355	838	44	838 ± 44	4465.2
Sertraline	pH 8		21	alive	15	21.PHION pH 8 Sertralin 96h #15 lebend	2.900	4747	819	41	819 ± 41	4364.0
Sertraline	pH 9	0.1	9	dead	8	9. PHION pH 9 tot Sertralin #8 96h	2.000	2213	553	33	553 ± 33	5576.0
Sertraline	pH 9		10	alive	11	10.PHION pH 9 Sertralin lebend 96h #11	3.000	5299	883	44	883 ± 44	8902.3
Sertraline	pH 9		11	alive	13	11.PHION pH 9 lebend Sertralin 96h #13	3.500	5191	742	35	742 ± 35	7475.2
Sertraline	pH 9		12	alive	13	12.PHION pH 9 lebend Sertralin 96h #13	3.700	5900	797	37	797 ± 37	8037.0

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Sertraline	pH 9		13	alive	13	13.PHION pH 9 Sertralin lebend 96h #13	3.300	5042	764	37	764 ± 37	7700.6
Sertraline	pH 9		14	alive	9	14.PHION pH 9 Sertralin lebend 96h #9	2.600	4063	781	41	781 ± 41	7877.0
Tetracaine	pH 5	170.13	1	dead	1	Nr. 1 pH 5 1.0051g+Tc 1Embryo	0.2421	<LOD	-	-	< LOD	-
Tetracaine	pH 5		5	dead	14	Nr. 5 pH 5 +14 Embryos Tc	3.3900	72	10.69	0.55	10.69 ± 0.55	0.07
Tetracaine	pH 5		10	alive	20	Nr. 10 pH 5 Alive Tc 20 Embryos	4.8428	81	8.37	0.39	8.37 ± 0.39	0.05
Tetracaine	pH 5		13	alive	21	Nr. 13 pH 5 Alive 21 Embryos Tc	5.0849	119	11.73	0.53	11.73 ± 0.53	0.08
Tetracaine	pH 6	59.2	2	dead	8	Nr. 2 1.005g+Tc 8 Embryos	1.9371	25	6.41	0.41	6.41 ± 0.41	0.12

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Tetracaine	pH 6		11	alive	3	Nr. 11 pH 6 <3 3 Embryos Tc	0.7264	45	30.7	3.5	30.7 ± 3.5	0.58
Tetracaine	pH 6		17	alive	20	Nr. 17 pH 6 Alive Tc 1.0044 g 20 Embryos	4.8428	142	14.67	0.68	14.67 ± 0.68	0.28
Tetracaine	pH 6		20	dead	15	Nr. 20 pH6 + 15 Embryos Tc	3.6321	81	11.19	0.56	11.19 ± 0.56	0.21
Tetracaine	pH 8	3.9	3	dead	5	Nr. 3 pH 8 1.0075 g + Tc 5 Embryos	1.2107	9	3.83	0.32	3.83 ± 0.32	1.86
Tetracaine	pH 8		6	dead	10	Nr. 6 pH 8 + 10 Embryos Tc	2.4214	49	10.09	0.59	10.09 ± 0.59	4.91
Tetracaine	pH 8		12	alive	20	Nr. 12 pH 8 <3 20 Embryos 1.071 g Tc	4.8428	238	24.6	1.1	24.6 ± 1.1	12.0
Tetracaine	pH 8		15	alive	20	Nr. 15 pH 8 <3 1.0035 20 Embryos Tc	4.8428	116	11.97	0.55	11.97 ± 0.55	5.82
Tetracaine	pH 8		18	alive	21	Nr. 18 pH 8 <3 21	5.0849	222	21.82	0.99	21.82 ± 0.99	10.62

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
						Embryos 1.0035 g Tc						
Tetracaine	pH 9	3.00	27	dead	2	Tetra 3 mg/L pH9 72hpf t2 27	1.8000	<LOD	-	-	< LOD	-
Tetracaine	pH 9		32	alive	20	Tetra 3 mg/L pH9 96hpf *20 32	5.9000	15	1.240	0.055	1.240 ± 0.055	1.09
Tetracaine	pH 9		33	alive	20	Tetra 3 mg/L pH9 96hpf *20 33	5.1000	30	2.92	0.13	2.92 ± 0.13	2.56
Tetracaine	pH 9		41	alive	20	Tetra 3 mg/L pH9 96hpf *20 41	4.9000	39	3.98	0.18	3.98 ± 0.18	3.50
Tetracaine	pH 9		43	dead	1	Tetra 3 mg/L pH9 48hpf t1 43	1.8000	<LOD	-	-	< LOD	-
Tetracaine	pH 9		45	alive	15	Tetra 3 mg/L pH9 96hpf *15 45	6.1000	22	1.785	0.078	1.785 ± 0.078	1.57

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Tetracaine	pH 9		46	alive	20	Tetra 3 mg/L pH9 96hpf *20 46	9.2000	26	1.400	0.057	1.400 ± 0.057	1.23
Tetracaine	pH 9		48	alive	8	Tetra 3 mg/L pH9 96hpf *8 48	3.4000	13	1.861	0.095	1.861 ± 0.095	1.63
Tramadol	pH 6	2750	1	alive	15	Tram pH 6 2750 mg/L 96 h 15 alive 1	3.900	6160	790	37	790 ± 37	0.5
Tramadol	pH 6		2	alive	15	Tram pH 6 2750 mg/L 96 h 15 alive 2	4.400	5070	576	26	576 ± 26	0.4
Tramadol	pH 6		3	alive	15	Tram pH 6 2750 mg/L 96 h 15 alive 3	5.100	6311	619	27	619 ± 27	0.4
Tramadol	pH 8	40	4	alive	15	Tram pH 8 40 mg/L 96 h 15 alive 4	4.100	4502	549	25	549 ± 25	31.6
Tramadol	pH 8		5	alive	15	Tram pH 8 40 mg/L 96 h 15 alive 5	4.300	4699	546	25	546 ± 25	31.5

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Tramadol	pH 8		6	alive	15	Tram pH 8 40 mg/L 96 h 15 alive 6	4.600	4361	474	21	474 ± 21	27.3
Tramadol	pH 9	15	6	dead	2	Tram 15 mg/L pH9 t2 6	9.600	552	28.7	1.1	28.7 ± 1.1	3.2
Tramadol	pH 9		12	alive	15	Tram 15 mg/L pH9 *15 12	5.100	6286	616	27	616 ± 27	68.7
Tramadol	pH 9		20	dead	6	Tram 15 mg/L 20t pH9 20	2.500	1150	230	13	230 ± 13	25.6
Tramadol	pH 9		21	alive	15	Tram 15 mg/L pH9 *15 21	4.700	4408	469	21	469 ± 21	52.3
Triclosan	pH 5	0.200	1	alive	20	Triclo pH 5 0.2 mg/L 20* 1	6.000	20.9	1.740	0.073	1.740 ± 0.073	81
Triclosan	pH 5		2	alive	20	Triclo pH 5 0.2 mg/L 20* 2	5.800	17.1	1.476	0.062	1.476 ± 0.062	68
Triclosan	pH 5		3	alive	20	Triclo pH 5 0.2 mg/L 20* 3	6.000	24.2	2.016	0.084	2.016 ± 0.084	93
Triclosan	pH 6	0.300	5	alive	20	Triclo pH 6 0.3 mg/L 20* 5	5.900	35.8	3.04	0.13	3.04 ± 0.13	82

