Hochschule für Angewandte Wissenschaften Hamburg
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# Development of acute toxicity tests on *Daphnia magna* for the determination of polycyclic aromatic hydrocarbons (PAHs) and phthalates in soft plastic baits

## Bachelor Thesis

at the

Department of Environment Engineering

of the Faculty of Life Sciences

at Hamburg University of Applied Sciences


Submitted by

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[redacted]


Hamburg the 31th of January 2020

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## SELF-DECLARATION

I, Alexander Ghrim, confirm that the following work was solely undertaken by myself and that no help was provided from other sources as those allowed. All sections of the thesis that use quotes or describe an argument or concept developed by another author have been referenced, including all secondary literature used, to show that this material has been adopted to support my thesis.

_____ _____

Place / Date Signature

# ACKNOWLEDGEMENTS

At first, I would like to express my great gratitude to my first referee Prof. Dr. rer. nat. habil. Gesine Witt for teaching me great knowledge on the subject of ecotoxicology and giving me the chance to write this bachelor thesis in her research group.

Further, I want to appreciate M.Sc. Moritz Kielmann, my second referee, for giving me valuable and useful advices during my work.

Moreover, I am thankful for the constructive criticism from M.Sc. Silja Denise Kröger. The helpful suggestions on form and content improved this thesis.

Also, thanks to Prof. Dr. Susanne Heise, M.Sc. Anne Kreutzer, Anne Below and Mika Stieler for the opportunity to use their *Daphnia magna* cultures and giving me a hand in the laboratory.

Finally, special thanks go to my parents for always encourage my decisions and being there for me, especially in times of need.

# ABSTRACT

Marine litter is a global problem that has been gaining attention in recent years. It threatens flora and fauna of sensible ecosystems. The major part of this litter is plastic material which is hardly degradable. Generally, the main diffuse input pathways are from landside sources or through rivers. Microplastic particles are found nearly everywhere nowadays, yet in deep sea.

The recreational fishing industry plays a part in this issue. Angler often make use of soft plastic lures. It is disputed that they have direct impacts on limnic and marine ecosystems. However, they have indirect impacts, because they include additives like softeners or attractants. These substances and other hazardous compounds might be released during fishing.

In this thesis, two compound classes are examined. Polycyclic aromatic hydrocarbons have toxicologic properties as being carcinogenic, mutagenic or teratogenic. Low-molecular phthalates are suspected to be endocrine disruptive. They were chosen as pollutants, because they both tend to be persistent and easily bioaccumulate. Their possible effects on the species *Daphnia magna* were investigated in this study. Besides, two methods on how to test soft plastic material in acute toxicity tests on *Daphnia magna* were developed and evaluated with two different soft plastic lures.

The developed plastic contact test method uses pestled plastic material debris. Dilution medium is added to it and everything is shaken for up to 24 hours. The fact that the material is not removed during testing leads to a constant release of pollutants. Contrary, leaching test uses 1 $cm^2$ great cubes which are also shaken for one day in dilution medium. However, the material is removed before start and only the leaching water is used in this toxicity test method.

$EC_{50}$ concentrations of 48 hours acute toxicity tests show that plastic lure Tiddler has greater effects on daphnids than Möhrchen. Additionally, gas chromatography analysis of these samples confirms this fact and suggests that hardly soluble phthalates emit easier due to solvents on or in the plastic material. Another aspect is the toxic effect addition of these determined substances. However, PAH values should be treated with caution, because only a

few reached the limit of quantitation. Moreover, average standard deviation values depict a greater stability and validity of plastic contact test compared with leaching test method. Considering the applicability with other materials, the plastic contact test is time consuming but more realistic and provides reliable results.

# ZUSAMMENFASSUNG

Meeresmüll ist ein globales Problem, dass in den letzten Jahren an Aufmerksamkeit gewonnen hat. Es bedroht sowohl Flora als auch Fauna von sensiblen Ökosystemen. Den größten Teil macht der Plastikmüll aus, der kaum abbaubar ist. Die Haupteintragspfade sind aus landbasierten Quellen oder diffus über in Meere mündende Flüsse. Mikroplastik Partikel sind bereits heute in einigen Teilen der Tiefsee zu finden.

Einen kleinen, aber nicht unerheblichen Teil trägt der Angelsport zu dem Problem bei. Hobbyangler benutzen nicht selten spezielle Gummifische als Köder. Es wird diskutiert, dass die Plastikfische direkten schädigenden Einfluss auf die limnischen oder marinen Ökosysteme haben. Jedoch ist klar, dass sie indirekten Einfluss haben, da sie einige Zusatzstoffe beinhalten. Zu diesen Stoffen gehören beispielsweise Weichmacher oder Lockstoffe, die während des Fischens an die wässrige Umgebung abgegeben werden.

Die vorliegende Arbeit untersucht explizit zwei Schadstoffklassen. Polyzyklische aromatische Kohlenwasserstoffe und einige Phthalate wurden als Schadstoffe ausgewählt, da sie persistent und bioakkumulativ sind. Zudem sind einige Verbindungen oder ihre Metabolite krebserregend, erbgutverändernd oder fortpflanzungsgefährdend. Phthalate stehen in der Kritik endokrin wirksam zu sein. Die möglichen toxischen Effekte auf die Spezies *Daphnia magna* waren Bestandteil der Untersuchungen. Ferner wurden zwei verschiedene Methoden zur Untersuchung von synthetischem Gummi in akuten Toxizitätstests an *Daphnia magna* anhand von zwei Gummiködertypen entwickelt und evaluiert, um Aussage über ihre Anwendbarkeit zu treffen.

Bei der entwickelten Kunststoffkontakt Methode wurde das Material klein gemörsert, Verdünnungsmedium hinzugefügt und verblieb nach einem 24-stündigen Schüttelvorgang im Testgefäß. Dies führt dazu, dass das Material auch während des Tests noch Schadstoffe abgeben kann. Anders war es beim Leaching Test, bei dem das Material in 1 $cm^2$ große Quader geschnitten wurde. Danach wurde es ebenfalls geschüttelt, jedoch wurde das Material vor Testbeginn entfernt und nur das Waschwasser im eigentlichen Toxizitätstest benutzt.

Die Ergebnisse zeigen, dass die $EC_{50}$ Werte der Proben nach 48 Stunden Testdauer deutlich geringer für den Gummiköder Tiddler als für Möhrchen sind. Somit hat Tiddler größere toxische Effekte auf die Daphnien. Die anschließenden Messungen mittels Gaschromatographie bestätigen diese Annahme. Außerdem lassen die gemessenen Konzentrationen der Phthalate im wässrigen Medium vermuten, dass Lösungsmittel die Schadstoffabgabe begünstigen. Eine weitere Erkenntnis ist die Addition der toxischen Wirkung dieser Stoffe. Des Weiteren zeigt eine geringere durchschnittliche Standardabweichung, dass die Kunststoffkontakt Testmethode eine bessere Aussagekraft besitzt und weniger fehleranfällig ist als die Leaching Methode. Die Anwendbarkeit der beiden Tests auf andere Stoffe ist demnach beim Kunststoffkontakt Test verlässlicher, da die Ergebnisse eher der Realität entsprechen.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACE | Acenaphthene |
| ACY | Acenaphthylene |
| ALEX | Automated liner exchange system |
| ANTH | Anthracene |
| BaA | Benzo[a]anthracene |
| BbF | Benzo[b]fluoranthene |
| BBP | Benzyl butyl phthalate |
| BghiP | Benzo[g,h,i]perylene |
| BkF | Benzo[k]fluoranthene |
| $C_{free}$ | Concentration of a freely dissolved substance |
| CHR | Chrysene |
| CIS | Cold injection system |
| D. magna | Daphnia magna |
| DBP | Dibutyl phthalate |
| DDT | Dichlorodiphenyltrichloroethane |
| DEHP | Diethylhexyl phthalate |
| DIDP | Diisodecyl phthalate |
| DINP | Diisononyl phthalate |
| $EC_{50}$ | Half maximal effective concentration |
| ECHA | European Chemicals Agency |
| EI | Electron impact |
| EQS | Environmental Quality Standards |
| FLUO | Fluoranthene |
| FLU | Fluorene |
| IND | Indeno[1,2,3-cd]pyrene |
| Log-$K_{OW}$ | Logarithm-transformed partition coefficient of octanol/water |
| LOQ | Limit of quantitation |
| MPS | Multi-purpose sampler |
| MSFD | Marine Strategy Framework Directive |
| m/z | Mass-to-charge ratio |

| | |
|---|---|
| NAPH | Naphthalene |
| PAHs | Polycyclic aromatic hydrocarbons |
| PCBs | Polychlorinated biphenyls |
| PHEN | Phenanthrene |
| PHTHs | Phthalates |
| POPs | Persistent organic pollutants |
| PVC | Polyvinyl chloride |
| PYR | Pyrene |
| SPLs | Soft plastic lures |
| SVHCs | Substances of very high concern |
| Tiddler | Soft plastic lure Tiddler Fast |

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# 1 INTRODUCTION

## 1.1 OVERVIEW

Marine litter is considered as a global problem because it affects flora and fauna of marine and coastal ecosystems equally. Cleaning of polluted areas is often expensive or impossible (Gall and Thompson, 2015; Bråte *et al.*, 2017). The majority of this marine litter is long-lasting plastic litter which originates from various sources. Macroplastic or microplastic is found in all oceans and marine biospheres (Otley and Ingham, 2003; McDermid and McMullen, 2004; Law *et al.*, 2014; Pham *et al.*, 2014; Jambeck and Johnsen, 2015; Auta, Emenike and Fauziah, 2017; Bergmann *et al.*, 2017). The greatest amount refers to landside sources, though a minor part comes from marine sources as well (Jambeck and Johnsen, 2015). Touristic beaches, waste disposal sites or the fishing industry threatens these sensitive marine ecosystems and more than 100.000 species of organisms (Galgani *et al.*, 2013; UBA, 2015; Moriarty *et al.*, 2016; Nelms *et al.*, 2017). Plastic waste can stay up to 500 years in waters until it is fully degraded (BMU, no date).

During degradation process, an emission of additives and other ingredients into the water, such as hazardous pollutants with different impacts on the environment, can happen. Therefore, some international agreements such as the Helsinki Convention, the OSPAR Convention and Marine Strategy Framework Directive (MSFD) are facing the problem (Helsinki Convention, 1980; OSPAR Convention, 1992; European Commission, 2014). Through mandatory regulations they target a conservation and improvement of a good ecological and chemical water condition (Helsinki Convention, 1980; OSPAR Convention, 1992; European Commission, 2014).

Even though industrial fishing may have a great impact on marine waste problem, there are also pathways which are unattended, yet. Sport and recreational angler often make use of so-called soft plastic baits or fishing lures (SPLs) for tackling (Kielmann, 2019). In this process SPLs can sever from fishing lines, get lost or discarded, consciously (Raison *et al.*, 2014). Snorkel surveys revealed that the deposition rate of SPLs at the researched lake was potentially as high as approximately 80 per km of shoreline per year (Raison *et al.*, 2014). Additionally, they have been found in aquatic environments and in the digestive tract of a

variety of fish species (Raison *et al.*, 2014). Compared to other marine pollutants, no restrictions for soft plastic baits were imposed yet, since the risk assessment database of them is small.

In this case, SPLs from different manufacturers are subject of investigations. Their risks and potential effects of their additives on crustaceans in acute toxicity tests is investigated further on.

Hence, two general pollutant classes were chosen as representative test substances. Both polycyclic aromatic hydrocarbons (PAHs) and phthalates (PHTHs) are ingredients of some SPLs (Kielmann, 2019). PHTHs function as plasticisers and PAHs are unwanted by-products during production. They are defined as persistent organic pollutants (POPs) (ECHA, 2006). POPs are chemical compounds with characteristics to bioaccumulate, resist degradation processes and travel long distances (Fitzgerald and Wikoff, 2014). Some of them are of natural origin and emit due to forest fires or volcanic eruptions, but the majority is man-made like polychlorinated biphenyls (PCBs) or dichlorodiphenyltrichloroethane (DDT) (Jones and De Voogt, 1999). Even though few substances have good features, like DDT as a biocide, most of them being discussed as carcinogenic, mutagenic or teratogenic (Nisbet and LaGoy, 1992). As an example, three phthalates and some PAHs are strictly forbidden in products such as plastic toys or baby articles (Leutert *et al.*, 2007)

## 1.2 AIM OF THE THESIS

The aim of this thesis is to discuss the potential impact and consequences of PAHs and PHTHs in SPLs on macrobenthos. Crustaceans, like *Daphnia magna* (*D. magna*), are highly sensitive to various pollutants in aqueous media and function as well-researched bioindicators. Additionally, acute toxicity biotests are an approved and established way of testing the toxic effects of chemicals (Persoone *et al.*, 2009) Concerning this, two test methods with different approaches on *D. magna* were developed to compare the pros and cons and if they qualify for further use. As test results, half maximal effective concentrations ($EC_{50}$ values) were calculated for each substance in the tests. Altogether 14 various PAHs and six PHTHs were compared among themselves.

In addition to that, the following tasks support and complete the studies:

1. Development of acute *D. magna* toxicity tests with two different approaches
2. Evaluation of the tests by reproductivity in regard to standard deviation
3. Calculation of $EC_{50}$ values on basis of the sample amount
4. Comparison of the two tests by means of PHTH and PAH concentrations at the end of the tests
5. Deduction of the risk assesment of SPLs in aquatic environment based on the study results

# 2 BACKGROUND AND STATUS OF RESEARCH

This section deals with the basics of acute toxicity tests on crustaceans. Furthermore, it describes and gives an overview of the dosing theory, that is used to develop both testing methods in this thesis. At last, the two pollutant classes of PAHs and PHTHs are introduced and presented.

## 2.1 ACUTE TOXICITY TESTS

In acute toxicity tests for crustaceans, the immobilisation rate after 24 or 48 hours is determined. These tests are standardised as OECD Guideline 202 or DIN EN ISO 6341. In a defined system with 5 daphnids various pollutants can be tested in aqueous media. The substance is given into the system in defined dosages and immobile daphnids are counted after a period. Several dilution stages give values between minimal ($EC_0$) and maximal ($EC_{100}$) effective concentrations. Thus, a relating concentration-response curve can be calculated for each pollutant. Generally, $EC_{50}$ concentrations are used as index, in order to better compare different compounds. The dosage method of acute toxicity tests can be established on standard or passive dosing theory. Both methods are explained further on.

On the basis of standard dosing and passive dosing theory, which are described in the sections below, two test methods are developed and evaluated.

### 2.1.1 STANDARD DOSING

Standard dosing describes a method on adding pollutants with defined concentrations into a test system (Konopka, 2013). The substances are dissolved in aqueous media and added once, mostly right at the beginning of the test. This method can be used in various toxicological biotests such as the acute toxicity test on *Daphnia magna*.

The main problem of this method are unprecise results and an underestimation of toxic impacts, especially with hydrophobic pollutants in hydrophilic media (Könemann, 1981; Konopka, 2013). Pollutants can evaporate, agglomerate or adsorb on test vessels instead of staying freely dissolved, thus the concentration ($c_{free}$) decreases during the test

(Figure 1) (Smith et al., 2010). That is the reason why a small amount could be unavailable for test organisms, hence this method is more sensitive to errors than passive dosing (see 2.1.2 Passive dosing) (Smith et al., 2010).

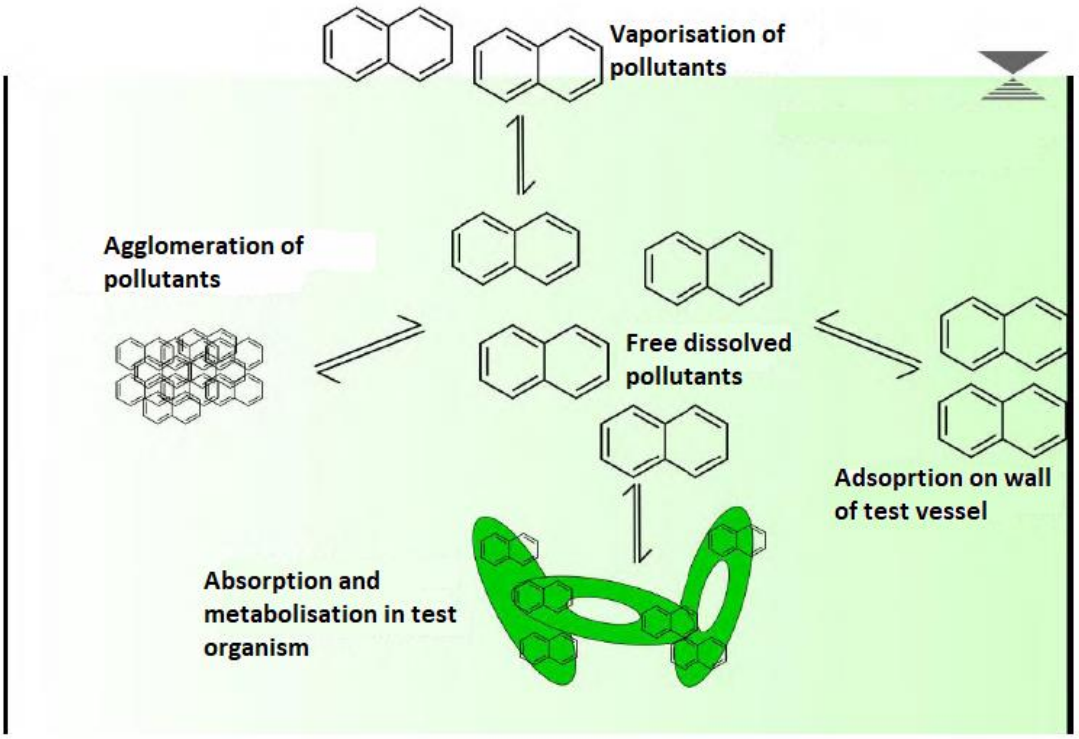


**Figure 1: Theory of standard dosing in a test vessel (Konopka, 2013)**

### 2.1.2 PASSIVE DOSING

The method of passive dosing bases on the equilibrium between a stationary phase and a mobile phase (Vrana *et al.*, 2005). At the beginning it is similar to the standard dosing method. The difference is a continuous diffusion of pollutants from a reservoir (stationary phase) into aqueous media (mobile phase) (Seiler *et al.*, 2014; Stibany *et al.*, 2017). Pollutant losses due to adsorption processes are compensated by the stationary phase. Therefore, $c_{free}$ stays constant during the test period. This advantage enables an analysis of both hydrophilic and lipophilic substances (Konopka, 2013). Figure 2 shows a set-up of a test operating with passive dosing.