Substance	pH	$C_{nominal}$ [mg/L]	Eppi No.	Status	Embryos No.	Sample Code	Weight of sample (mg)	$C_{measured}$ [mg/L]	$C_{internal}$ [mg kg^{-1}]	± SD	$C_{internal}$ [mg kg^{-1}] ± SD	BCF [L kg^{-1}]
Triclosan	pH 6		6	alive	20	Triclo pH 6 0.3 mg/L 20* 6	5.900	51.0	4.32	0.18	4.32 ± 0.18	117
Triclosan	pH 6		7	alive	20	Triclo pH 6 0.3 mg/L 20* 7	14.900	53.5	1.795	0.065	1.795 ± 0.065	49
Triclosan	pH 8	0.350	9	alive	20	Triclo pH 8 0.35 mg/L 20* 9	5.900	58.6	4.97	0.21	4.97± 0.21	70
Triclosan	pH 8		10	alive	20	Triclo pH 8 0.35 mg/L 20* 10	5.400	74.3	6.88	0.30	6.88 ± 0.30	97
Triclosan	pH 8		11	alive	20	Triclo pH 8 0.35 mg/L 20* 11	5.900	84.2	7.14	0.30	7.14 ± 0.30	101
Triclosan	pH 9	0.600	13	alive	15	Triclo pH 8 0.6 mg/L 15* 13	4.100	79.5	9.70	0.45	9.70 ± 0.45	68
Triclosan	pH 9		14	alive	15	Triclo pH 8 0.6 mg/L 15* 14	3.900	106.1	13.60	0.64	13.60 ± 0.64	95
Triclosan	pH 9		15	alive	16	Triclo pH 8 0.6 mg/L 16* 15	3.800	92.9	12.23	0.58	12.23 ± 0.58	86

Source: Own depiction

A.1 Supplementary text A1: Chemicals and Reagents

All the analytical standards used were of high-purity grade. Many of them were purchased from Sigma-Aldrich (Steinheim, Germany). The standards Sertraline HCl, Fluoxetine, Ketotifen, Metoprolol, Naproxen and Tramadol were purchased from Analytical Standard Solutions (A2S) (France). Moreover, the standards Acetylsalicylic acid, Lidocaine and Pentachlorophenol were purchased from HPC Standards GmbH (Borsdorf, Germany). The isotope-labelled internal standards (IS), Triclosan-D3, Citalopram-D4, Propranolol-D7, Sertraline-D3, Tetracaine-D6, Ibuprofen-D3, Lidocaine-D10, Metoprolol-D7 and Fluoxetine-D5 were purchased from Analytical Standard Solutions (A2S) (France). Furthermore, the internal standards Diclofenac-D4, Naproxen-D3 and Acetylsalicylic acid-D4 were purchased from HPC Standards GmbH (Borsdorf, Germany). The IS, Amitriptyline-D3 was purchased from Cerilliant (Round Rock, TX) and Tramadol-D6 was purchased from Toronto Research Chemicals (TRC)(Canada). Stock standard solutions of individual compounds (1000 µg mL^{-1}) were prepared in MeOH and stored at −20 °C in amber glass bottles to prevent photo-degradation.

All the solvents were UPLC-MS grade. Methanol (MeOH) was purchased from Merck (Darmstadt, Germany) and distilled water (H_2O) was provided by a Milli-Q purification apparatus (Direct-Q UV; Millipore, Bedford, MA, USA).

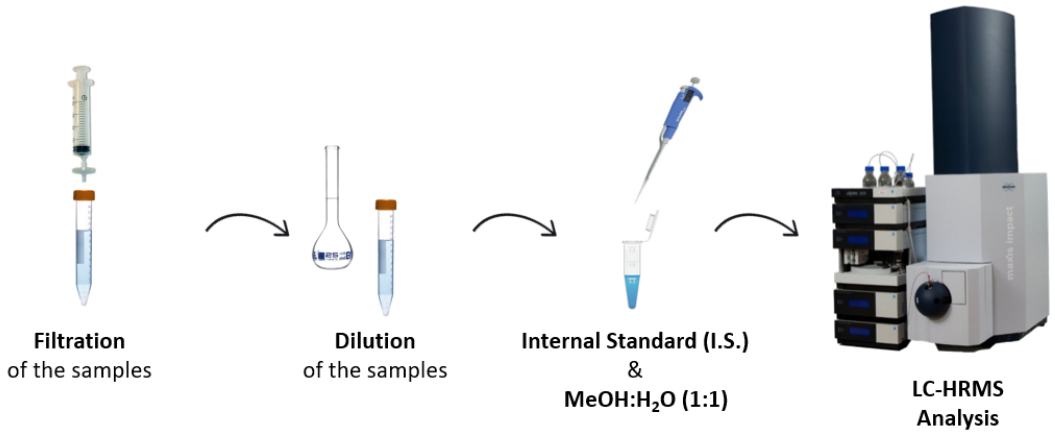
The additives of the mobile phases, ammonium formate (≥ 99.0%), ammonium acetate (99%), and formic acid (99%) were all purchased from Fluka (Buchs, Switzerland).

Regenerated cellulose syringe filters used during the sample preparation (RC, pore size 0.2 µm, diameter 15mm) were purchased from Phenomenex (Torrance, CA, USA).

A.2 Supplementary text A2: Sample preparation

The exposure media samples were filtered through 0.2 µm RC syringe filters and diluted based on the preliminary test results. Afterwards, equal amounts of MeOH and the diluted samples were transferred into glass vials to reach a final constitution of 1:1 v/v MeOH:H_2O. Finally, the samples were spiked with stable isotope-labeled internal standards (IS) and stored at -80 ˚C until the LC-HRMS analysis. Then stable isotope-labelled internal standards were added. The IS were used to account for potential insufficiencies of the whole analytical procedure (sample preparation & instrumental analysis). The Figure below illustrates the steps of the applied sample preparation procedure for the water samples (Figure to text A2).

Figure to text A2: Sample preparation of the water samples from exposure experiments



Source: Own depiction

A.3 Supplementary text A3: LC-HRMS analysis

The analysis of the exposure media samples was carried out using a UHPLC-QTOF-MS system, equipped with a UHPLC apparatus (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific, Dreieich, Germany), consisting of a solvent rack degasser, auto-sampler, a binary pump with solvent selection valve and a column oven coupled to the QTOF-MS mass analyzer (Maxis Impact, Bruker Daltonics, Bremen, Germany).

The QTOF-MS system was equipped with an electrospray ionization (ESI) source, operating in positive and negative ionization mode.

The samples were were analyzed with reversed phase liquid chromatography (RPLC).

In RPLC mode, an Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 µm) from Thermo Fisher Scientific (Dreieich, Germany), preceded by an ACQUITY UPLC BEH C18 1.7 µm, VanGuard Pre-Column from Waters (Dublin, Ireland), and thermostated at 30 °C, was used.

For positive ionization mode, the aqueous phase consisted of H_2O:MeOH = 90:10 (solvent A) and the organic phase was MeOH (solvent B) both amended with 5 mM ammonium formate and 0.01% formic acid.

For negative ionization mode, the aqueous phase consisted of H_2O:MeOH = 90:10 (solvent A) with 5 mM ammonium acetate and the organic phase was MeOH (solvent B) with 5 mM ammonium acetate.

The adopted elution gradient for both ionization modes in RP mode started with 1% of organic phase (flow rate 0.2 mL min^{-1}) for one minute, increasing to 39 % by 2 min (flow rate 0.2 mL min^{-1}), and then to 99.9 % (flow rate 0.4 mL min^{-1}) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.48 mL min^{-1}) and then initial conditions were restored within 0.1 min, kept for 3 min and then the flow rate decreased to 0.2 mL min^{-1} for the last minute. The injection volume was set to 5 µL.

The operation parameters of ESI were the following: capillary voltage, 2500 V for positive and 3500 V for negative mode; end plate offset, 500 V; nebulizer pressure, 2 bar (N2); drying gas, 8 L min−1 (N2); and drying temperature, 200 °C.

In HILIC chromatographic separation, the corresponding MS parameters were: capillary voltage, 3500 V for positive and 2500 V for negative mode; end plate offset, 500 V; nebulizer pressure, 2 bar (N2); drying gas, 10 L min−1 (N2); and drying temperature, 200 °C.

The QTOF-MS system was operating in broadband collision-induced dissociation (bbCID, data-independent) acquisition mode and recorded spectra over the range m/z 50–1000 with a scan rate of 2 Hz. This mode provides MS and MS/MS spectra at the same time, working at two different collision energies; at low collision energy (4 eV), MS spectra were acquired. At high collision energy (25 eV), no isolation is taking place at the quadrupole, and the ions from the preselected mass range are fragmented at the collision cell. A second analysis was performed in AutoMS acquisition mode. Concerning AutoMS mode (Data-dependent), the collision energy applied was set to predefined values, according to the mass and the charge state of every ion.

A QTOF-MS external calibration was daily performed with a sodium formate solution (10 mM sodium formate in a mixture of H2O:isopropanol (1:1)), using a calibrant injection at the beginning of each run. Fourteen cluster ions of the calibrant solution Na(NaCOOH)1-14 in the range of 50-1000 Da were used for calibration. The instrument provided a typical resolving power of 36000–40000 during calibration.

Data treatment and evaluation were processed with DataAnalysis 5.3 and TASQ 2.1 Client (Bruker Daltonics, Bremen, Germany).

A.4 Supplementary text A4: Screening and quantification

The following steps were applied to the raw data of the reference standard solutions, to obtain the analytical evidence that was mandatory for the identification of each analyte in the respective samples:

Firstly, internal mass calibration of the raw data files was performed with a calibration list to minimize potential mass errors. The retention time (RT) of each analyte was determined, by creating its extracted ion chromatogram (EIC) with a mass window of ± 5 mDa. Furthermore, the isotopic pattern fitting was evaluated. In addition, the MS and MS/MS spectra were processed to determine the precursor and qualifier ions of each analyte.

An in-house database containing all the analyte was compiled. This database contained information over the RT, precursor, and qualifier ions (fragments) of the parent compounds. In the following figure (Table to text A4), the data contained in the in-house database for some of the analyte are presented below:

Table to text A4: Analytical evidence data included in the in-house database for the identification of the tested compounds