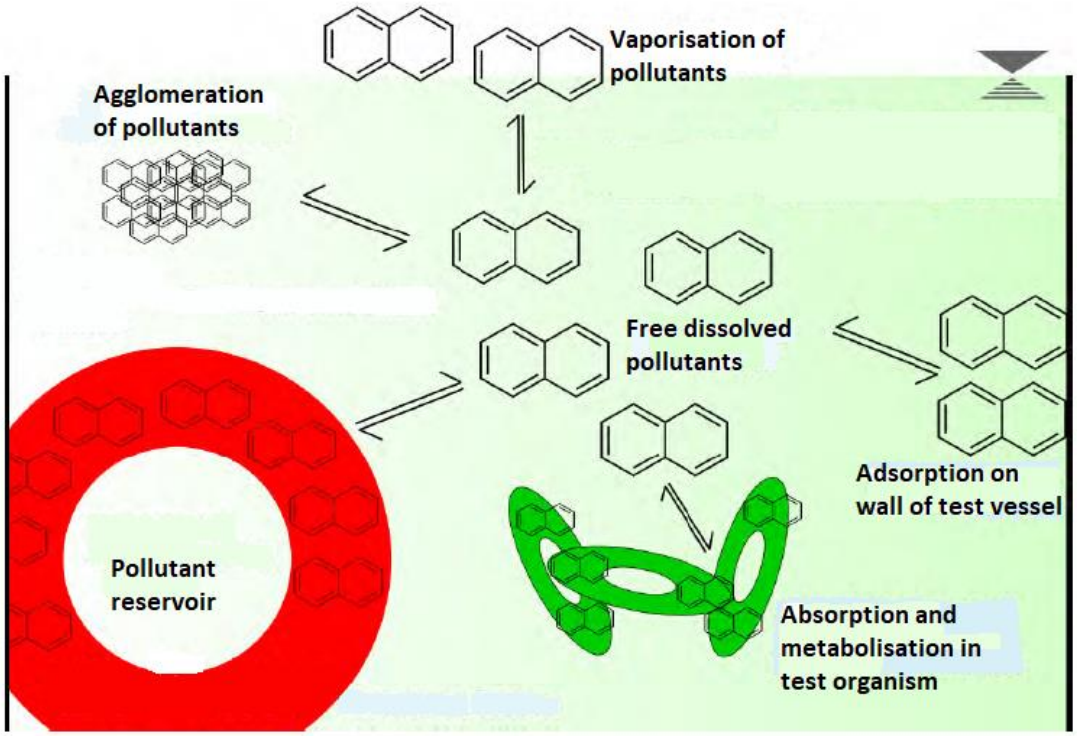


**Figure 2: Theory of passive dosing in a test vessel (Konopka, 2013)**

## 2.2 THE TEST SUBSTANCES

### 2.2.1 POLYCYCLIC AROMATIC HYDROCARBONS

PAHs are a group of organic chemical compounds with at least two fused benzene rings. As a result of the increase of carbons, the molecules get more covalent and insoluble in water. Naphthalene ($C_{10}H_8$) (Figure 3) is the simplest PAH with two benzene rings (Zander, 1995). Furthermore, the most rings consist of methyl groups, but some carbons also bind oxygen or nitrogen atoms, that is why approximately 10.000 different compounds exist (Zander, 1995; Brandt and Einhenkel-Arle, 2016).

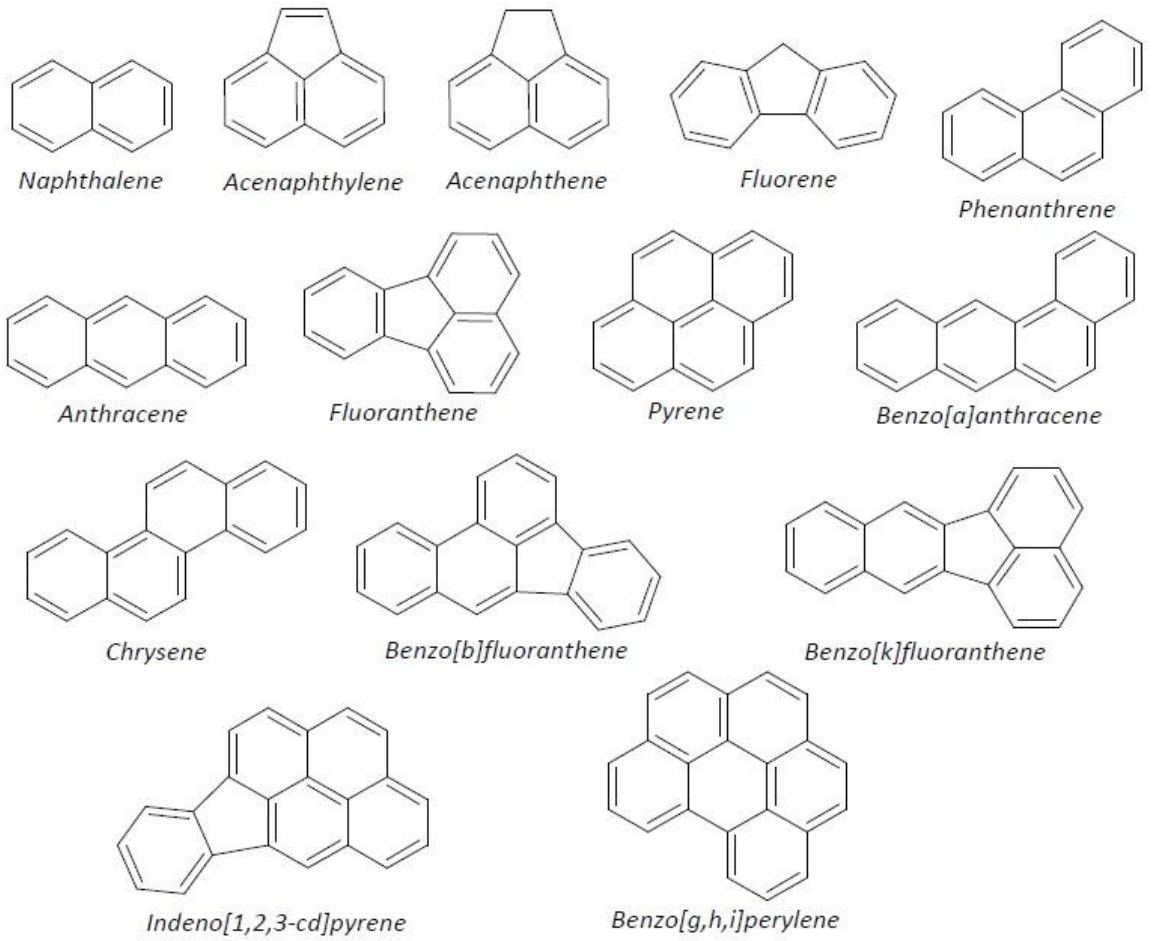


**Figure 3: Overview of structural formulas of determined PAHs**

The chemical properties of PAHs vary among themselves. It is noticeable, that on the one hand the solubility in water decreases and on the other hand the solubility in fat increases with higher amount of benzene rings. That is why, especially long-chain PAHs, accumulate in organisms fatty tissues easily (Brandt and Einhenkel-Arle, 2016). Additionally, Table 1 gives information about the log-transformed partition coefficient (Log-$K_{OW}$ value) in an octanol/water system (Sverdrup, Nielsen and Krogh, 2002). This is a parameter on the solubility of a substance in similar mixtures like hexane with water and furthermore a reference for bioaccumulation (Sangster, 1997; Fent, 2007). Values from 0 to 1 indicate a better solubility in aqueous media and higher numbers in organic media. Moreover, some of these compounds, especially their metabolites, can have carcinogenic, mutagenic or teratogenic characteristics, therefore PAHs are defined as substances of higher concern (SVHCs) (Nisbet and LaGoy, 1992; ECHA, 2006). The REACH-regulation (EC No. 1907, 2006) defines SVHCs as chemical compounds with identified hazardous characteristics on humans and environment (ECHA, 2006).

PAHs generally originate from anthropogenic sources like incomplete combustion processes of organic matter (Brandt and Einhenkel-Arle, 2016). Naturally, these compounds are released due to volcanic eruption or wildfires (Zander, 1995). Although PAHs are air pollutants, they are even traceable in soil and water.

In environmental samples the concentration of Benzo[a]pyrene (BaP) always functions as a lead substance (Zander, 1995). BaP is the best researched PAH, because of the carcinogenic effects of its derivates (Schrenk *et al.*, 2000). As defined in environmental quality standards (EQS) for several substances in surface waters (BGBI I Nr. 28, S. 1373), an average yearly value of 0.05 µg/L BaP should not be exceeded (UBA, 2016). In this thesis, 14 PAHs were measured with gas chromatography (GC-MS) analysis (Table 1).

**Table 1: PAHs measurable with used GC-MS analysis. The Log-$K_{OW}$ values originate from Sverdrup et al. (2002)**

| PAH | Abbreviation | Chemical formula | Log-$K_{OW}$ value |
|---|---|---|---|
| Naphthalene | NAPH | $C_{10}H_8$ | 3.32 |
| Acenaphthylene | ACY | $C_{12}H_8$ | 4.07 |
| Acenaphthene | ACE | $C_{12}H_{10}$ | 3.94 |
| Fluorene | FLU | $C_{13}H_{10}$ | 4.23 |
| Phenanthrene | PHEN | $C_{14}H_{10}$ | 4.50 |
| Anthracene | ANTH | $C_{14}H_{10}$ | 4.60 |
| Fluoranthene | FLUO | $C_{16}H_{10}$ | 5.20 |
| Pyrene | PYR | $C_{16}H_{10}$ | 5.20 |
| Benzo[a]anthracene | BaA | $C_{18}H_{12}$ | 5.66 |
| Chrysene | CHR | $C_{18}H_{12}$ | 5.80 |
| Benzo[b]fluoranthene | BbF | $C_{20}H_{12}$ | 6.40 |
| Benzo[k]fluoranthene | BkF | $C_{20}H_{12}$ | 6.40 |
| Indeno[1,2,3-cd]pyrene | IND | $C_{22}H_{12}$ | 6.70 |
| Benzo[g,h,i]perylene | BghiP | $C_{22}H_{12}$ | 6.90 |

### 2.2.2 PHTHALATES

The esters of phthalic acid, also known as phthalates, have generally the same chemical structure which contains a benzene ring and an ester with different alcohol groups

(Figure 4) (EPA, 2012). Phthalates are mainly applied in polyvinyl chloride (PVC) products to plasticise them. They are added in 65 – 70% of all PVC products (Saechtling *et al.*, 2013). The most used ones are Diisodecyl phthalate (DIDP), Diisononyl phthalate (DINP), Diethylhexyl phthalate (DEHP), Dibutyl phthalate (DBP) and Benzyl butyl phthalate (BBP) (Leutert *et al.*, 2007).

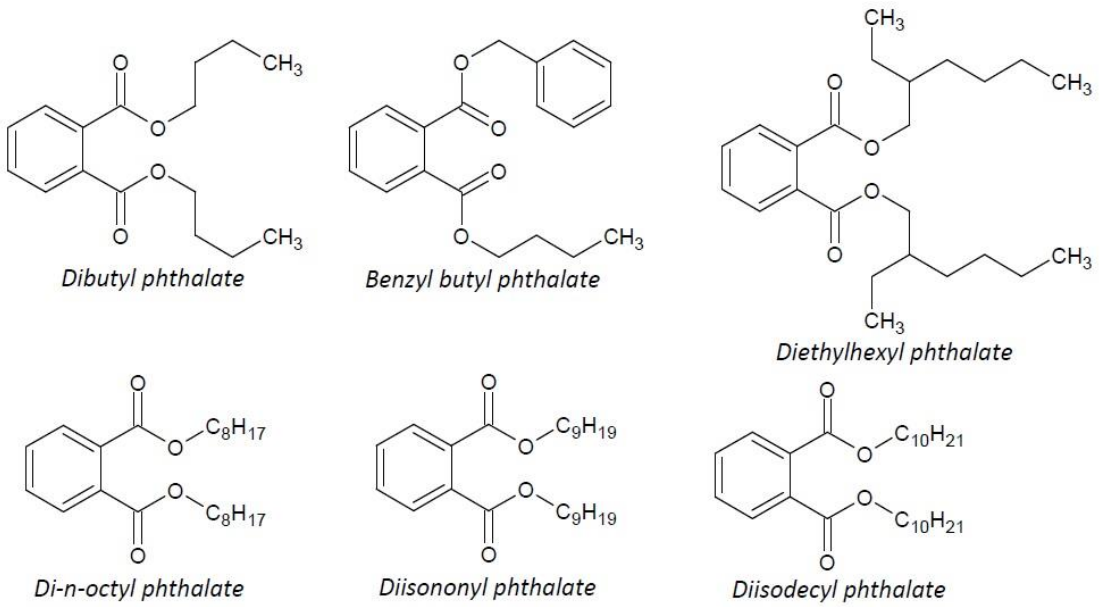


**Figure 4: Overview of structural formulas of determined PHTHs**

Phthalates have a wide range of applications for example in food packages, PVC floor coatings or wallpapers. Consequently, humans cannot prevent contact with these substances. Especially low-molecular type PHTHs are emitted, washed out or rubbed off from plastic products, hence nearly every human has got them or their metabolic products in their urine or blood (Leutert *et al.*, 2007).

They ecotoxicologically belong to POPs and even three of them are also listed as SVHCs because of their degradation persistent characteristics (European Parliament, 2006). Even at low concentrations they are defined as substances with endocrine effects on organisms (Oehlmann *et al.*, 2009).

Three PHTHs (DEHP, DBP and BBP) are forbidden in products such as soft plastic baits, because of teratogenic characteristics. Their substitutes DINP and DIDP are also discussed as problematic, because of possible liver-toxic effects (Leutert *et al.*, 2007). The toxicological assessment of PHTHs still considers single compounds which is controversial.

Because of a possible addition of toxic impacts, similar phthalates should be tested together (Leutert *et al.*, 2007).

Six PHTHs, as shown in Table 2, are object of determination in this thesis.

**Table 2: PHTHs measurable with used GC-MS method. The log-$K_{OW}$ values originate from various sources**

| PHTH | Abbreviation | Chemical formula | Log-$K_{OW}$ value |
|---|---|---|---|
| Dibutyl phthalate | DBP | $C_{16}H_{22}O_4$ | 4.50 (Ellington and Floyd, 1996) |
| Benzyl butyl phthalate | BBP | $C_{19}H_{20}O_4$ | 4.73 (Ellington and Floyd, 1996) |
| Diethylhexyl phthalate | DEHP | $C_{24}H_{38}O_4$ | 7.60 (De Bruijn *et al.*, 1989) |
| Di-n-octyl phthalate | DNOP | $C_{24}H_{38}O_4$ | 8.10 (Ellington and Floyd, 1996) |
| Diisononyl phthalate | DINP | $C_{26}H_{42}O_4$ | 9.37 (O'Neil, 2013) |
| Diisodecyl phthalate | DIDP | $C_{28}H_{46}O_4$ | 9.46 (Cousins and Mackay, 2000) |

# 3 MATERIALS AND METHODS

At first, the following chapter describes the crustacea *Daphnia magna* and the reason why it is an often-used test organism. Moreover, advices on the maintenance after standardised conditions are mentioned. The presentation of the two compared soft plastic lures follows, subsequently. In addition, the pre-tests with their resulting developed test method procedures are introduced and explained. New methods are developed and tested, because there is no standardised procedure on how to test various plastic materials in acute toxicity tests, yet. In the end, the measurement of PAH and PHTH concentrations takes place with GC-MS. Before this, the samples have to be prepared in a multi-step extraction process.

## 3.1 TEST ORGANISM *DAPHNIA MAGNA*

The great water flea *Daphnia magna* (Straus, 1820) belongs to the taxonomic group of zooplankton. This species plays a significant role investigating pelagic ecosystems (Fent, 2007). Mainly it lives in calm limnetic water bodies with low current. *D. magna* has an oval body shape with up to 6 mm in size for female adults and 2 mm for male adults (Streble and Krauter, 1988). Furthermore, it has a bivalve carapace made of chitin and on the front of its head two antennas and a compound eye for orientation (Figure 5 (A)).

*D. magna* is a filter feeder, which means that it filters phytoplankton like green algae from water current. Therefore, water fleas have an important role in the food chain as secondary producers. For this process it moves their mouthparts to generate a current and absorb plankton with a mesh size of 1 µm (Fent, 2007). To protect from predators, *D. magna* stays in deep water layers during the day and wanders up to higher layers in the night (Ebert, 2005).

In common, the sexual reproduction is one of the advantages why *D. magna* is a popular species in biotests (Mitchell and Lampert, 2000). There are two types of reproduction, the sexual and the parthenogenetic cycle. In a parthenogenetic period, the female adults lay clone eggs which are genetically identic (DIN EN ISO 6341, 2013). Thus, it is guaranteed that in a new generation only female offsprings are born. In addition, in a sexual period of reproduction, female *D. magna* can produce new male or female neonates bisexual by

laying ephippial eggs, as shown in Figure 5 (B) (Streble and Krauter, 1988; Fent, 2007). These eggs can survive adverse conditions and normally they are laid in autumn (Fent, 2007).