Precursor ion — Qualifier ions

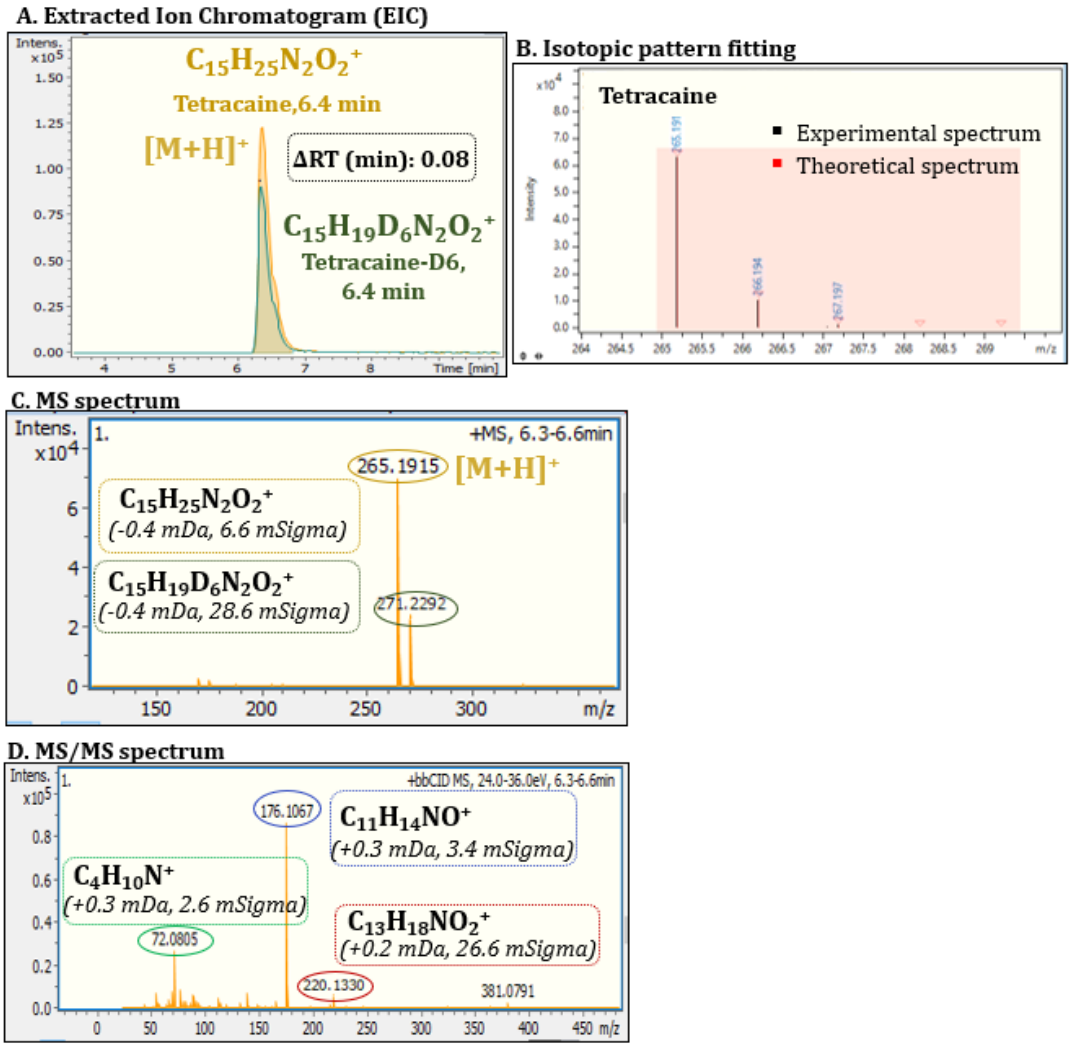
IS →

m/z	rt	name	formula	Neutral formula	CAS	Qual1	Qual2	Qual3	Qual4	Qual5	Qual1 form	Qual2 for	Qual3 for	Qual4 formula	Qual5 formula
260.1645	6.39	Propranolol	C16H22NO2^1+	C16H21NO2	(525-66-6)	74.06	56.0495	116.107	183.0804	98.0964	C3H8NO^1	C3H6N^1+	C6H14NO	C13H11O^1+	C6H12N^1+
267.2084	6.33	Propranolol-D7	C16H15D7NO2^1+	C16H14D7NO2											
265.1911	6.43	Tetracaine	C15H25N2O2^1+	C15H24N2O2	(94-24-6)	176.107	72.0808	220.1332			C11H14NO	C4H10N^1	C13H18NO2^1+		
271.2287	6.43	Tetracaine-D6	C15H19D6N2O2^1+	C15H18D6N2O2	(80404-52-0)										
278.1903	8.20	Amitriptyline	C20H24N1^1+	C20H23N1	(50-48-6)	91.0542	105.0699	117.0699	233.1325	218.109	C7H7^1+	C8H9^1+	C9H9^1+	C18H17^1+	C17H14^1+
281.2092	8.21	Amitriptyline-D3	C20H21D3N1^1+	C20H20D3N1	(342611-00-1)										
306.0811	8.85	Sertraline	C17H18N1Cl2^1+	C17H17N1Cl2	(79617-96-2)	158.9763	129.0699	275.0389	91.0542		C7H5Cl2^1+	C10H9^1+	C16H13Cl2	C7H7^1+	
309.100	8.84	Sertraline-D3	C17H15D3Cl2N^1+	C17H14D3Cl2N	(1217741-83-7)										
406.1932	10.35	Enclomiphene	C26H29ClNO^1+	C26H28ClNO	(14158-65-7)	100.112076	72.080776	58.065126	297.1274		C6H14N^1+	C4H10N^1	C3H8N^1+	C22H17O^1+	
372.2322	10.75	Tamoxifen	C26H30NO^1+	C26H29NO	(10540-29-1)	72.080776	129.06988	207.11683	327.1743		C4H10N^1+	C10H9^1+	C16H15^1	C24H23O^1+	

Source: Own depiction

Samples were screened utilizing the in-house built database. The detection of the analyte was based on specific screening parameters. In the following figure (Figure to text A4), the identification procedure for the analyte tetracaine in the samples is exemplified as an example:

Figure to text A4: Identification workflow for the analyte tetracaine



A: Extracted ion chromatogram (EIC) of tetracaine and tetracaine-IS (Tetracaine-D6), and retention time difference between the experimental and the theoretical retention time (ΔRT). B: Isotopic pattern fitting of tetracaine. The theoretical spectrum is presented with black color and the corresponding experimental spectra with red C: MS spectrum of the molecular ion of tetracaine ([M+H]+). Principal ion: yellow color, Ion of IS: green color (Information in brackets: mass accuracy, isotopic pattern fitting) D: MS/MS spectrum of tetracaine. Qualifier ions are presented in frames with blue, green, and red color. 3 qualifier ions are detected (Information in brackets: mass accuracy, isotopic pattern fitting).
Source: Own depiction

The quantification of the chemicals was performed by using standard addition calibration curves. Calibration curves were constructed with reference standard solutions at different concentration levels. The instrument provides a linear signal response at a certain concentration range for each analyte. Reference standard solutions were used, for the determination of this concentration range for each analyte. The stable isotopically labeled internal standards (IS) were spiked to the samples in different concentration for each analyte. They were used in order to achieve reliable and accurate quantitative results and for correcting potential insufficiencies of the sample preparation, ion suppression phenomena and for improving the quantification confidence. For that reason, relative areas have been used, namely, the absolute area of each analyte has been divided with the area of the respective IS.

The calibration curves were constructed using the linear regression model. The regression lines (Equation [S1]) were determined by the least-squares method, and were of the form:

$$y = (a \pm S_a) * C + (b \pm S_b) \quad [S1]$$

in which,

y: relative peak area of each analyte

a: the slope,

b: the intercept

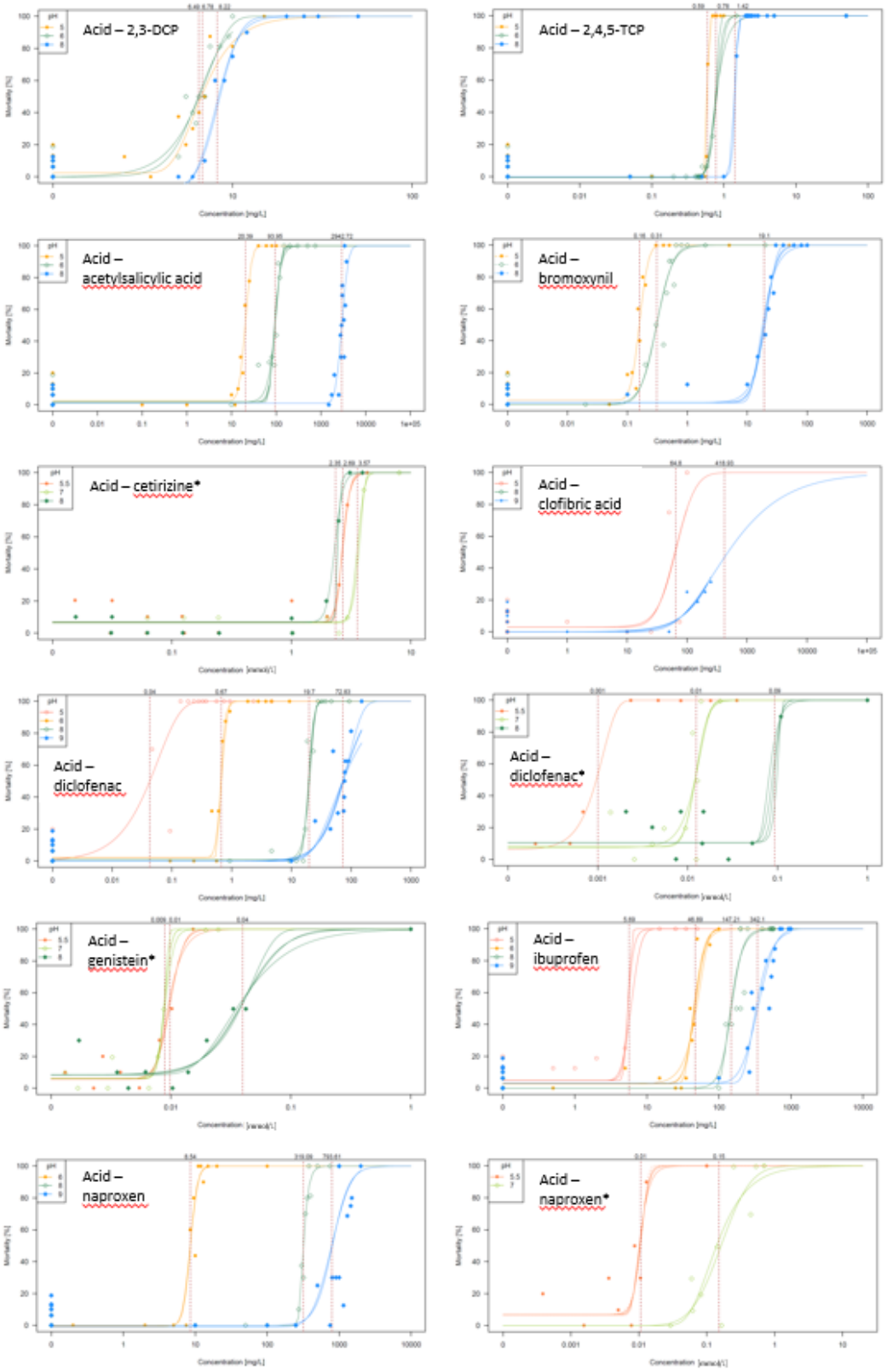
C: concentration of analyte

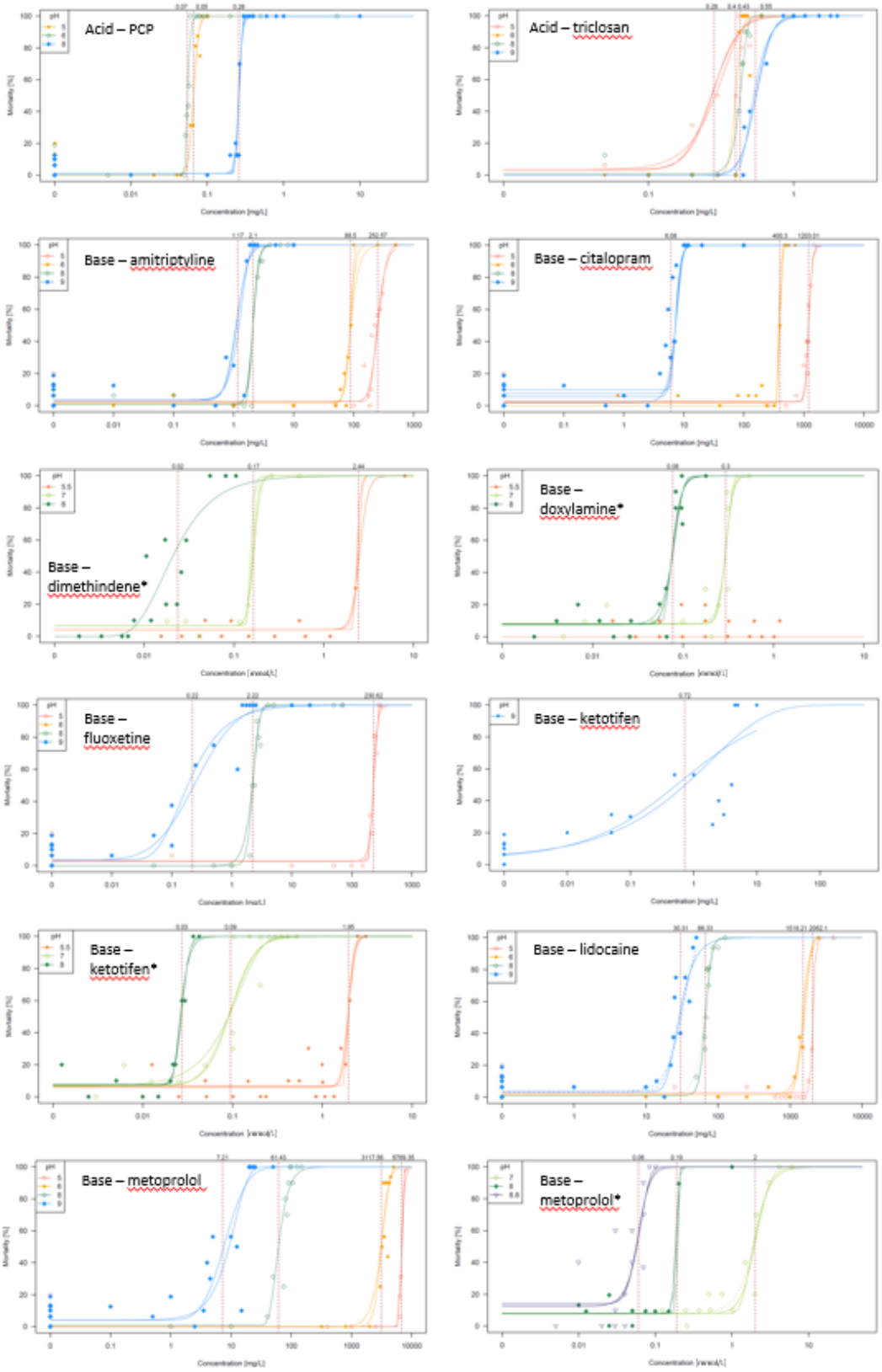