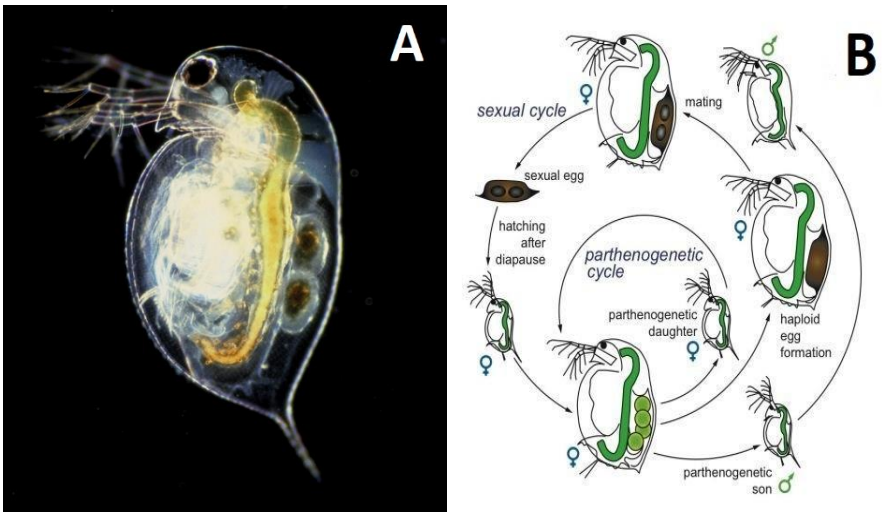


**Figure 5: (A): Body shape of an adult *D. magna* under a dark-field microscope (Hebert, 2005) (B): Reproduction cycles of *D. magna* (Ebert, 2005)**

As described in the OECD Guideline 202 (2004) only neonates younger than 24 hours were used for testing. Moreover they must not be from the first hatch generation of an adult mother and not show any symptoms of stress or disease (DIN EN ISO 6341, 2013). *Daphnia magna* is a popular model organism when testing a substance in acute toxicity tests. Because of its cyclic parthenogenetic reproduction cycle, their easy breeding and handling and their wide range of habitats, they are adapted in several interdisciplinary biotests (Persoone *et al.*, 2009). Additionally, that is one of the reasons why they were chosen as test organisms in this work. Even though they are genetically all the same, it is important to mention that not every individual shows the same reaction on testing pollutants due to biotic and abiotic factors (Fent, 2007).

## 3.2 MAINTENANCE OF *DAPHNIA MAGNA* FOR BIOTESTS

For the maintenance, the daphnids were cultured in 1000 mL glass beakers (Simax borosilicate, Bohemia Cristal) with 800 mL of Elendt M4-breeding medium (see annex 8.2). The breeding medium had a pH value of 7.8 ± 0.5. Each culture contained ten female and adult daphnids which were maintained in an incubator with a light-dark cycle of 8:16 hours and a constant temperature of 20 °C ± 1 °C (Figure 6). Once a week, the cultures were fed

with 1.5 mL of dry yeast dissolved in M4-medium with a concentration of 0.1 g yeast on 10 L medium. Additionally, 800 µL of green algae *Chlorella vulgaris* were added every day.

**Figure 6: Incubator with constant breeding temperature and a light-dark cycle**

Furthermore, the breeding medium was changed every week. Firstly, new Elendt M4-breeding medium was prepared and young daphnids were separated from adults. Secondly, the used glass beakers were rubbed clean and filled with approximately 400 – 450 mL of new M4-medium. Finally, all adult daphnids were put back gently into the glass beakers and the rest volume was filled up to 800 mL with used breeding water for acclimation.

Regarding DIN EN ISO 6341 (2013) the dilution medium, which was used for biotests, contained the substances which are shown in Table 3. For the leaching tests and the plastic contact tests, it was freshly prepared with ultrapure water every week and autoclaved (DE-23, Systec), if not used the same day.

**Table 3: Chemicals for the preparation of dilution medium**

| Chemical | Concentration [g·L$^{-1}$] |
|---|---|
| Calcium chloride | 11.76 |
| Magnesium sulphate | 4.93 |
| Sodium bicarbonate | 2.59 |
| Potassium chloride | 0.23 |

## 3.3 SOFT PLASTIC BAITS MÖHRCHEN AND TIDDLER FAST

In this thesis two different SPLs were investigated in acute toxicity tests. Relating to the results of Kielmann (2019), they both depicted extrema as the lowest and the highest total phthalate concentrations, that is why these baits were chosen. Besides, there are many more SPLs from various manufacturers, but reviewing more is not the focus of this work.

The first bait is called Möhrchen and is invented and produced by a company named Lieblingsköder in Germany. Its head Jens Puhle wanted to develop the best fishing bait for zander fishes in every situation. Therefore, he elaborated soft plastic bait prototypes together with some experts on the subject (Puhle, 2012). Möhrchen is a bait designed for codfish (Puhle, 2012). It is provided in different lengths from 7.5 cm to 20 cm and equipped with dynamic stripes and a tail for a better generation of streamed water (Puhle, 2012). The specification is a better fish attraction through ultra-violet sensitive substances inside and its typical orange colour (Puhle, 2012). Lengths from 12.5 cm to 15 cm (Figure 7) were used for the tests. The reason why Möhrchen was chosen as a test bait is the presumed fact that it contains only few dangerous PHTHs or PAHs (Kielmann, 2019).

The second SPL from the company Fox International is called Tiddler Fast (Tiddler) which is produced in Great Britain. In contrast to Lieblingsköder, Fox International is a well-established British manufacturer of angling equipment since the 1960's (*Fox Rage About us*, no date). The Tiddler Fast has roughly the same shape as Möhrchen with a flat tail and rippled surface. Moreover, the baits are loaded with attractants to get a higher chance catching fishes like basses (Strozyk, no date). It is presumed that this SPL contains PAHs and phthalates, that is why it was also chosen for the following tests (Kielmann, 2019).

A length of 9 cm was used in the pilot test series and 18 cm (Figure 7) in the main tests, because of another supplier.


**Figure 7: The soft plastic baits Tiddler Fast Hot Tiger (left) and Möhrchen (right)**

## 3.4 OPERATING PROCEDURE FOR LEACHING TESTS

Before starting the main leaching test series, some pre-tests were made. They were necessary to find out which dilution stages show no effect on immobilisation and if the two different acute toxicity tests run stable with laboratory conditions. The main required information for the determination of an $EC_{50}$ concentration of a substance were the monitoring of immobilisation during these tests. On basis of a paper from Lithner et. al (2009), which in turn is grounded on EN 12457-4 (2003), the following leachate method was used.

Preparing the pilot tests, a liquid to solid ratio of 10 $L \cdot kg^{-1}$ was used. Therefore, approximately 20 g of each SPL was cut into 10·10 mm pieces and put into two 250 mL glass bottles (Schott Duran), subsequently. Table 20 and Table 23 in annex shows the true values of the weighed baits. Afterwards each bottle was filled up with 200 mL ultrapure water, plastic caps were screwed on top and everything was fixed on a horizontal shaker table (Promax 1020, Heidolph). In comparison to the method of Lithner et. al (2009) the shaker table speed was set to 120 $r \cdot min^{-1}$ instead of 90 $r \cdot min^{-1}$. This ensures that plastic pieces do not agglomerate. Finally, the bottles stayed on the shaker table for at least 24 hours, which was necessary to guarantee that the lipophilic pollutants diffused into the mobile phase.

The following step was the removing of the soft plastic bait pieces from the glass bottles and the preparation of the six-well-plates. Hence, leaching water and dilution medium was pipetted into wells with descending concentrations, as shown in Table 4.

**Table 4: Dilution stages of the pilot leaching test with related volumes of the containing substances**

| Dilution stage | Volume dilution medium [mL] | Volume leaching water [mL] |
|---|---|---|
| 1:1 | - | 10.0 |
| 1:2 | 5.0 | 5.0 |
| 1:4 | 7.5 | 2.5 |
| 1:8 | 8.75 | 1.25 |
| 1:16 | 9.375 | 0.625 |
| 1:32 | 9.6875 | 0.3125 |

After completing the pilot test series, the dilutions 1:16 and 1:32 were not examined in biotests, as they did not cause any reactions to the daphnids (Table 16 and Table 17 annex). Another second pilot leaching test showed that a dilution of 1:1.5 and 1:3 is also not required because of mostly same results as in 1:1 and 1:2 (Table 18 and Table 18 annex). On the one hand most of the operating procedure, as it worked out for the pilot tests, was used for main tests as well. The cutting of the soft plastic baits and filling it into 250 mL glass bottles were exact the same steps (Figure 8). On the other hand, instead of using ultrapure water, this time the same amount of dilution medium was filled into the bottles. This change allows the neonates to better acclimate when they are put into the wells. Additionally, it minimises possible sources of error using the same medium during the toxicity test. Furthermore, every dilution step had four replicates with five daphnids each in it, to get plausible results and to base on DIN EN ISO 6341 (Figure 9). Finally, it has to be mentioned that leaching test method follows the standard dosing procedure, because the dissolved mixed pollutants were put into the system only at the beginning.

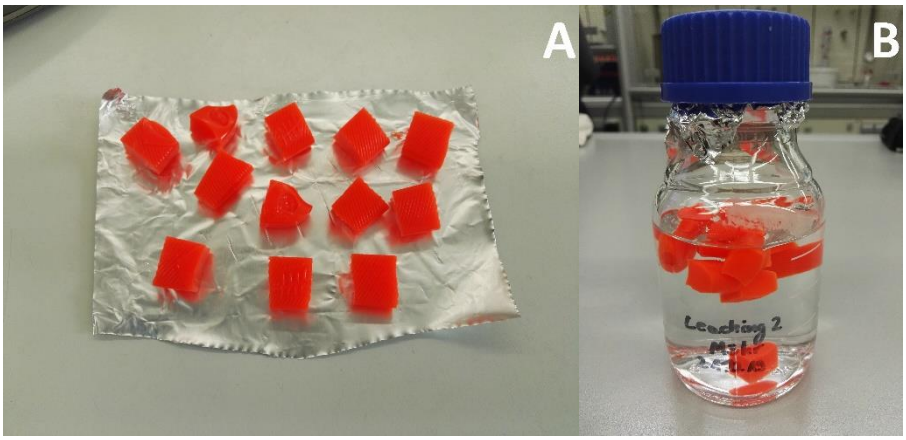


**Figure 8: (A): SPL Möhrchen cut into pieces. (B): SPL put into glass bottles with dilution medium**

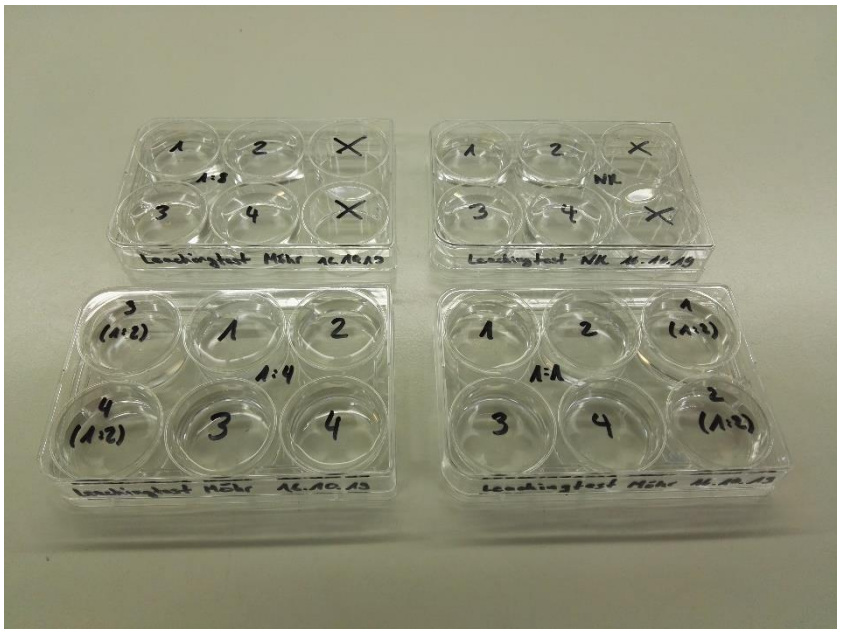

**Figure 9: Set up of a leaching test for Möhrchen before putting in the *Daphnia magna*. Each dilution step has four replicates**

## 3.5 OPERATING PROCEDURE FOR PLASTIC CONTACT TESTS

In contrast to the leaching test, a plastic contact test was prepared simultaneously. In this case the two soft plastic baits were cut into small pieces and spilled with liquid nitrogen. Immediately after that, the frozen and brittle material has been stamped with a pestle in a mortar, so the particle size was lower than 5 mm. This necessary step was done to allow comparison with average microplastic particles in natural environment. The shredded and ground plastic baits were put into six-well-plates, afterwards. For each dilution step, as shown in Table 5, one well was used. In addition, the same liquid to solid ratio of 10 $L \cdot kg^{-1}$ as in the leaching sample was continued. Furthermore, a stainless grid with a mesh size of 1 mm was cut into round shape and put on the bait fraction to prevent the plastic from

floating up to the water surface. Besides, another six-well plate with four wells and the same grid for a negative control was prepared. In the end, every well was filled up with 10 mL dilution medium and placed on an orbital shaker table (Multi Shaker DOS 10L, neoLab) with 120 $r \cdot min^{-1}$ for 24 hours.

**Table 5: Dilution stages of the pilot plastic contact test with related weights of soft plastic debris**

| Dilution stage | Sample weight [g] |
|---|---|
| 1:1 | 1.0 |
| 1:2 | 0.5 |
| 1:4 | 0.25 |
| 1:8 | 0.125 |
| 1:16 | 0.0625 |
| 1:32 | 0.03125 |

For both tests and the negative control, five neonates were put into each well. Afterwards, everything was stored in an incubator for 24 hours until they were ready to be analysed.

As opposed to the pre-tests some points were also changed in the main plastic contact tests. In general, most of the operating procedure did not change unless the change of stainless grid. During pilot tests, a grid with a mesh size of approximately 1 mm was used. This led to death of some daphnids, because they swam through the grid holes and got stuck between plastic pieces under it. In order to avoid the problem another stainless grid with a mesh size of 0.25 mm was used. The prepared six-well plates, as shown in Figure 10, were put onto the shaker table, immediately. Table 21 until Table 25 in annex show the results of pilot plastic contact test series.

Contrary to the leaching method, the plastic contact test approaches with passive dosing. The SPL debris stay in the test vessels during the whole test. Even though the six-well plates shake for at least 24 hours the plastic pieces can still emit pollutants. As a result, there is probably no equilibrium between the mobile and stationary phase. This aspect makes up the difference to the passive dosing method.

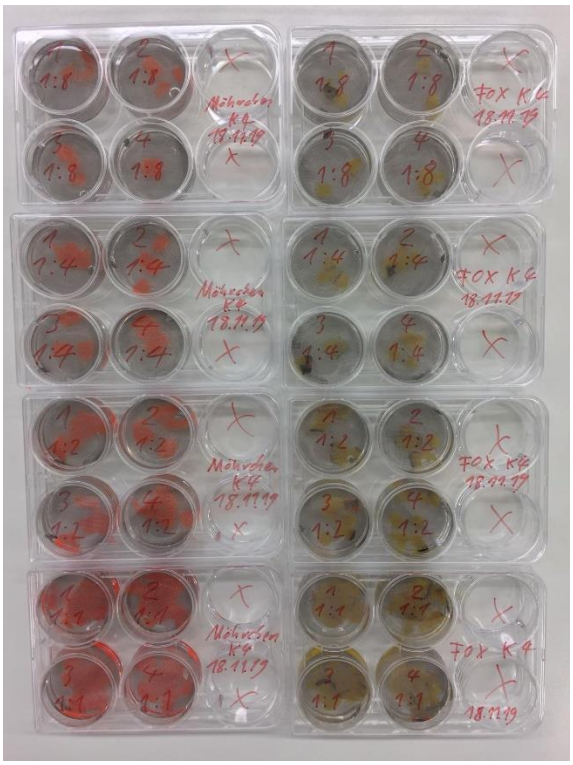

**Figure 10: Set up of a plastic contact test with Möhrchen (left) and Tiddler (right). Each dilution step has its own six-well plate (Kielmann, 2019)**

## 3.6 GC-MS MEASUREMENT

### 3.6.1 GC-MS THEORY

Measurements with gas chromatographs are precise, fast and efficient (Chauhan, 2014). That is why many laboratories make use of this method (Chauhan, 2014). Compared with other methods like liquid chromatography this analysis method has a significant advantage. The pollutants which are analysed in this present thesis are mostly elusive, so it is important to have a mobile phase consisting of gas instead of liquid, thus GC-MS analysis was chosen.

The schematic build-up of a gas chromatograph, as shown in Figure 11, begins with the injector port, where the sample is transferred into a thermostatic column through a carrier gas (Schwedt and Vogt, 2010). Inside the column, the substances are heated up to 350 °C where thus an equilibrium between the mobile and the solid phase establishes (Schwedt and Vogt, 2010). The fractionation happens through temporally different adsorption of the molecules on the interface layer. Generally, the smaller the fractions are, the later they adhere on the solid phase.

Afterwards, the separation of the substances is taking place in the mass spectrometer. Therefore, the most common system is the EI (electron impact) chamber. An electron stream puts the molecules on a higher energy level, thus positive charged ions are the result (Bienz *et al.*, 2016). They are accelerated and focused, subsequently (Bienz *et al.*, 2016)

Additionally, the ions are segmented after their m/z (mass-to-charge ratio) in the mass analyser. This step enables a separation of substances with same retention times. At the end, a detector generates an electronic signal what can be processed further with a computer. The result is a chromatogram in which different peaks are shown.

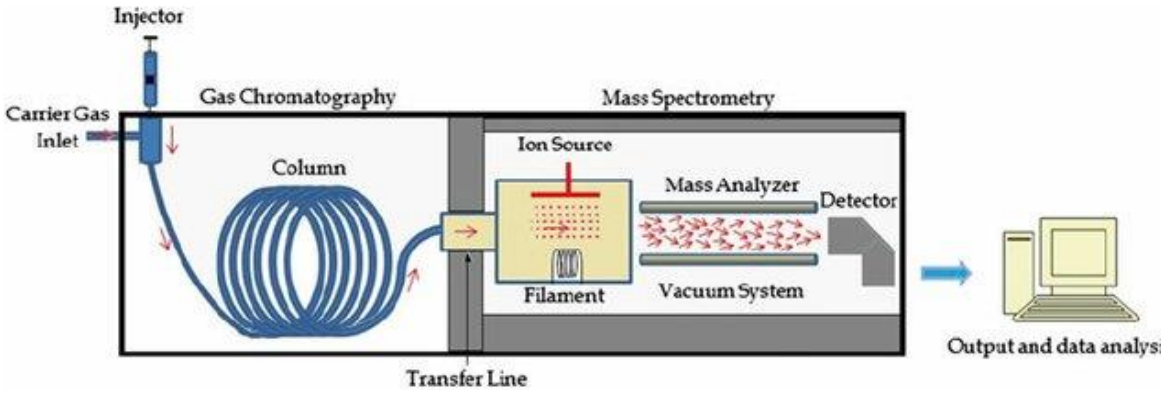


**Figure 11: Diagram of a coupled GC-MS System**

The chemical analysis of the samples took place with a gas chromatograph (7890A GC system, Agilent Technologies) coupled with a mass spectrometer (5975C Inert XL MSD with Triple-Axis Detector). The whole system was equipped with a multi-purpose sampler (MPS) autosampler (MPS 2XL-Twister, Gerstel) where an automated liner exchange system (ALEX) (Gerstel) was integrated. At first, the sample was given onto the cold injection system (CIS). The carrier gas, consisting of helium, transferred the substances through the capillary column (HP-5MS, 325 °C: 30 m x 250 µm x 0.25 µm, Agilent Technologies) where they were inspected in a quadrupole mass analyser, afterwards. In Table 6 to Table 8 the adjustments for PAH and PHTH sample measurements are shown.