Sb: standard deviation of the intercept

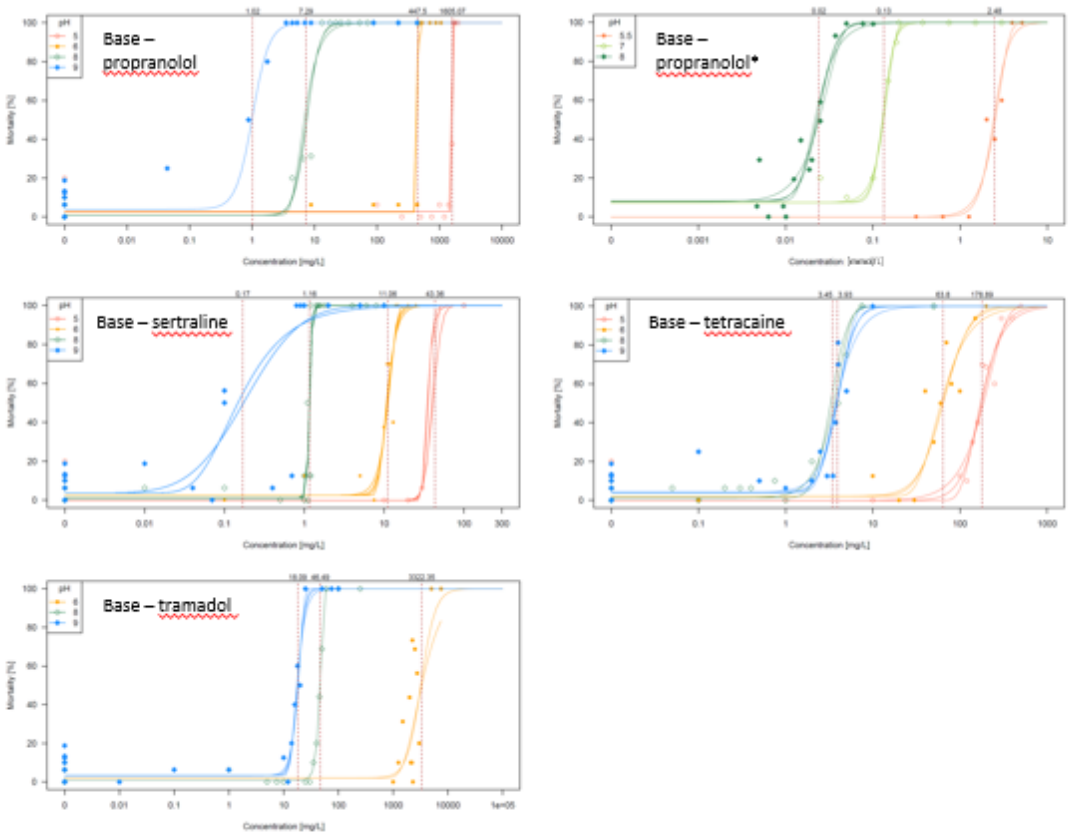
Sa: standard deviation of the slope

The concentration of each analyte in the samples was determined by using the corresponding equation [S1] with the relative peak areas (y).

Figure A2: Concentration-response curves for all chemicals tested

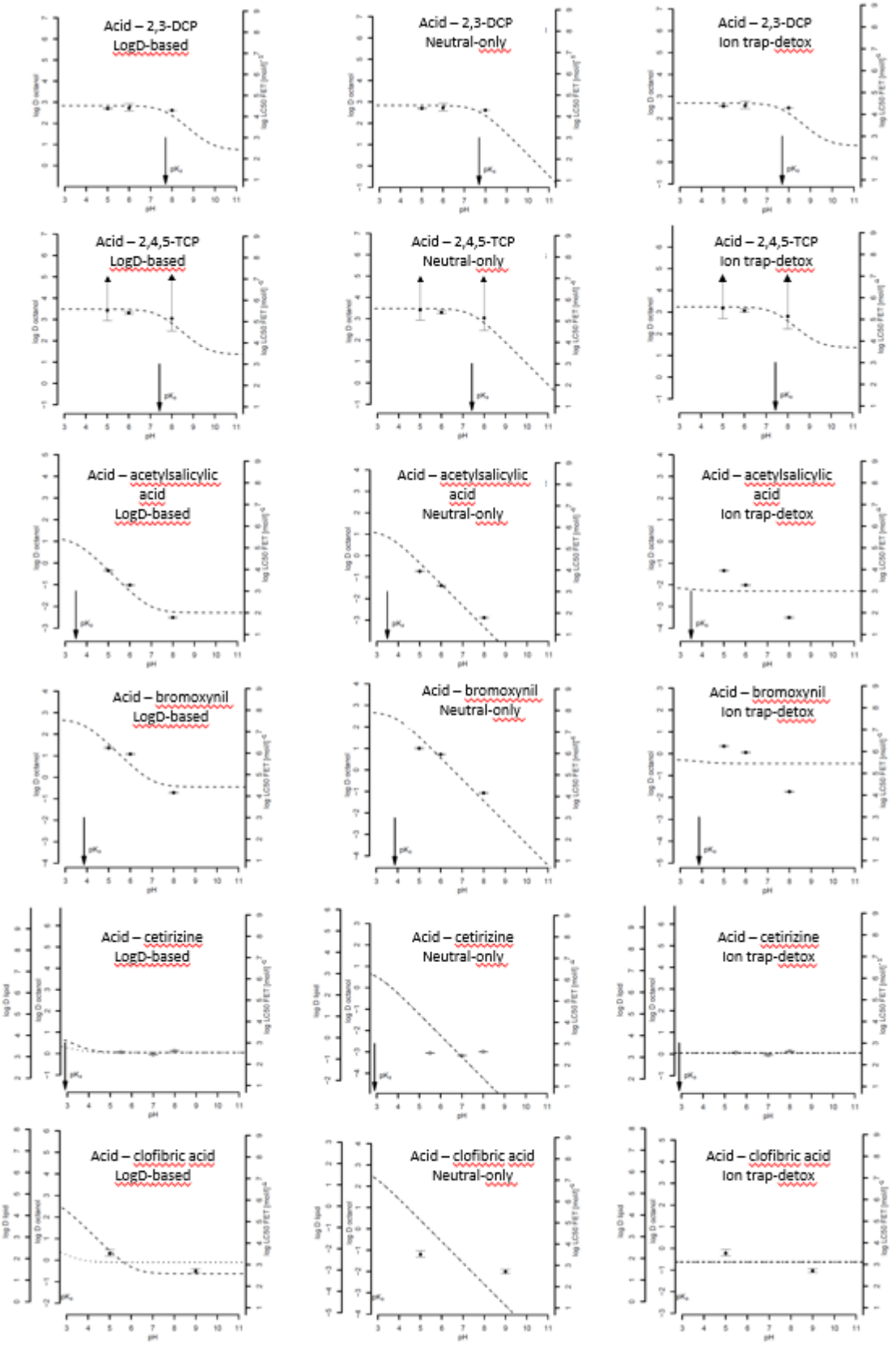


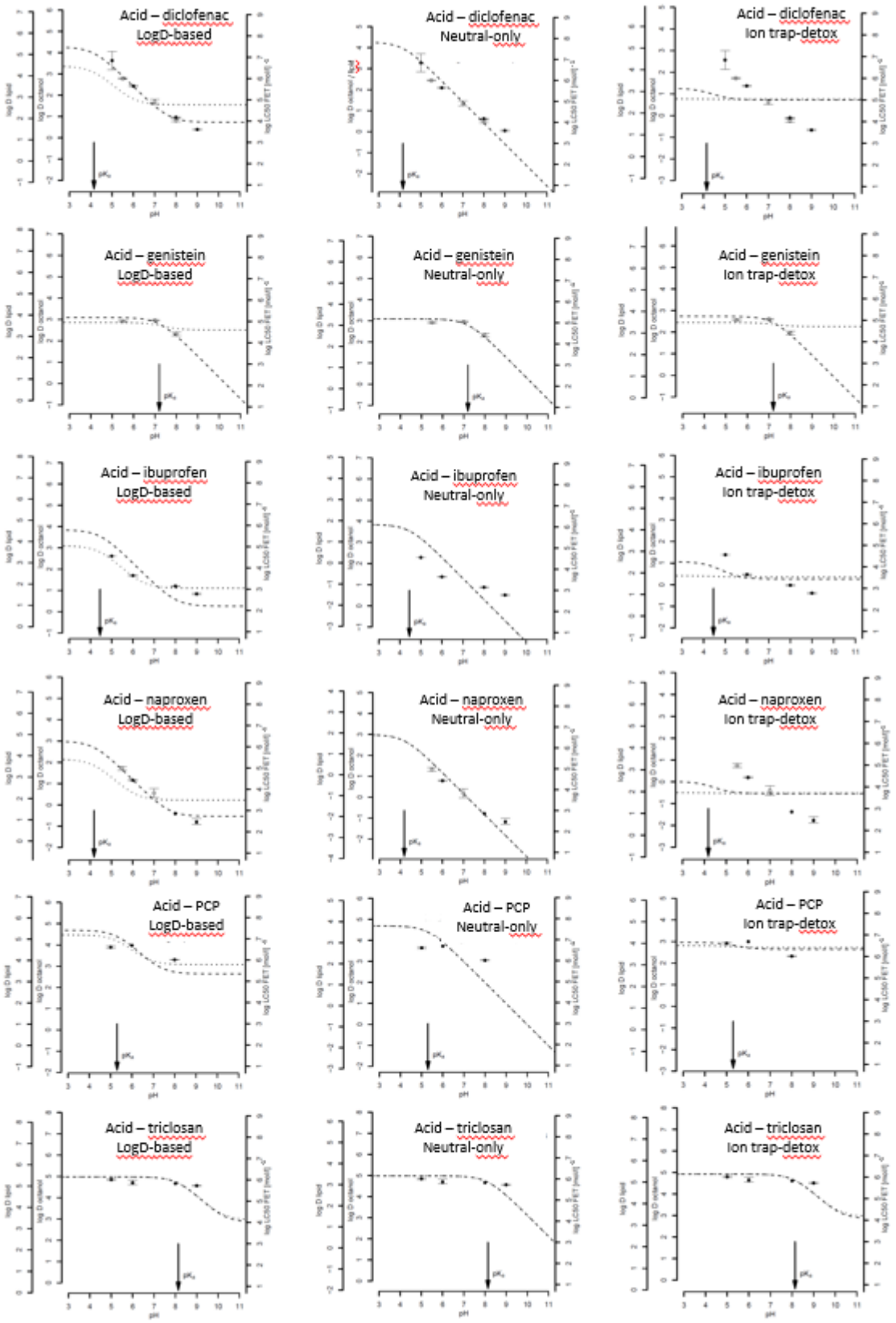

Acid – PCP
Acid – triclosan
Base – amitriptyline
Base – citalopram
Base – dimethindene*
Base – doxylamine*
Base – fluoxetine
Base – ketotifen
Base – ketotifen*
Base – lidocaine
Base – metoprolol
Base – metoprolol*