**Table 6: CIS temperature program (Kielmann, 2019)**

| | Step | Description | Temperature |
|---|---|---|---|
| **PAHs** | 1 | Start temperature | 35 °C |
| | 2 | Heating-up | 35 °C to 290 °C with 12 °C·min$^{-1}$ |
| | 3 | 15 minutes hold time | 290 °C |
| **PHTHs** | 1 | Start temperature | 35 °C |
| | 2 | Heating-up | 35 °C to 290 °C with 12 °C·min$^{-1}$ |
| | 3 | 13 minutes hold time | 290 °C |

**Table 7: GC oven temperature program (Kielmann, 2019)**

| Step | Description | Temperature |
|---|---|---|
| 1 | Start temperature | 50 °C |
| 2 | Heating-up | 50 °C to 280 °C with 30 °C·min$^{-1}$ |
| 3 | 0 minutes hold time | 280 °C |
| 4 | Heating-up | 280 °C to 310 °C with 15 °C·min$^{-1}$ |
| 5 | 4 minutes hold time | 310 °C |

**Table 8: MS parameter settings (Kielmann, 2019)**

| Description | Value |
|---|---|
| MS Transfer Line Temperature | 280 °C |
| Source Temperature | 230 °C |
| Quadrupole Temperature | 150 °C |
| Ionisation energy | 70 eV |
| Solvent delay | 6 minutes |
| Dwell time | 80 milliseconds |
| Resolution | High |

### 3.6.2 SAMPLE PREPARATION

Before the samples were analysed by GC-MS, they had to be prepared and cleaned up in different steps. Because of a poorer volatility of water contrary to n-hexane the substances had to be carried from a hydrophilic into a lipophilic phase by liquid extraction. Additionally, the samples would not have been measurable in water, because it damages the GC-MS system.

The first step was to absorb the mixed pollutants in the water phase into a phase containing hexane as a solvent by shaking. For this reason, 20 mL of each sample with the highest concentrations were put into a 50 mL separation funnel. 30 mL of n-hexane was pipetted on top (Figure 12 (A)). Afterwards, the separation funnels were put on a horizontal shaker table (Promax 1020, Heidolph) with 250 $r \cdot min^{-1}$ for at least 5 minutes. This ensured a fast transition of the analyte from water into hexane through a great contact area of both phases. When shaking stopped, the lighter phase got separated from the heavier phase, subsequently (Figure 12 (B)). The separation funnels were fixed on brackets, where the heavier phase got drained through an outlet into new empty 50 mL funnels. Now the hexane was transferred into Erlenmeyer flasks with a total volume of 250 mL. The procedure was made three times, to ensure the whole solute is washed out of the water phase.

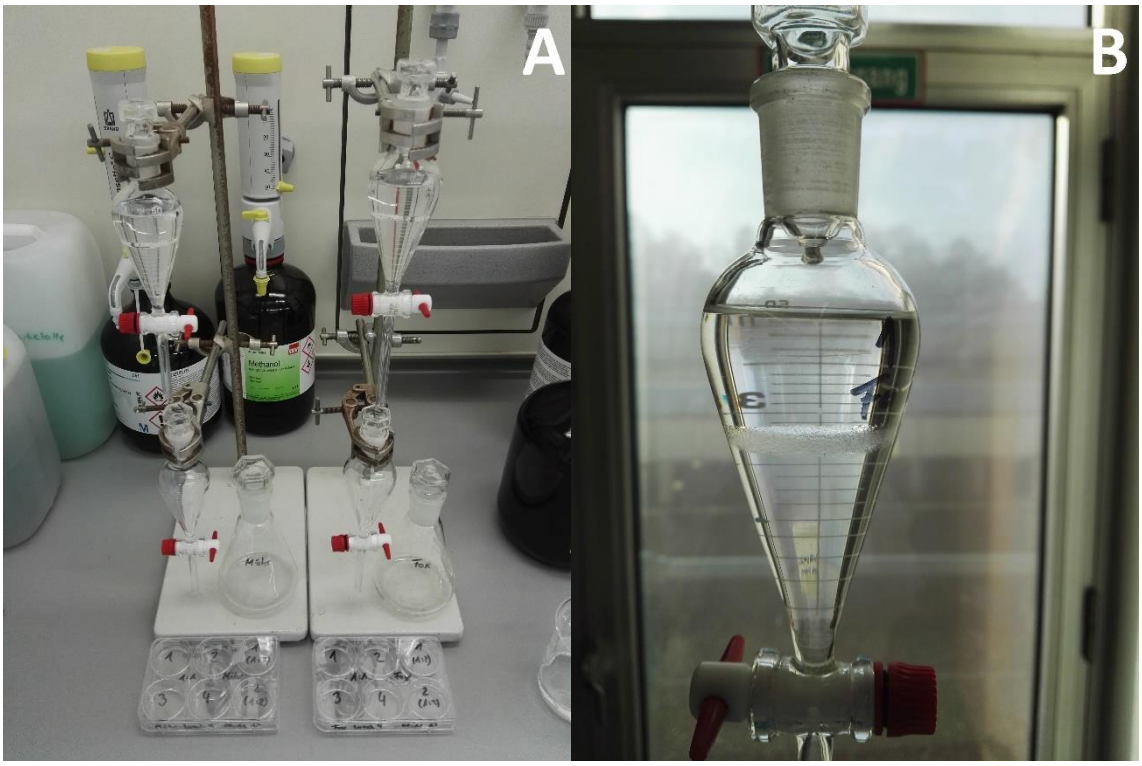


**Figure 12: (A): Set up of the sample preparation before shaking them. (B): A separation funnel after shaking it**

The aim of the second step was the concentration of the dissolved analyte for further processing. Sodium sulphate ($Na_2SO_4$) was put into the Erlenmeyer flasks until it was flocculating when shaken, in order to dry the hexane from remaining water. Afterwards, the sodium sulphate was filtered in a column processor (Baker SPE – 12G, J.T.Baker) with a glass fibre filter on top of it (Figure 13 (A)). The resulting cleaned sample is called eluate. In this case it was the hexane which was caught in a 100 mL flask. Finally, the Erlenmeyer flask was rinsed three times with hexane, to ensure that no analyte remained in the flask.

The third and last step, the eluate evaporated in a rotary evaporator (Laborata 4000 efficient, Heidolph) with 150 $r \cdot min^{-1}$ in a 50 °C water quench and approximately 330 mbar ambient pressure (Figure 13 (B)). The reason is to reduce the hexane and concentrate the mixed pollutants. The liquid was reduced until there was only a tip left. The remaining rest was transferred into a 50 mL flask with tighter tips. The flask was put back on the rotary evaporator to repeat the step. At the end the whole solution was injected into a 1.5 mL GC vial by a 100 µL syringe (Hamilton Company). The extracts were filled up with hexane to 800 µL. The prepared GC vials were stored in a fridge at 4 °C.

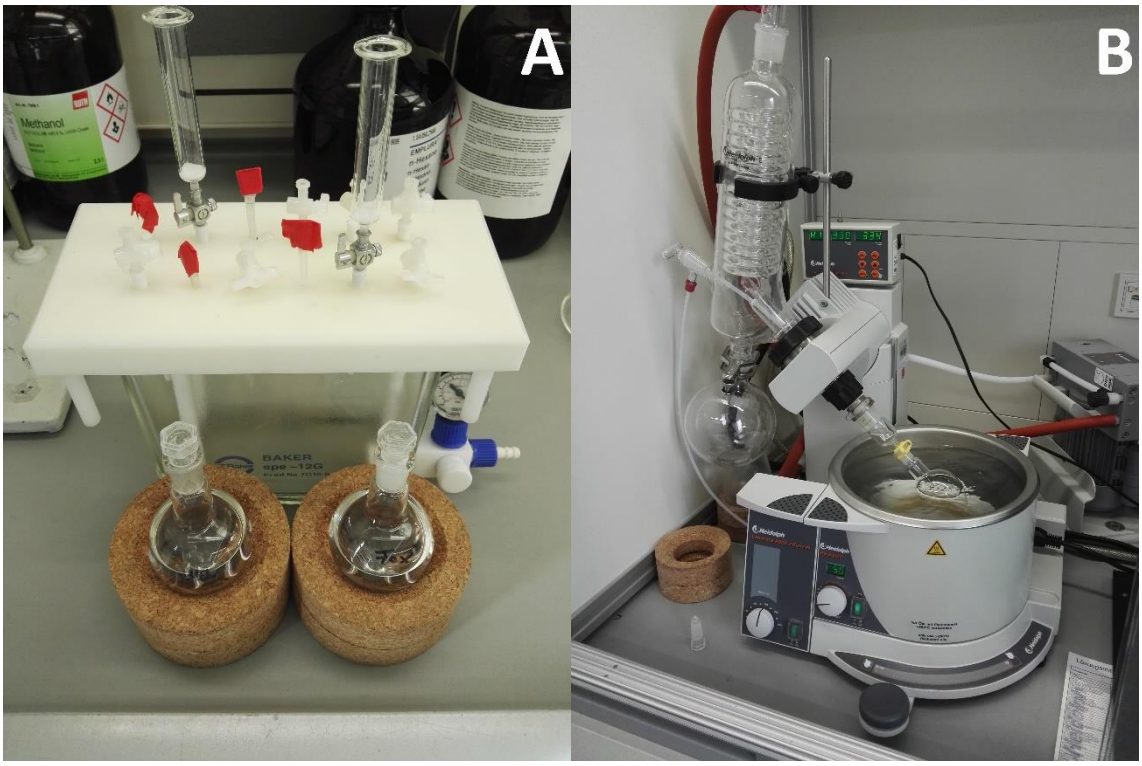


**Figure 13: (A): Baker Bond SPE vacuum basin with glass fibre filters and 100 mL flasks. (B): Set up of a rotary evaporator**

### 3.6.3 STANDARD PREPARATION

The prepared samples were compared with standard solutions with defined concentrations. A mixed PAH standard (PAH Mix 9, Dr. Ehrenstorfer) and a mixed phthalate standard (1 mL Appendix IX Phthalate Mix, AccuStandard) with six compounds was used. Additionally, a dilution series, as shown in Table 9, was made out of it. Afterwards, the standard solutions were pipetted into 1.5 mL GC vials. The vials got filled up with n-hexane to a total volume of 1 mL, subsequently. Caps were screwed on the vials and they were stored at 4 °C for further measurements.

**Table 9: Concentrations of the dilutions and stock solutions of PAH and phthalate standard**

| | **Concentration of PAH standard [pg·mL$^{-1}$]** | **Concentration of PHTH standard [pg·mL$^{-1}$]** |
|---|---|---|
| **Stock solution** | **10,000** | **1,000,000** |
| Dilution 1 | 10 | 10 |
| Dilution 2 | 100 | 100 |
| Dilution 3 | 200 | 1,000 |
| Dilution 4 | - | 10,000 |
| Dilution 5 | - | 100,000 |

# 4 RESULTS

The previous sections describe the theoretical basics, methods and materials that are used in order to get the following displayed results. Leaching and plastic contact tests passed through pre-test series and were optimised, subsequently. First, leaching and afterwards plastic contact test results are shown. Further samples and test results are abbreviated for easier handling, which is explained below.

## 4.1 SAMPLE NOTATION

The notation of the samples is abbreviated and follows a definite pattern. The first two letters in “HT_24hLeach_1” indicate if it is a HT (main test) or VT (pre-test). The middle part names the moment of counting (after 24 hours or 48 hours) and the test type. This can be either “Leach” (leaching test) or “K-K” (plastic contact test). Finally, “1” gives information about the number of repetitions, so “VT_K-K_2” is the second plastic contact test in the pilot test series.

## 4.2 LEACHING TESTS

SPLs from two different manufacturers with predicted mixed toxics were tested on *D. magna* in developed acute leaching test series. The figures below show the immobilisation rates of the daphnids after 24 hours and 48 hours exposure with various calculated concentration of the SPLs.

All four independent tests fulfilled the validity criteria as the oxygen concentration in the negative controls was above 3 $mg \cdot L^{-1}$ and less than 10 % of *D. magna* were immobile. In Figure 14 to Figure 29 the calculated concentrations of solid plastic material after their original weights are plotted against the immobilisation number of *D. magna*. Both, immobilisation and concentration are pictured in a sigmoidal concentration-response curve model with variable slope (GraphPad Prism 8.3.0, San Diego California).

### 4.2.1 1ST LEACHING TEST

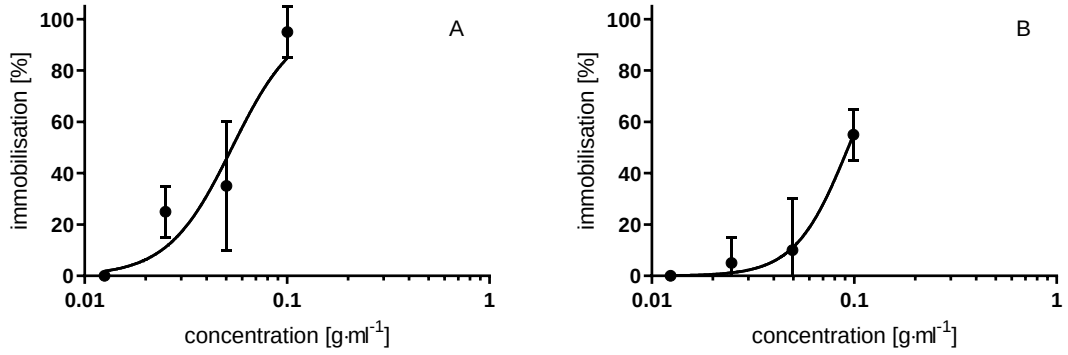


**Figure 14: Concentration-response curves of HT_24hLeach_1 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

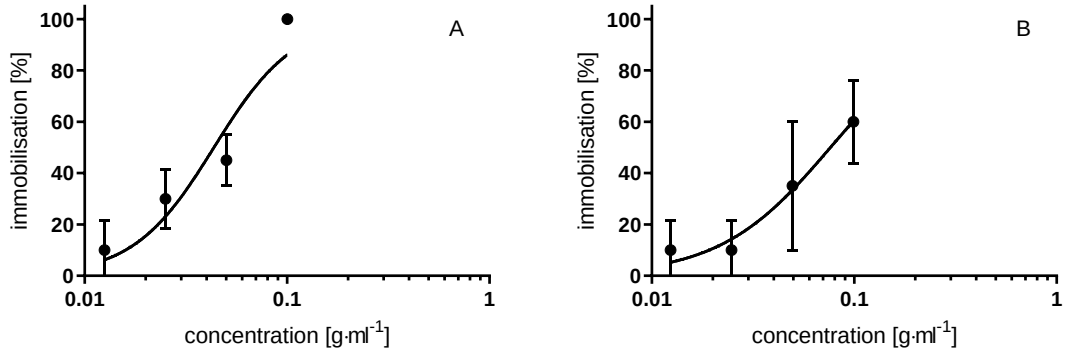


**Figure 15: Concentration-response curves of HT_48hLeach_1 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

In Figure 14 and Figure 15 the results of the first leaching test after 24 hours and 48 hours exposure with soft plastic bait Tiddler (A) and Möhrchen (B) are shown. Basically, all curves approached a sigmoidal curve shape. Four dilution stages with each four replicates were performed in the test series. As a result, the four marked points depicted a conclusion of these independent replicates with various standard deviations which are displayed as error bars.

The tests after 24 hours exposure (Figure 14) depicted $EC_{50}$ values of 0.05 g·mL$^{-1}$ for Tiddler and 0.09 g·mL$^{-1}$ for Möhrchen. The 95 % confidence interval of this test was 0.04 g·mL$^{-1}$ to 0.06 g·mL$^{-1}$ for Tiddler and 0.08 g·mL$^{-1}$ to 0.10 g·mL$^{-1}$ for Möhrchen.

The tests in Figure 15 resulted in $EC_{50}$ values of 0.04 g·mL$^{-1}$ for Tiddler and 0.08 g·mL$^{-1}$ for Möhrchen. The 95 % confidence interval of this test was 0.04 g·mL$^{-1}$ to 0,05 g·mL$^{-1}$ for Tiddler and 0.05 g·mL$^{-1}$ to 0.11 g·mL$^{-1}$ for Möhrchen.