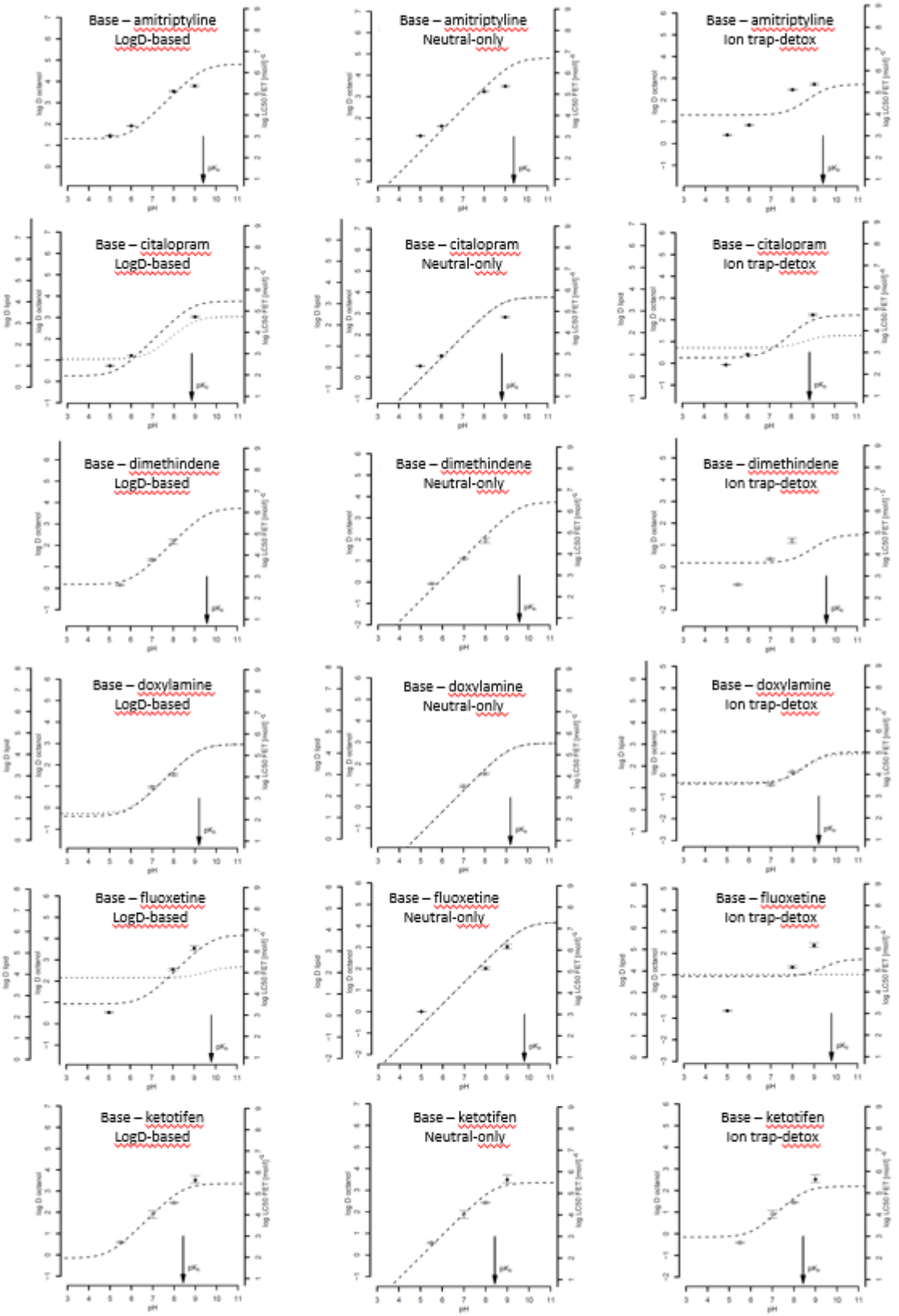


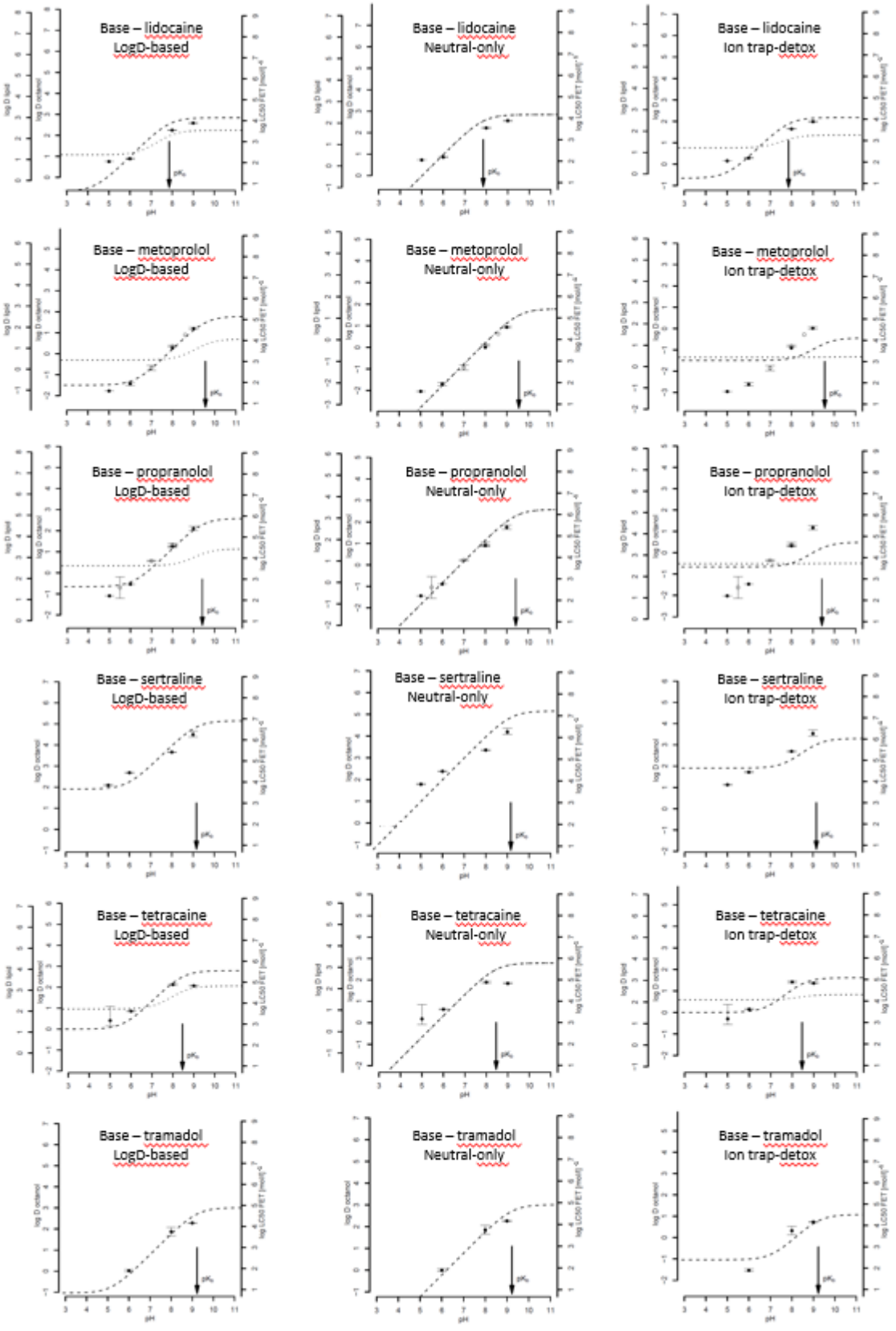
Determination of LC50 for all ionizable chemicals tested, acids (top plots) and bases (bottom plots) at different pH by nonlinear regression analysis. Mortality in the FET vs. nominal concentrations in mg/L (own data) or in m mol/L (*: data taken from Bittner et al. (2018), Bittner et al. (2019a) or Bittner et al. (2019b)) in the aqueous medium. Data obtained for pH5, pH 5.5, pH6, pH7, pH8 and pH9 (colours and symbols refer to the respective legends in the top left corner of each plot). LC50 data are symbolized by red vertical dashed lines and given at the top of the graphs. Whenever two or more equally well-fitting regression lines could be used for LC50 determination, preference was given to the lowest LC50 value. Source: Own depiction

Figure A3: Models applied to mortality data




Acid – diclofenac
LogD-based
Acid – diclofenac
Neutral-only
Acid – diclofenac
Ion trap-detox
Acid – genistein
LogD-based
Acid – genistein
Neutral-only
Acid – genistein
Ion trap-detox
Acid – ibuprofen
LogD-based
Acid – ibuprofen
Neutral-only
Acid – ibuprofen
Ion trap-detox
Acid – naproxen
LogD-based
Acid – naproxen
Neutral-only
Acid – naproxen
Ion trap-detox
Acid – PCP
LogD-based
Acid – PCP
Neutral-only
Acid – PCP
Ion trap-detox
Acid – triclosan
LogD-based
Acid – triclosan
Neutral-only
Acid – triclosan
Ion trap-detox


Base – amitriptyline
LogD-based
Base – amitriptyline
Neutral-only
Base – amitriptyline
Ion trap-detox
Base – citalopram
LogD-based
Base – citalopram
Neutral-only
Base – citalopram
Ion trap-detox
Base – dimethindene
LogD-based
Base – dimethindene
Neutral-only
Base – dimethindene
Ion trap-detox
Base – doxylamine
LogD-based
Base – doxylamine
Neutral-only
Base – doxylamine
Ion trap-detox
Base – fluoxetine
LogD-based
Base – fluoxetine
Neutral-only
Base – fluoxetine
Ion trap-detox
Base – ketotifen
LogD-based
Base – ketotifen
Neutral-only
Base – ketotifen
Ion trap-detox



Models fitted to experimentally generated mortality data, expressed as log LC_{50}, for for all ionizable chemicals tested at different pH in the FET, acids (top plots) and bases (bottom plots). Black dots: own data, circles: data generated from data of Bittner et al. (2018), Bittner et al. (2019a) or Bittner et al. (2019b). The three columns correspond to the three types of model sets, logD-based (left), neutral-only (middle) and ion trap-detox (right). The vertical scaling for the modelled putatively effected logD data the at the left side of each plot was adapted to the experimentally generated data in such a

way that the best possible agreement of the models with the real data was achieved. Dashed lines represent the models based on log P_{ow}, dotted lines those based on log P_{lipw} (in the case of identical curves for both models, only the dashed curve is shown). The vertical arrows point to the pKa of the substances.
Source: Own depiction