### 4.2.2 2ND LEACHING TEST

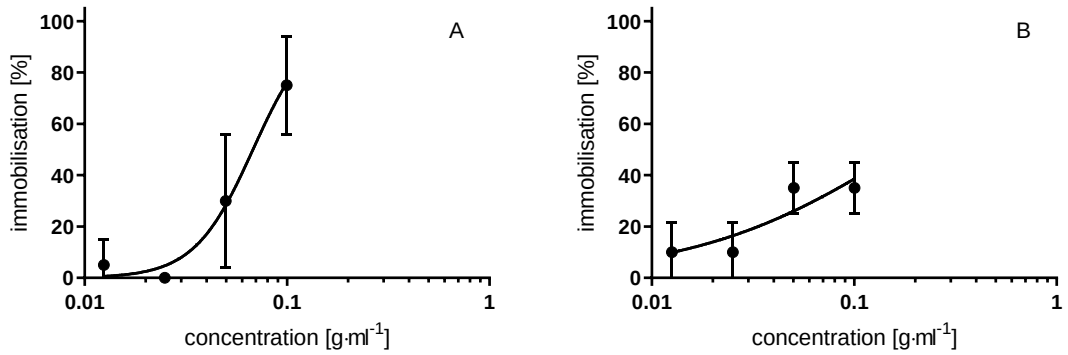


**Figure 16: Concentration-response curves of HT_24hLeach_2 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

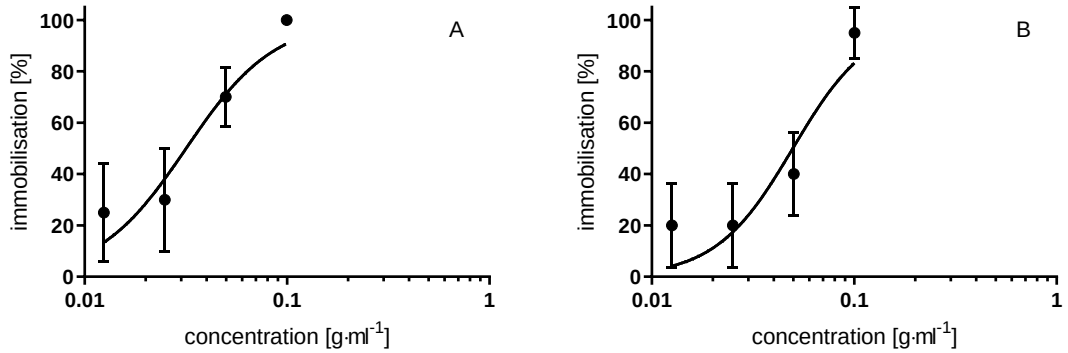


**Figure 17: Concentration-response curves of HT_48hLeach_2 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

Figure 16 and Figure 17 show the results of the second leaching test. $EC_{50}$ values of 0.07 g·mL$^{-1}$ for Tiddler and 0.17 g·mL$^{-1}$ for Möhrchen were determined after 24 hours exposure time (Figure 16). The 95 % confidence interval for Tiddler was between 0.06 g·mL$^{-1}$ and 0.08 g·mL$^{-1}$ and for Möhrchen between 0.07 g·mL$^{-1}$ and 0.42 g·mL$^{-1}$.

$EC_{50}$ values of 0.03 g·mL$^{-1}$ for Tiddler and 0.05 g·mL$^{-1}$ for Möhrchen were determined after 48 hours (Figure 17). The resulting 95 % confidence interval for Tiddler was 0.03 g·mL$^{-1}$ to 0.04 g·mL$^{-1}$ and for Möhrchen from 0.04 g·mL$^{-1}$ to 0.07 g·mL$^{-1}$.

### 4.2.3 3RD LEACHING TEST

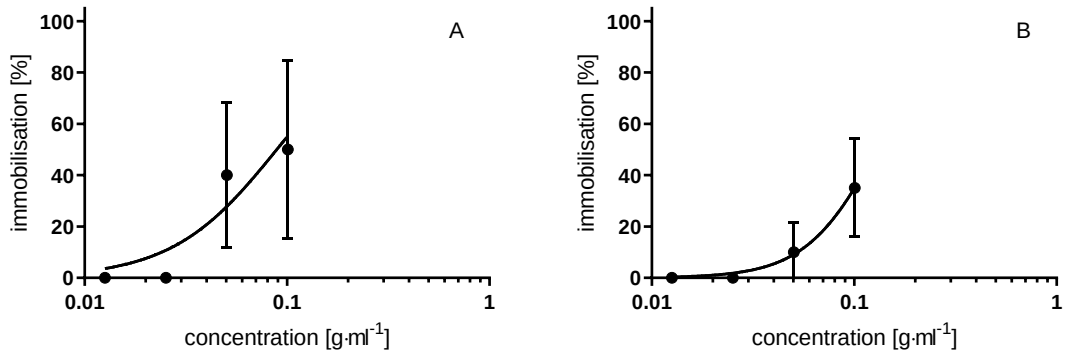


**Figure 18: Concentration-response curves of HT_24hLeach_3 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

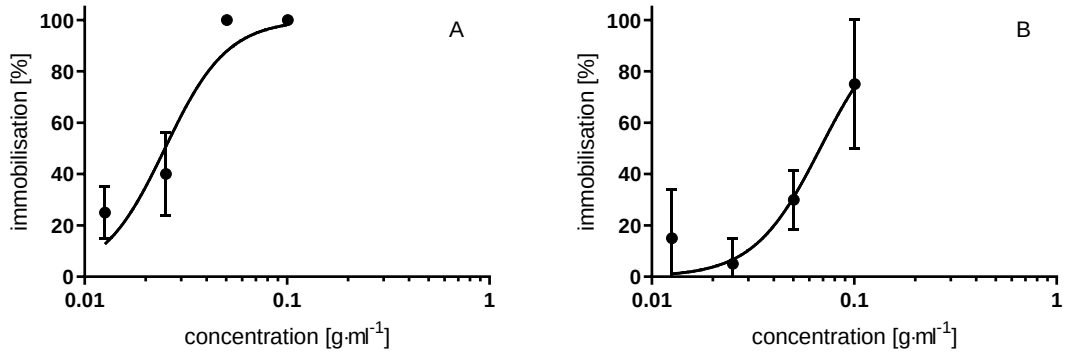


**Figure 19: Concentration-response curves of HT_48hLeach_3 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

In the third leaching test after 24 hours the *D. magna* were immobile at a half maximal effect concentration of 0.09 g·mL$^{-1}$ for Tiddler and 0.13 g·mL$^{-1}$ for Möhrchen (Figure 18). The 95 % confidence interval for Tiddler varied from 0.05 g·mL$^{-1}$ to 0.15 g·mL$^{-1}$ and for Möhrchen from 0.09 g·mL$^{-1}$ to 0.18 g·mL$^{-1}$.

Additionally, the results after 48 hours, as shown in Figure 19, depicted an $EC_{50}$ value of 0.03 g·mL$^{-1}$ for Tiddler and 0.07 g·mL$^{-1}$ for Möhrchen. For Tiddler a 95 % confidence interval from 0.02 g·mL$^{-1}$ to 0.03 g·mL$^{-1}$ and an interval from 0.05 g·mL$^{-1}$ to 0.09 g·mL$^{-1}$ was calculated for Möhrchen.

### 4.2.4 4TH LEACHING TEST

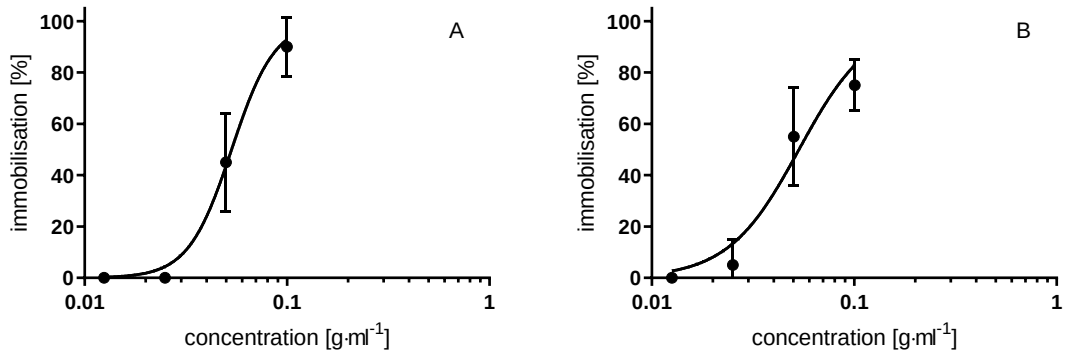


**Figure 20: Concentration-response curves of HT_24hLeach_4 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

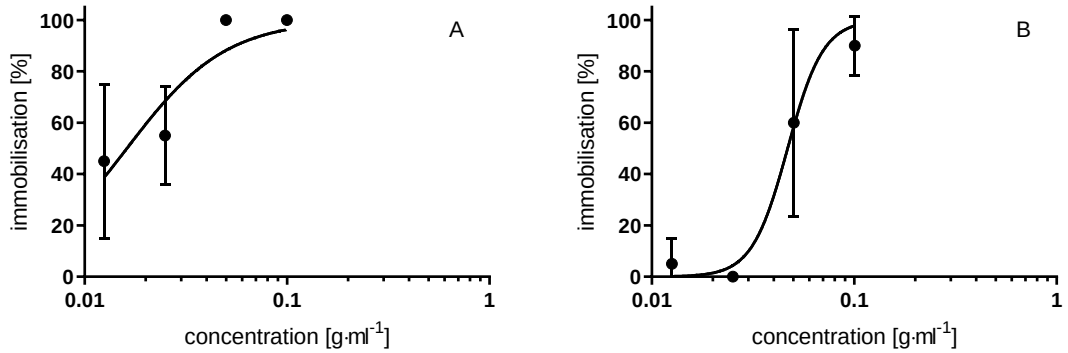


**Figure 21: Concentration-response curves of HT_48hLeach_4 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates**

For both Tiddler and Möhrchen an $EC_{50}$ value of 0.05 g·mL$^{-1}$ and a 95 % confidence interval for Tiddler of 0.05 g·mL$^{-1}$ to 0.06 g·mL$^{-1}$ and 0.04 g·mL$^{-1}$ to 0.06 g·mL$^{-1}$ for Möhrchen after 24 hours exposure was calculated (Figure 20).

After 48 hours the effective concentrations for Tiddler and Möhrchen varied (Figure 21). Thus, *D. magna* showed an $EC_{50}$ value of 0.02 g·mL$^{-1}$ with Tiddler and 0.05 g·mL$^{-1}$ with Möhrchen. The resulting 95 % confidence interval for Tiddler diversified from 0.01 g·mL$^{-1}$ to 0.02 g·mL$^{-1}$. For Möhrchen it varied between 0.04 g·mL$^{-1}$ to 0.06 g·mL$^{-1}$.

## 4.3 PLASTIC CONTACT TESTS

The acquisition of results stays the same as in the leaching test series (see 4.2). Soft plastic baits called Tiddler and Möhrchen from two different manufacturers were used in a developed acute plastic contact test series. The only difference to leaching test series is the fact that every replicate in each dilution stage has its own SPL debris weight.

This gives sixteen various original sample weights in a single test. For that reason, mean values were calculated for each dilution stage.

The figures below show the results of the plastic contact test series with 24 hours and 48 hours exposure time displayed in sigmoidal concentration-response curves. With an immobilisation rate less than 10 % and an oxygen concentration higher than 3 mg·L$^{-1}$ after 48 hours in the negative controls the tests were all valid.

### 4.3.1 1ST PLASTIC CONTACT TEST

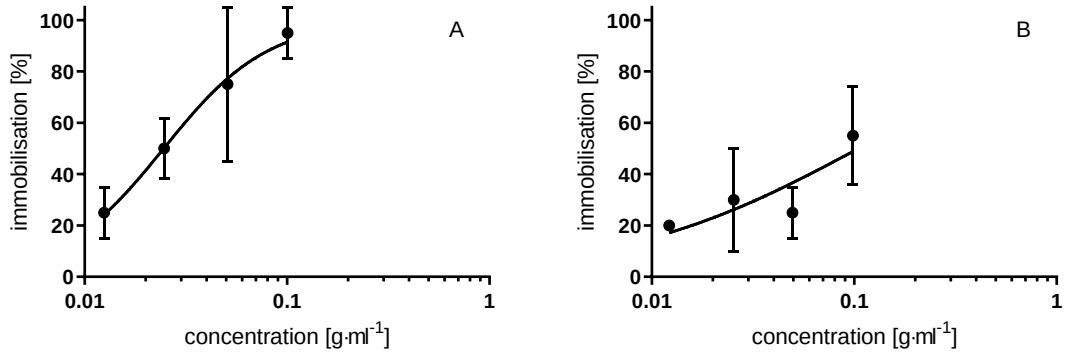


**Figure 22: Concentration-response curves of HT_24hK-K_1 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

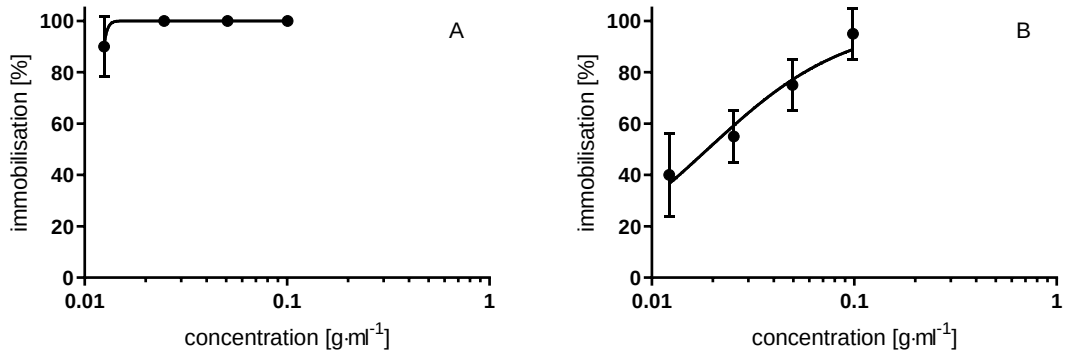


**Figure 23: Concentration-response curves of HT_48hK-K_1 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

In Figure 22 the 24 hours test results are shown. Only graph A approaches to a sigmoidal curve shape. $EC_{50}$ values of 0.02 g·mL$^{-1}$ for the SPL Tiddler and 0.11 g·mL$^{-1}$ for Möhrchen. Moreover, the confidence interval with a 95 % estimation for Tiddler started at a concentration of 0.02 g·mL$^{-1}$ and ended at 0.03 g·mL$^{-1}$. The 95 %confidence interval for Möhrchen ranged from 0.06 g·mL$^{-1}$ to 0.24 g·mL$^{-1}$.

Figure 23 shows the first plastic contact test results after 48 hours. For Tiddler, an approximate $EC_{50}$ concentration of 0.01 was determined. This was the first test, where

nearly every neonate was immobile after 48 hours. Hence, the calculation of an exact $EC_{50}$ value and 95 % confidence interval was not possible. Contrary, a more precise $EC_{50}$ concentration of 0.02 g·mL$^{-1}$ was calculated for Möhrchen. A corresponding 95 % confidence interval of ranged from 0.01 g·mL$^{-1}$ to 0.03 g·mL$^{-1}$.

### 4.3.2 2ND PLASTIC CONTACT TEST

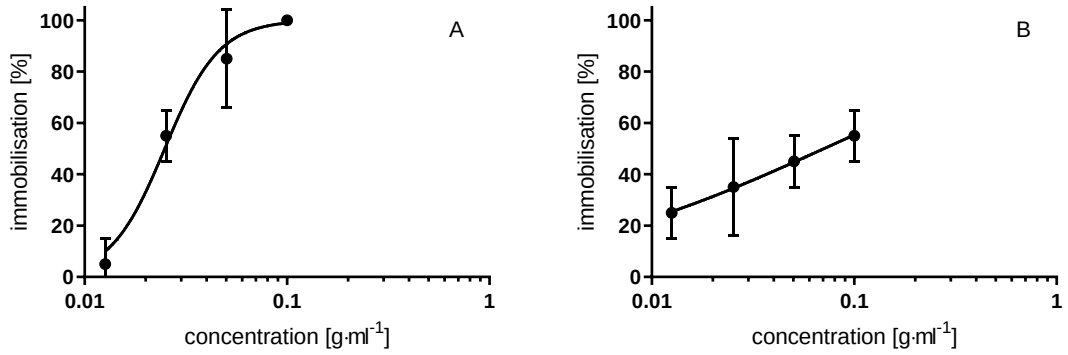


**Figure 24: Concentration-response curves of HT_24hK-K_2 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

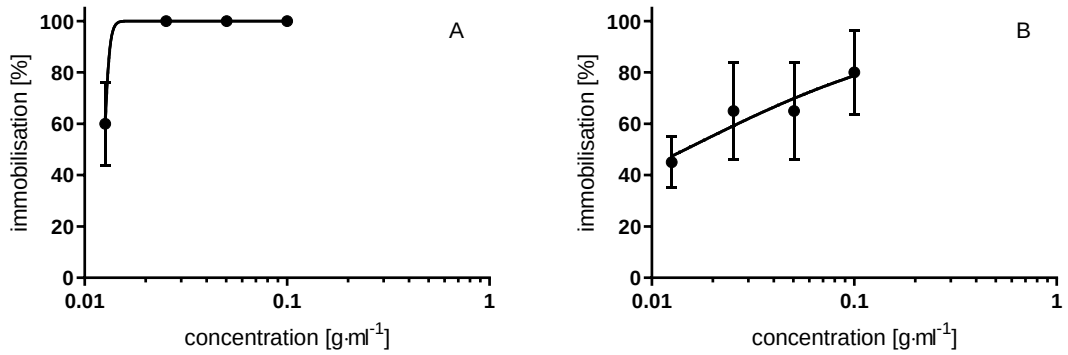


**Figure 25: Concentration-response curves of HT_48hK-K_2 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

The second plastic contact test resulted in 24 hours $EC_{50}$ values of 0.03 g·mL$^{-1}$ for Tiddler and 0.07 g·mL$^{-1}$ for Möhrchen (Figure 24). The 95 % confidence intervals were from 0.02 g·mL$^{-1}$ to 0.03 g·mL$^{-1}$ for Tiddler and from 0.04 g·mL$^{-1}$ to 0.13 g·mL$^{-1}$ for Möhrchen.

According to the first graph in Figure 25, the $EC_{50}$ value for Tiddler was also approximate due to a high immobilisation rate giving a concentration of 0.01 g·mL$^{-1}$ and no 95 % confidence interval. In the 48 hours test results Möhrchen had an exact $EC_{50}$ value of 0.01 g·mL$^{-1}$. The 95 % confidence interval varied from 0.01 g·mL$^{-1}$ to 0.03 g·mL$^{-1}$.

### 4.3.3 3RD PLASTIC CONTACT TEST

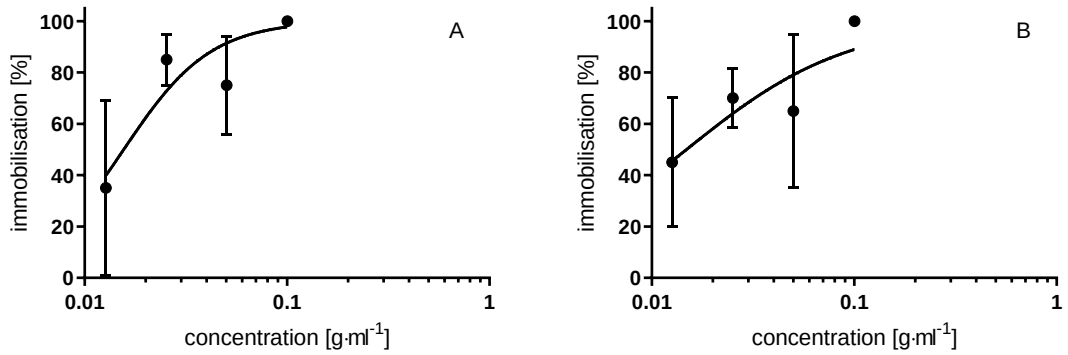


**Figure 26: Concentration-response curves of HT_24hK-K_3 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

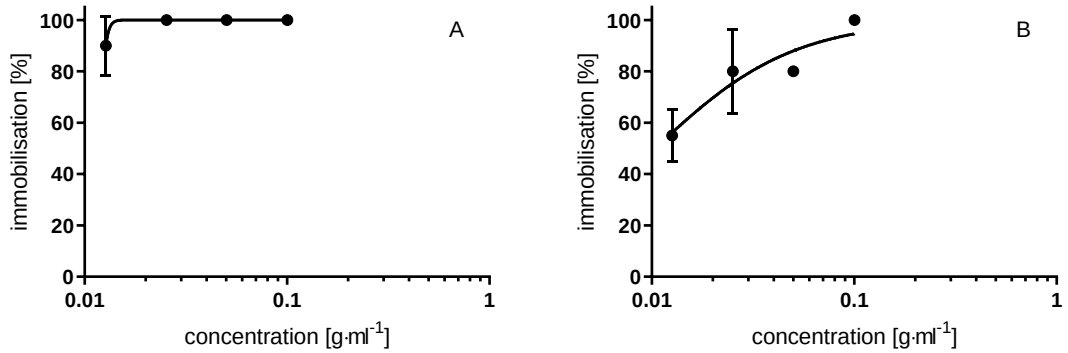


**Figure 27: Concentration-response curves of HT_48hK-K_3 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

The third independent plastic contact test resulted in half maximal effective concentrations of 0.02 g·mL$^{-1}$ for Tiddler and 0.01 g·mL$^{-1}$ for Möhrchen (Figure 26). For Tiddler a 95 % confidence interval of 0.01 g·mL$^{-1}$ to 0.02 g·mL$^{-1}$ was calculated. Furthermore, an interval from 0.01 g·mL$^{-1}$ to 0.03 g·mL$^{-1}$ was determined for Möhrchen.

Figure 27 shows no precise $EC_{50}$ value for Tiddler. Thus, there was no confidence interval calculated. The rough $EC_{50}$ value is 0.01 g·mL$^{-1}$. Contrary, for Möhrchen an exact $EC_{50}$ concentration of 0.01 g·mL$^{-1}$ was determined with a corresponding 95 % confidence interval from 0.01 g·mL$^{-1}$ to 0.02 g·mL$^{-1}$.

### 4.3.4 4TH PLASTIC CONTACT TEST

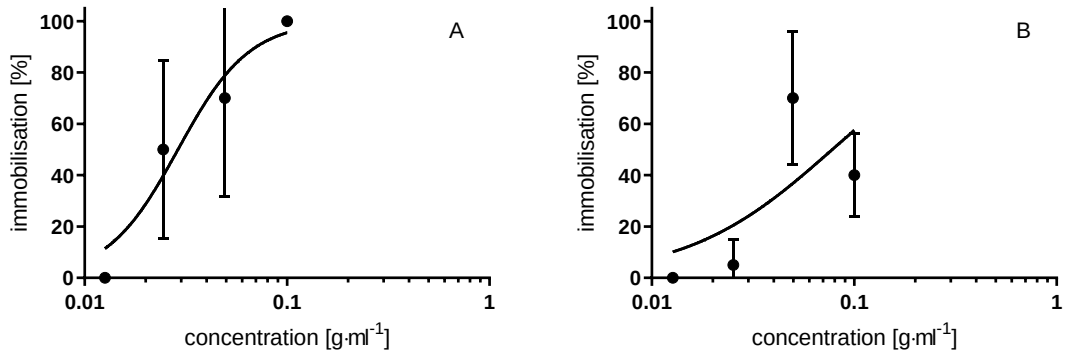


**Figure 28: Concentration-response curves of HT_24hK-K_4 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

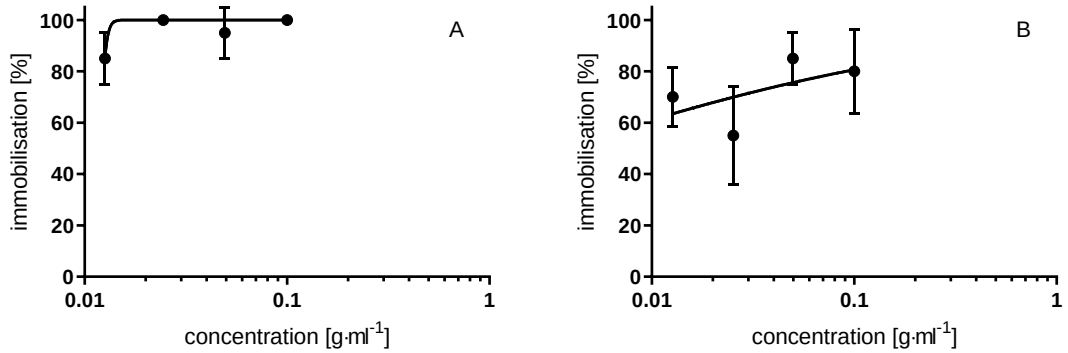


**Figure 29: Concentration-response curves of HT_48hK-K_4 with Tiddler (A) and Möhrchen (B). Plotted are the mean immobilisation rate and the standard deviations of four independent replicates with various original weights**

The last plastic contact test showed 24 hours $EC_{50}$ values of 0.03 g·mL$^{-1}$ for Tiddler and 0.08 g·mL$^{-1}$ for Möhrchen. The 95 % confidence intervals for Tiddler ranged from 0.02 g·mL$^{-1}$ to 0.04 g·mL$^{-1}$ and from 0.04 g·mL$^{-1}$ to 0.16 g·mL$^{-1}$ for Möhrchen (Figure 28).

Like other results for Tiddler after 48 hours exposure in plastic contact test series, there was an estimated $EC_{50}$ value of 0.01 g·mL$^{-1}$ and no calculated 95 % confidence interval. Additionally, also the Möhrchen test results had no precise $EC_{50}$ of approximately 0.003 g·mL$^{-1}$ with a determined 95 % confidence interval of 0.00 g·mL$^{-1}$ to 0.09 g·mL$^{-1}$ (Figure 29).

## 4.4 COMPARISON OF BOTH TESTS WITH $EC_{50}$ VALUES

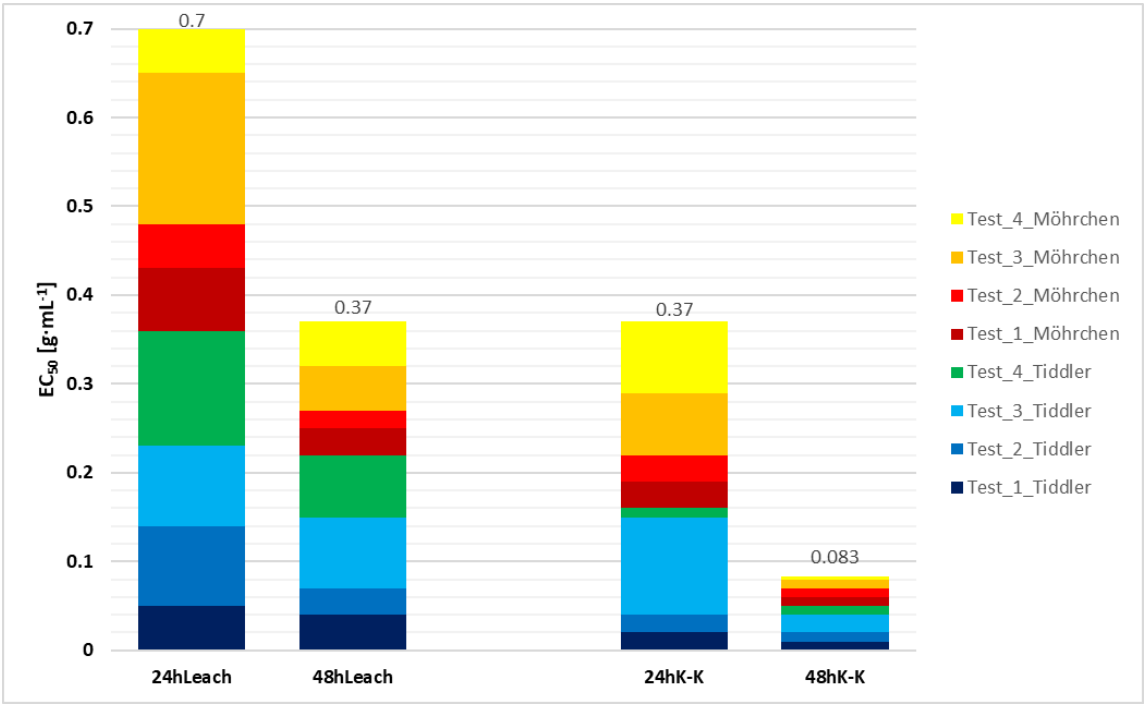


**Figure 30: Representation of EC50 values in leaching and plastic contact test. Plotted are 24 hours and 48 hours values relating to original sample weights for all four tests in each bar**

Figure 30 represents $EC_{50}$ values of leaching and plastic contact test series in a stacked bar graph. The sum of the values is displayed on top of each bar. Values of 48 hours are less than 24 hours exposure time for both tests. Furthermore, leaching tests $EC_{50}$ are greater than for plastic contact tests.

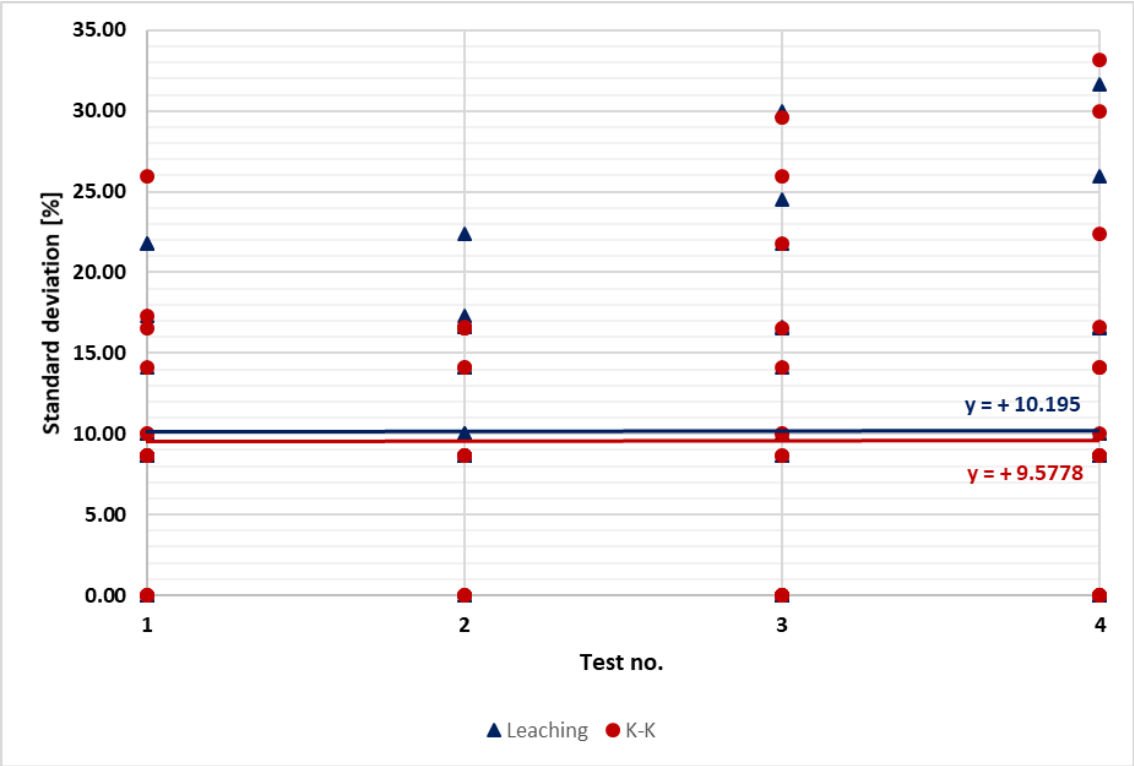


**Figure 31: Summary of standard deviations in leaching and plastic contact test series. Shown are values after 24 hours as well as 48 hours exposure with straight lines relating to the average values**

In Figure 31 the standard deviations of immobilisation rate in leaching and plastic contact test are shown, percentagewise. A relating straight line signifies the average values of the calculated deviations. This line has its y-axis intersection at approximately 10.20 % in leaching test and roughly 9.58 % in plastic contact test series. As a result, four leaching tests had a greater average standard deviations value than plastic contact tests.

## 4.5 COMPARISON OF SOFT PLASTIC BAITS WITH $EC_{50}$ VALUES

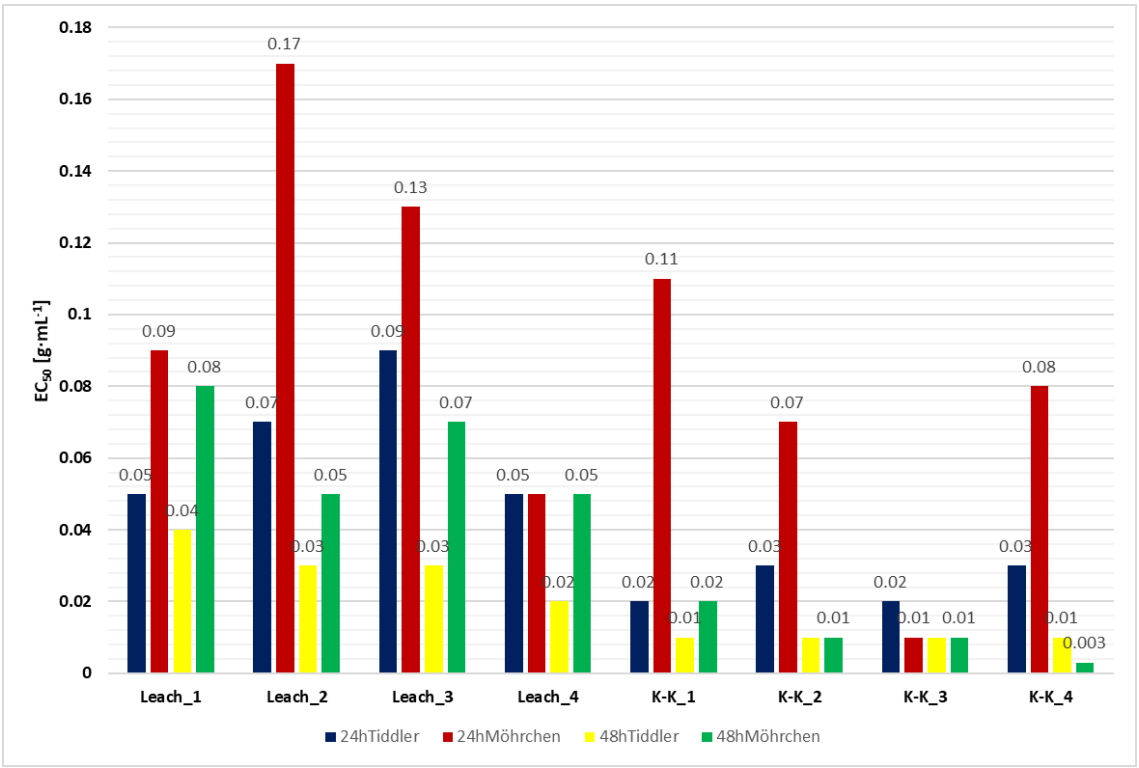


**Figure 32: Comparison of SPL with 24 hours and 48 hours $EC_{50}$ values**

The bar graph in Figure 32 above shows 24 hours as well as 48 hours $EC_{50}$ concentrations of Tiddler and Möhrchen in each test. The highest measured concentration amounts to 0.17 $g \cdot mL^{-1}$ whereas the lowest was 0.003 $g \cdot mL^{-1}$. Mostly, Möhrchen depicted greater $EC_{50}$ values compared to Tiddler, especially after 24 hours test time. Outliers are K-K_3 and K-K_4. Möhrchen had lower or same $EC_{50}$ values than Tiddler in these tests.

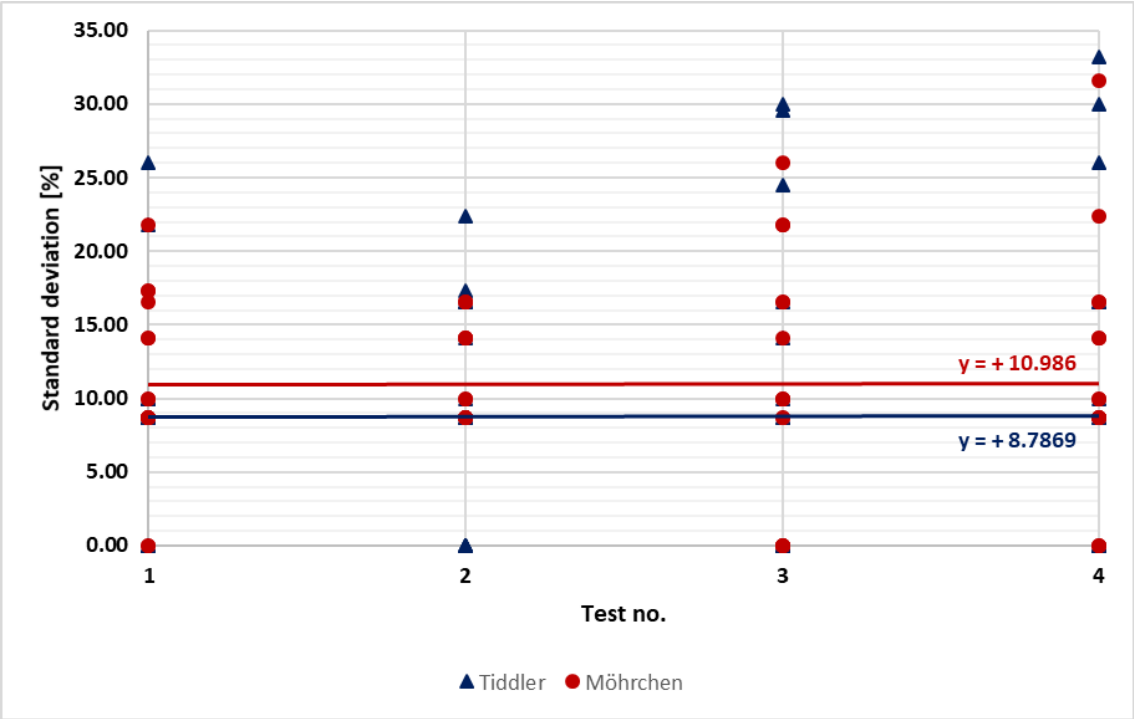


**Figure 33: Summary of standard deviations for Tiddler and Möhrchen. Shown are values after 24 hours as well as 48 hours exposure with straight lines relating to the average values**

A graphical overview of standard deviations of both SPL is shown in Figure 33. The shown points are percental standard deviations of immobilisation rates in each test. Two straight lines were plotted as well. The lower one refers to the average standard deviation of Tiddler with approximately 8.79 %. The other line has its y-axis intersection at 10.99 % and bases on values from SPL Möhrchen.

## 4.6 GC-MS RESULTS

All GC-MS measurement results refer to a 48 hours exposure time for a dilution stage of 1:1. After each acute toxicity test was finished, the samples were prepared for GC-MS analysis, subsequently. Afterwards they were stored in a refrigerator and used on various dates.

Table 10 and Table 11 show the samples which had at least one value over the practical limit of quantitation (LOQ). Table 50 and Table 51 in annex contain all measured values of PAH and PHTH investigations for a complete overview. The LOQ value makes three times the average blank value for each pollutant. As an example, the following equation depicts the LOQ for Naphthalene.

$$\mathrm{LOQ_{NAPH}} = 3 \cdot \overline{\mathrm{Blank}}_{\mathrm{NAPH}}$$

### 4.6.1 PAH INVESTIGATION RESULTS

**Table 10: Samples of PAH measurement. Only samples with values over the LOQ are shown**

| | Measured value [µg·L$^{-1}$] | | | |
|---|---|---|---|---|
| **Sample** | **BaA** | **CHR** | **BkF** | **BghiP** |
| HT_48hK-K_3_Tiddler | 0.3 | 0.2 | 0.9 | 0.1 |
| HT_48hLeach_4_Möhrchen | | | 0.2 | |

For PAH investigation results, as shown in Table 10, only two samples reached values over the LOQ. The results for HT_48hK-K_3_Tiddler show that this sample contained higher BaA, Chrysene, BkF and BghiP concentrations. The substance BkF was traced in the HT_48hLeach_4_Möhrchen sample with 0.2 µg·L$^{-1}$.

### 4.6.2 PHTHALATE INVESTIGATION RESULTS

**Table 11: Samples of PHTH measurement. Only values over the LOQ are shown**

| | Measured value [µg·L$^{-1}$] | | | | | |
|---|---|---|---|---|---|---|
| **Sample** | **DBP** | **BBP** | **DEHP** | **DNOP** | **DINP** | **DIDP** |
| HT_48hK-K_1_Tiddler | | 1 | 9 | 5 | | |
| HT_48hK-K_1_Möhrchen | | 14 | 13 | 11 | | |
| HT_48hK-K_2_Tiddler | 125 | 77 | 60 | 58 | 42 | 39 |
| HT_48hK-K_2_Möhrchen | | 24 | 19 | 8 | | |
| HT_48hK-K_3_Tiddler | | 4 | 18 | 8 | 75 | |
| HT_48hK-K_3_Möhrchen | | 5 | 17 | 6 | 22 | |
| HT_48hLeach_1_Tiddler | | 2 | 82 | 12 | 94 | |
| HT_48hLeach_1_Möhrchen | | 32 | 34 | 17 | 26 | |
| HT_48hLeach_2_Tiddler | 53 | 115 | 82 | 64 | 49 | 44 |
| HT_48hLeach_2_Möhrchen | | 12 | 15 | 4 | | |
| HT_48hLeach_3_Tiddler | | 68 | 49 | 25 | 82 | 17 |
| HT_48hLeach_3_Möhrchen | | 9 | 14 | 4 | | |
| HT_48hLeach_4_Tiddler | | 49 | 35 | 15 | 57 | |
| HT_48hLeach_4_Möhrchen | | 4 | 13 | 11 | | |

All samples depict various phthalate compounds with concentrations over the LOQ which are shown in Table 11. DBP was found in two samples and both from Tiddler with the highest amount of 125 $\mu g \cdot L^{-1}$. BBP, DEHP and DNOP were traced in all samples with various concentrations. Moreover, DEHP provided the highest amount of these three with 460 $\mu g \cdot L^{-1}$ in total. In nearly all test results, Tiddler showed higher phthalate concentrations than Möhrchen, except in HT_48hK-K_1.

# 5 DISCUSSION

## 5.1 DISCUSSION OF $EC_{50}$ VALUES

At first, the $EC_{50}$ values of both Tiddler and Möhrchen must be discussed. As shown in Figure 32, Möhrchen has greater $EC_{50}$ values than Tiddler by trend, except two outliers. So Möhrchen has less effects in acute biotests on *D. magna*. In turn, this fact suggests that Tiddler releases a higher amount of mixed toxic substances into dilution medium. The two outliers are both from plastic contact test (HT_24hK-K_3 and HT_48hK-K_4). In these results Tiddler has greater $EC_{50}$ values than Möhrchen, however the deviation is much smaller than in the other tests. It is likely that these outliers are caused due to random errors during preparation or counting the immobile neonates.

Another trend of $EC_{50}$ values is observable when comparing leaching and plastic contact test. Both summaries of 24 hours and 48 hours values of leaching test series are higher compared to plastic contact test series, as depicted in Figure 30. Because of smaller values it could be argued that plastic contact test is more sensitive than leaching test. Another aspect is the time of pollutant release. Plastic debris functions as a reservoir and secures constant pollutant release in plastic contact test. By contrast, in leaching test there is no reservoir, thus the total amount of toxic substances decreases faster.

Even though the calculated $EC_{50}$ values, in this present thesis, probably relate to an addition of toxic effects from more than one substance, however they should be compared with values taken from literature sources (Könemann, 1981). The following Table 12 and Table 13 represent $EC_{50}$ concentrations of several substances tested on crustacea and calculated average half effect concentrations in leaching and plastic contact tests.

**Table 12: $EC_{50}$ values taken from literature source (GESTIS-Stoffdatenbank). For the missing PAHs and PHTHs no data was found**

| | | Minimum [mg·L$^{-1}$] | Maximum [mg·L$^{-1}$] | Median [mg·L$^{-1}$] |
|---|---|---|---|---|
| **PAH** | **NAPH** | 1.60 | 6.47 | 3.60 |
| | **ACE** | 1.28 | 3.45 | 2.36 |
| | **FLU** | 0.21 | 11.60 | 1.12 |
| | **PHEN** | 0.11 | 3.23 | 0.52 |
| | **ANTH** | 0.01 | 0.75 | 0.05 |
| | **FLUO** | 0.00398 | 0.19 | 0.02 |
| | **PYR** | 0.00433 | 12.30 | 0.05 |
| | **BaA** | 0.000959 | 0.00148 | 0.00122 |
| | **BghiP** | 0.000133 | 0.00104 | 0.000587 |
| **PHTH** | **DBP** | 2.90 | 2.90 | 2.90 |
| | **BBP** | 1.00 | 4.70 | 1.80 |
| | **DEHP** | 0.13 | 2.00 | 1.07 |

**Table 13: Mean values of $EC_{50}$ concentrations from both tests**

| | Calculated $EC_{50}$ [mg·L$^{-1}$] |
|---|---|
| **24hLeach** | $8.75 \cdot 10^{-8}$ |
| **48hLeach** | $4.63 \cdot 10^{-8}$ |
| **24hK-K** | $4.63 \cdot 10^{-8}$ |
| **48hK-K** | $1.04 \cdot 10^{-8}$ |

As expected, the literature values are obviously higher than the calculated ones. This emphasises the fact, that a toxic mixture causes immobilisation on *D. magna* in both tests.

Finally, it is important to mention that all calculated $EC_{50}$ concentrations have to be treated with caution. They can be defective and deviate from true values. As an example, some sigmoidal curves in concentration-response graphs do not reach an immobilisation rate of 50 %, thus the graph-plotting program only estimates an $EC_{50}$ value (see Figure 16(B) or Figure 23(A)). In order to get more significant and less defective results, it could be better adding two or more dilution stages.

## 5.2 DISCUSSION OF GC-MS RESULTS

GC-MS analysis was used to confirm the reproducibility of the dissolved exposure concentrations for the individual PAHs. For PAH investigations only two samples had measurable concentrations over the LOQ. As shown in Table 10, HT_48hK-K_3_Tiddler had the highest value with 0.9 $\mu g \cdot L^{-1}$ of BkF. All traced PAHs have 18 or more carbon atoms, so they are counted as higher-molecule compounds. Additionally, their Log-$K_{OW}$ values are 5,66 or greater, thus they are hardly soluble in water. The hydrophobic properties of PAHs and PHTHs make them difficult to dissolve with increasing number of benzene rings and to maintain constant exposure concentrations, subsequently (Rojo-Nieto *et al.*, 2012). Resulting sorptive losses are highly compound-specific, which can affect the proportions between the compounds in the mixture (Rojo-Nieto *et al.*, 2012). This suggests the presence of solvents, which support faster release of these compounds out of the plastic material. The SPL Tiddler showed a greater variety of PAHs than Möhrchen, so it is likely that it contains solvents. The fish oil addition during production can be determining due to this fact. These results do not correlate with the data of Kielmann (2019). His results showed that Möhrchen generally includes more PAH compounds than Tiddler. Perhaps, the PAHs were distributed inhomogeneous, so lower concentrations were measured for Möhrchen in this work. Additionally, Kielmann's (2019) results show greater standard deviations, so they have to be treated with caution and thus a comparison is not meaningful. Finally, looking at the concentration-response curves of the two samples in Figure 21(B) and Figure 27(A) it is noticeable that both curves have high slopes which can be a reason for a quick intake, metabolism and toxic effect of the pollutants on the daphnids (Fent, 2007).

Regarding to Table 11, all investigated samples contain at least one phthalate compound. Again, Tiddler shows greater variety of traced compounds than Möhrchen. And even considering the summation of PHTH concentrations found in both SPL, Tiddler evidently sticks out. As expected, these results verify the acute toxicity on *D. magna*. Crustaceans appear to be especially sensitive to PHTHs by affecting reproduction, even at low concentrations (Oehlmann *et al.*, 2009). Moreover, by interfering with the functioning of various hormone systems, most plasticisers appear to act hazardous on *D. magna*, but some PHTHs have wider pathways of disruption (Oehlmann *et al.*, 2009).

The two controversial phthalates DBP and BBP might be significant, because they both have the highest concentrations with 125 µg·$L^{-1}$ of DBP, 115 µg·$L^{-1}$ of BBP, for instance. Moreover, Log-$K_{OW}$ values of 4.50 (DBP) and 4.73 (BBP) indicate a higher chance being released, and thus available for daphnids.

Comparing both test methods, it is noticeable that plastic contact test had on one hand a greater measurable number of PAHs. On the other hand, leaching test supplied more various PHTHs with a greater total amount of measured concentrations than plastic contact test, which is unexpected. Theoretically, plastic contact test samples must contain more traceable pollutants than the other method, because of approaching to passive sampling method. These results can have several reasons. Leaching test samples are probably easier to prepare and less error-prone for further GC-MS analysis. Due to a larger amount of greater plastic particles in plastic contact test, which can falsify the measurement process, it is possible not to find expected substances in appropriate concentrations. Larger particles are more likely to get lost in extraction process, because of a better agglomeration with other compounds or an adsorption on test vessel walls.

Another reason might be the validity of three samples for each method. Additionally, these samples are of dilution stage 1:1. For further investigations it might be concise to analyse more than three samples and to measure other dilution stages as well to get a precise overview.

## 5.3 LEACHING COMPARED TO PLASTIC CONTACT TEST

Firstly, comparing both test methods must be viewed with caution. For plastic contact test, chopped material smaller than 5 mm was used for easier weighing. The difference is another surface area than in leaching test, in which the SPL got chopped into 10 mm x 10 mm cubes. Nevertheless, it is essential to examine both test methods to qualify them for further investigations.

Considering the standard deviations of leaching compared with plastic contact tests in Figure 31, it is noticeable that plastic contact test has a lower standard deviation rate. The values diversify with less deviation (9.58 %) around the average value. As a result, the test reproductivity is higher in this testing method. It can be interpreted as more valid and

stable and less error-prone than leaching tests. The benefits of passive dosing, which can relate to plastic contact test method as well, are a maintenance of constant exposure concentrations and a relative increase in toxicity (Rojo-Nieto *et al.*, 2012).

Looking at the pros and cons of both tests, the advantages of leaching test are a shorter preparation time, an easier counting of immobile daphnids at the end and cost-efficiency due to less material usage than plastic contact test. Furthermore, acute toxicity tests of plastic product leachates were found to be useful for screening purposes for differentiating between toxic and non-toxic products (Lithner *et al.*, 2009). A disadvantage of leaching test is the validity of results, because of a higher sensitiveness to errors due to $c_{free}$ decrease. Contrary, a greater approach to passive dosing method and realistic environment conditions improves the validity of plastic contact tests. Nevertheless, usual deviations that result from weighing and pipetting errors must not be unattended for both test methods.

Another important aspect is the applicability with other plastic materials in both test methods. On the one hand, leaching test can be versatile and adaptable with other types of macroplastic, because it makes no difference during preparation process. In case, the leachate duration in dilution medium is relevant. On the other hand, in plastic contact test it can be a problem using other plastic material. In order to get precise dilution stages, it is substantial to weigh the particles. Not every plastic is as soft as the fishing lures, so the applicability can be limited to soft plastics.

## 5.4 TIDDLER COMPARED TO MÖHRCHEN

The first aspect are the standard deviations of the two SPL in four independent repetitions, as shown in Figure 33. The immobilisation rates differ less from the average value for Tiddler than for Möhrchen. In this case, Tiddler depicts a better value when looking at the aspect of test reproductivity with an average standard deviation of 8.79 %. Contrary, Möhrchen relates to a deviation rate of 10.98 %. Also, the fluctuation of $EC_{50}$ values, as shown in Figure 32, is higher for Möhrchen than for Tiddler. It can be assumed that toxic effects, caused by Möhrchen, vary within production process due to contaminants.

Another fact is the watching, that chopped pieces of Möhrchen mostly float at the surface, whereas Tiddler sinks to ground. This is unexpected, because Tiddler is covered with real

fish oil which must let it float instead of sinking to the ground. It might be that it has a greater density than water, what is intended. In fishing sport, it is not unusual letting the bait sink to the ground and increasing the chance to catch fishes by jigging method, for example.

Moreover, the physical differences between SPL Tiddler and Möhrchen must be discussed. Tiddler is a plastic bait that is produced in the United Kingdom. It is available in various colours and lengths. Additionally, it is covered with real fish oil which should increase the chance of catches. The other bait Möhrchen is made in Germany. Its feature is the orange colour, so it is more visible for predator fishes. A study revealed that the chance of catches was similar across individual colours and categories (Moraga, Wilson and Cooke, 2015). The colour only had a small influence on the size of captured fish (Moraga, Wilson and Cooke, 2015). An additional feature of Möhrchen is the ultra-violet sensitivity, thus this bait is all-weather applicable (Puhle, 2012). Both baits have similar shape to create specific water currents. When SPLs get lost on water grounds or get stuck in fish digestive tract, they can increase an average of 61 % in weight and 19 % in length in cold water, while an increase of 205 % in weight and 39 % in length in warm water can happen (Raison *et al.*, 2014). Relating to the potential risk having negative effects on *D. magna*, none of both SPL can be recommended. Nevertheless, Möhrchen is probably the better choice, because of less released phthalates into aqueous media and thus less effects on biological and chemical water quality during fishing.

# 6 CONCLUSION AND OUTLOOK

The aim of this thesis was the development of two different acute toxicity testing methods to *D. magna* with soft plastic lures. In order to observe validity of the results and evaluate the reproductivity, $EC_{50}$ concentrations and standard deviations of four independent test repetitions were determined. Additionally, GC-MS analysis took place considering the possible toxic effects of PAHs and phthalates in mixtures. Finally, the pros and cons of the two baits were discussed.

The results of both test methods showed that on the one hand leaching test is cost-efficient due to less material usage and easier to prepare, therefore time saving and better evaluable. On the other hand, plastic contact tests are more sensitive due to lower $EC_{50}$ concentrations. Its results can be interpreted as more valid and stable. Another advantage is a probably higher total amount of available pollutants for the daphnids in the system. Unexpectedly, leaching test samples contain greater variety and concentrations of phthalates. Though this fact must be verified in further experiments, because it bases on values of only three samples each method with a dilution stage of 1:1. At latest, the applicability to other types of plastic or materials is limited in both methods. It should be examined whether other materials provide similar results.

Most samples depict PAH concentrations under the LOQ. Contrary, phthalates are traceable in every sample. Even though measured PAH and PHTH concentrations probably make up a small part of toxic mixture, they are likely to increase their effects the more various compounds are available. Moreover, an expected trend of better solubility with less benzene rings is observable for PAHs, whereas PHTHs show greater solubility with growing alcohol chain lengths.

Comparing the SPLs, Tiddler generally shows to have greater toxic effect on *D. magna* than Möhrchen. Furthermore, the average standard deviations of Tiddler are lower, thus its results are more reliable and valid. Looking at the pros and cons of both SPLs it can be concluded that Möhrchen might be the better choice relating to the potential risks in aquatic environment. Nevertheless, both baits contain special additives as fish oil or ultra-violet sensitive substances, thus their possible impacts must be screened in further

experiments. In addition, the tackle industry should continue to investigate SPLs that are less likely to be pulled off by fish or that degrade rapidly.

The most striking gaps in current knowledge on the impacts of plasticisers on wildlife are the lack of data for long-term exposures to environmentally relevant concentrations and their ecotoxicity when part of complex mixtures. Furthermore, the hazard of plasticisers has been investigated in annelids, molluscs and arthropods only, and given the sensitivity of some invertebrates, effects assessments are warranted in other invertebrates as well.

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# 8 ANNEX

## 8.1 USED CHEMICALS AND DEVICES

| Chemicals and devices | Description |
|---|---|
| aluminium foil | thickn. 30 µm, L 100 m x B 500 mm, Carl Roth |
| n-hexane | ROTISOLV® ≥99 %, Carl Roth |
| glass column processor | Baker spe-12G, J.T. Baker |
| rotary evaporator | Laborata 4000 efficient, Heidolph |
| microliter syringe | 100 µL, Hamilton Bonaduz |
| gc-vial with cap | 1.5 mL ROTILABO®, Carl Roth |
| glass pasteur pipette | 2 mL, L 230 mm, neoLab |
| oxygen probe | ProfiLine™ Oxi 3210, WTW™ |
| brown glass bottle | 250 mL baked out 9 h at 300 °C, Schott Duran |
| round flask | 50 mL, 100 mL, Schott Duran |
| separation funnel | 50 mL, 100 mL, Schott Duran |
| erlenmeyer flask | 250 mL with polished neck, Schott Duran |
| glass fibre filter | diameter 12.3 mm, BEKOlut® GF Filter, BEKOlut |
| six-well plate | Macro plate PS 6 F with lid, boettger |
| SPL Tiddler | L 18 cm, Hot Tiger, Fox Rage |
| SPL Möhrchen | L 12.5 – 15 cm, Lieblingsköder |
| orbital shaker table | Multi Shaker, platform 409 x 297 mm, neoLab |
| horizontal shaker table | Unimax 1010, Heidolph |
| ultra-sonic bath | S 30 H Elmasonic, Omnident |

## 8.2 INGREDIENTS OF ELENDT-M4 MEDIUM

For the preparation of Elendt-M4 breeding medium it is necessary to produce stock solution 1 first. Afterwards M4-medium is made by adding stock solution 2, a macro nutrient stock solution and a combined vitamin stock solution.

**Table 14: Ingredients of stock solution 1 and 2**

| Stock solution 1 | Amount added to ultrapure water [mg·L$^{-1}$] | Volume of stock solution 1 added to ultrapure water [mL·L$^{-1}$] (gives stock solution 2) |
|---|---|---|
| $H_3BO_3$ | 57190 | 1.0 |
| $MnCl_2 \cdot 4H_2O$ | 7210 | 1.0 |
| LiCl | 6120 | 1.0 |
| RbCl | 1420 | 1.0 |
| $SrCl_2 \cdot 6H_2O$ | 3040 | 1.0 |
| NaBr | 320 | 1.0 |
| $Na_2MoO_4 \cdot 2H_2O$ | 1230 | 1.0 |

| | | |
|---|---|---|
| $CuCl_2 \cdot 2H_2O$ | 335 | 1.0 |
| $ZnCl_2$ | 260 | 1.0 |
| $CoCl_2 \cdot 6H_2O$ | 200 | 1.0 |
| KI | 65 | 1.0 |
| $Na_2SeO_3$ | 43.8 | 1.0 |
| $NH_4VO_3$ | 11.5 | 1.0 |
| $Na_2EDTA \cdot 2H_2O$ | 5000 | - |
| $FeSO_4 \cdot 7H_2O$ | 1991 | - |
| $Na_2EDTA \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ are poured together and autoclaved straight away. This gives the following product: | | |
| 21 Fe-EDTA solution | | 20.0 |

**Table 15: Ingredients of macro nutrient stock solution and combined vitamin stock solution**

| | **Amount added to ultrapure water [mg·L$^{-1}$]** | **Amount of stock solution 2 added to have M4-medium [mL·L$^{-1}$]** |
|---|---|---|
| Stock solution 2 | | 50 |
| Macro nutrient stock solution | | |
| $CaCl_2 \cdot 2H_2O$ | 293800 | 1.0 |
| $MgSO_4 \cdot 7H_2O$ | 246600 | 0.5 |
| KCl | 58000 | 0.1 |
| $NaHCO_3$ | 64800 | 1.0 |
| $Na_2SiO_3 \cdot 9H_2O$ | 50000 | 0.2 |
| $NaNO_3$ | 2740 | 0.1 |
| $KH_2PO_4$ | 1430 | 0.1 |
| $K_2HPO_4$ | 1840 | 0.1 |
| Combined vitamin stock | - | 0.1 |
| The following 3 vitamins are added to 1 L ultrapure water: | | |
| Thiamine hydrochloride | 750 | |
| Cyanocobalamine ($B_{12}$) | 10 | |
| Biotine | 7.5 | |

## 8.3 PILOT TEST RESULTS

**Measurements of plastic bait weights for pilot leaching test no. 1:**

| | |
|---|---|
| Tiddler [g]: | 20.15 |
| Möhrchen [g]: | 20.11 |

**Table 16: Results of pilot leaching test no. 1 after 24 hours. The results of every dilution stage and a negative control are shown. Every neonate that is able to swim gets counted**

| VT_24hLeach_1 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| | Tiddler 1:1 | 0 | Möhrchen 1:1 | 0 |
| | Tiddler 1:2 | 2 | Möhrchen 1:2 | 2 |
| | Tiddler 1:4 | 2 | Möhrchen 1:4 | 3 |
| | Tiddler 1:8 | 5 | Möhrchen 1:8 | 5 |
| | Tiddler 1:16 | 5 | Möhrchen 1:16 | 5 |
| | Tiddler 1:32 | 5 | Möhrchen 1:32 | 5 |

**Table 17: Results of pilot leaching test no. 1 after 48 hours**

| VT_48hLeach_1 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| | Tiddler 1:1 | 0 | Möhrchen 1:1 | 0 |
| | Tiddler 1:2 | 0 | Möhrchen 1:2 | 1 |
| | Tiddler 1:4 | 2 | Möhrchen 1:4 | 3 |
| | Tiddler 1:8 | 4 | Möhrchen 1:8 | 4 |
| | Tiddler 1:16 | 5 | Möhrchen 1:16 | 5 |
| | Tiddler 1:32 | 5 | Möhrchen 1:32 | 5 |

**Measurements of plastic bait weights for pilot leaching test no. 2:**

| | |
|---|---|
| Tiddler [g]: | 20.23 |
| Möhrchen [g]: | 20.21 |

**Table 18: Results of pilot leaching test no. 2 after 24 hours**

| VT_24hLeach_2 | 1 | 2 | | |
|---|---|---|---|---|
| negative control | 5 | 5 | | |
| | Tiddler 1:1 | 4 | Möhrchen 1:1 | 2 |
| | Tiddler 1:1.5 | 2 | Möhrchen 1:1.5 | 2 |
| | Tiddler 1:2 | 2 | Möhrchen 1:2 | 3 |
| | Tiddler 1:3 | 1 | Möhrchen 1:3 | 3 |
| | Tiddler 1:4 | 5 | Möhrchen 1:4 | 4 |
| | Tiddler 1:8 | 5 | Möhrchen 1:8 | 5 |

**Table 19: Results of pilot leaching test no. 2 after 48 hours**

| VT_48hLeach_2 | 1 | 2 | | |
|---|---|---|---|---|
| negative control | 5 | 5 | | |
| | Tiddler 1:1 | 0 | Möhrchen 1:1 | 1 |
| | Tiddler 1:1.5 | 0 | Möhrchen 1:1.5 | 1 |
| | Tiddler 1:2 | 0 | Möhrchen 1:2 | 2 |
| | Tiddler 1:3 | 0 | Möhrchen 1:3 | 2 |
| | Tiddler 1:4 | 0 | Möhrchen 1:4 | 3 |
| | Tiddler 1:8 | 4 | Möhrchen 1:8 | 4 |

**Table 20: Measurements of plastic bait weights for pilot contact test no. 1 in gram**

| VT_K-K_1 | [g] | | [g] |
|---|---|---|---|
| Tiddler 1:1 | 1.0022 | Möhrchen 1:1 | 0.9994 |
| Tiddler 1:2 | 0.6689 | Möhrchen 1:2 | 0.6610 |
| Tiddler 1:4 | 0.5015 | Möhrchen 1:4 | 0.5009 |
| Tiddler 1:8 | 0.3359 | Möhrchen 1:8 | 0.3365 |
| Tiddler 1:16 | 0.2535 | Möhrchen 1:16 | 0.2548 |
| Tiddler 1:32 | 0.1266 | Möhrchen 1:32 | 0.1240 |

**Table 21: Results of pilot plastic contact test no. 1 after 24 hours**

| VT_24hK-K_1 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 | 5 | 4 |
| | Tiddler 1:1 | 0 | Möhrchen 1:1 | 2 | | |
| | Tiddler 1:2 | 0 | Möhrchen 1:2 | 1 | | |
| | Tiddler 1:4 | 1 | Möhrchen 1:4 | 3 | | |
| | Tiddler 1:8 | 4 | Möhrchen 1:8 | 5 | | |
| | Tiddler 1:16 | 5 | Möhrchen 1:16 | 5 | | |
| | Tiddler 1:32 | 5 | Möhrchen 1:32 | 5 | | |

**Table 22: Results of pilot plastic contact test no. 1 after 48 hours**

| VT_48hK-K_1 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 | 5 | 4 |
| | Tiddler 1:1 | 0 | Möhrchen 1:1 | 1 | | |
| | Tiddler 1:2 | 0 | Möhrchen 1:2 | 1 | | |
| | Tiddler 1:4 | 1 | Möhrchen 1:4 | 3 | | |
| | Tiddler 1:8 | 4 | Möhrchen 1:8 | 5 | | |
| | Tiddler 1:16 | 4 | Möhrchen 1:16 | 5 | | |
| | Tiddler 1:32 | 5 | Möhrchen 1:32 | 5 | | |

**Table 23: Measurements of plastic bait weights for pilot contact test no. 2 in gram**

| VT_K-K_2 | [g] | | [g] |
|---|---|---|---|
| Tiddler 1:1 | 0.9992 | Möhrchen 1:1 | 1.0000 |
| Tiddler 1:1.5 | 0.6600 | Möhrchen 1:1.5 | 0.6683 |
| Tiddler 1:2 | 0.5030 | Möhrchen 1:2 | 0.5010 |
| Tiddler 1:3 | 0.3330 | Möhrchen 1:3 | 0.3306 |
| Tiddler 1:4 | 0.2520 | Möhrchen 1:4 | 0.2510 |
| Tiddler 1:8 | 0.1272 | Möhrchen 1:8 | 0.1279 |

**Table 24: Results of pilot plastic contact test no. 2 after 24 hours**

| VT_24hK-K_2 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 | 5 | 5 |
| | Tiddler 1:1 | 0 | Möhrchen 1:1 | 1 | | |
| | Tiddler 1:1.5 | 0 | Möhrchen 1:1.5 | 1 | | |
| | Tiddler 1:2 | 2 | Möhrchen 1:2 | 2 | | |
| | Tiddler 1:3 | 3 | Möhrchen 1:3 | 2 | | |
| | Tiddler 1:4 | 2 | Möhrchen 1:4 | 3 | | |
| | Tiddler 1:8 | 5 | Möhrchen 1:8 | 5 | | |

**Table 25: Results of pilot plastic contact test no. 2 after 48 hours**

| VT_48hK-K_2 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 | 5 | 4 |
| | Tiddler 1:1 | 0 | Möhrchen 1:1 | 0 | | |
| | Tiddler 1:1.5 | 0 | Möhrchen 1:1.5 | 0 | | |
| | Tiddler 1:2 | 1 | Möhrchen 1:2 | 1 | | |
| | Tiddler 1:3 | 3 | Möhrchen 1:3 | 2 | | |
| | Tiddler 1:4 | 2 | Möhrchen 1:4 | 2 | | |
| | Tiddler 1:8 | 4 | Möhrchen 1:8 | 4 | | |

## 8.4 LEACHING TEST RESULTS

**Table 26: Measurements of plastic bait weights for leaching test no. 1 in gram**

| | |
|---|---|
| Tiddler [g]: | 20.09 |
| Möhrchen [g]: | 19.82 |

**Table 27: Results of leaching test no. 1 after 24 hours. The results of four replicates for every dilution stage and a negative control are shown. Every neonate that is able to swim gets counted**

| HT_24hLeach_1 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 1 | 0 | 0 |
| Tiddler 1:2 | 3 | 3 | 2 | 5 |
| Tiddler 1:4 | 3 | 4 | 4 | 4 |
| Tiddler 1:8 | 5 | 5 | 5 | 5 |
| Möhrchen 1:1 | 2 | 2 | 3 | 2 |
| Möhrchen 1:2 | 5 | 5 | 3 | 5 |
| Möhrchen 1:4 | 5 | 5 | 4 | 5 |
| Möhrchen 1:8 | 5 | 5 | 5 | 5 |

**Table 28: Results of leaching test no. 1 after 48 hours**

| HT_48hLeach_1 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 3 | 3 | 2 | 3 |
| Tiddler 1:4 | 3 | 4 | 4 | 3 |
| Tiddler 1:8 | 4 | 4 | 5 | 5 |
| Möhrchen 1:1 | 2 | 1 | 3 | 2 |
| Möhrchen 1:2 | 3 | 5 | 2 | 3 |
| Möhrchen 1:4 | 5 | 5 | 4 | 4 |
| Möhrchen 1:8 | 4 | 5 | 5 | 4 |

**Table 29: Measurements of plastic bait weights for leaching test no. 2 in gram**

| | |
|---|---|
| Tiddler [g]: | 19.91 |
| Möhrchen [g]: | 20.10 |

**Table 30: Results of leaching test no. 2 after 24 hours**

| HT_24hLeach_2 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 2 | 2 | 1 | 0 |
| Tiddler 1:2 | 3 | 4 | 5 | 2 |
| Tiddler 1:4 | 5 | 5 | 5 | 5 |
| Tiddler 1:8 | 4 | 5 | 5 | 5 |
| Möhrchen 1:1 | 3 | 3 | 4 | 3 |
| Möhrchen 1:2 | 4 | 3 | 3 | 3 |
| Möhrchen 1:4 | 4 | 4 | 5 | 5 |
| Möhrchen 1:8 | 4 | 4 | 5 | 5 |

**Table 31: Results of leaching test no. 2 after 48 hours**

| HT_48hLeach_2 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 2 | 1 | 1 | 2 |
| Tiddler 1:4 | 3 | 3 | 3 | 5 |
| Tiddler 1:8 | 3 | 5 | 4 | 3 |
| Möhrchen 1:1 | 1 | 0 | 0 | 0 |
| Möhrchen 1:2 | 4 | 3 | 3 | 2 |
| Möhrchen 1:4 | 4 | 3 | 5 | 4 |
| Möhrchen 1:8 | 3 | 4 | 5 | 4 |

**Table 32: Measurements of plastic bait weights for leaching test no. 3 in gram**

| | |
|---|---|
| Tiddler [g]: | 20.17 |
| Möhrchen [g]: | 20.14 |

**Table 33: Results of leaching test no. 3 after 24 hours**

| HT_24hLeach_3 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 6 | 5 |
| Tiddler 1:1 | 2 | 1 | 2 | 5 |
| Tiddler 1:2 | 1 | 3 | 4 | 4 |
| Tiddler 1:4 | 5 | 5 | 5 | 5 |
| Tiddler 1:8 | 5 | 5 | 5 | 5 |
| Möhrchen 1:1 | 2 | 4 | 4 | 3 |
| Möhrchen 1:2 | 5 | 5 | 4 | 4 |
| Möhrchen 1:4 | 5 | 5 | 5 | 5 |
| Möhrchen 1:8 | 5 | 5 | 5 | 5 |

**Table 34: Results of leaching test no. 3 after 48 hours**

| HT_48hLeach_3 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 6 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 0 | 0 | 0 |
| Tiddler 1:4 | 2 | 3 | 3 | 4 |
| Tiddler 1:8 | 4 | 4 | 4 | 3 |
| Möhrchen 1:1 | 0 | 1 | 3 | 1 |
| Möhrchen 1:2 | 3 | 4 | 3 | 4 |
| Möhrchen 1:4 | 4 | 5 | 5 | 5 |

| | | | | |
|---|---|---|---|---|
| Möhrchen 1:8 | 4 | 5 | 3 | 5 |

**Table 35: Measurements of plastic bait weights for leaching test no. 4 in gram**

| | |
|---|---|
| Tiddler [g]: | 19.96 |
| Möhrchen [g]: | 20.12 |

**Table 36: Results of leaching test no. 4 after 24 hours**

| HT_24hLeach_4 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 0 | 1 | 1 |
| Tiddler 1:2 | 4 | 3 | 2 | 2 |
| Tiddler 1:4 | 5 | 5 | 5 | 5 |
| Tiddler 1:8 | 5 | 5 | 5 | 5 |
| Möhrchen 1:1 | 1 | 1 | 2 | 1 |
| Möhrchen 1:2 | 3 | 2 | 1 | 3 |
| Möhrchen 1:4 | 4 | 5 | 5 | 5 |
| Möhrchen 1:8 | 5 | 5 | 5 | 5 |

**Table 37: Results of leaching test no. 4 after 48 hours**

| HT_48hLeach_4 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 4 | 5 | 4 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 0 | 0 | 0 |
| Tiddler 1:4 | 3 | 3 | 1 | 2 |
| Tiddler 1:8 | 4 | 2 | 4 | 1 |
| Möhrchen 1:1 | 0 | 0 | 1 | 1 |
| Möhrchen 1:2 | 1 | 0 | 3 | 4 |
| Möhrchen 1:4 | 5 | 5 | 5 | 5 |
| Möhrchen 1:8 | 5 | 5 | 4 | 5 |

## 8.5 PLASTIC CONTACT TEST RESULTS

**Table 38: Measurements of plastic bait weights for plastic contact test no. 1 in gram**

| HT_K-K_1 | 1 [g] | 2 [g] | 3 [g] | 4 [g] |
|---|---|---|---|---|
| Tiddler 1:1 | 1.0143 | 1.0146 | 0.9968 | 1.0000 |
| Tiddler 1:2 | 0.5024 | 0.5198 | 0.5031 | 0.5086 |
| Tiddler 1:4 | 0.2517 | 0.2463 | 0.2458 | 0.2435 |
| Tiddler 1:8 | 0.1288 | 0.1231 | 0.1240 | 0.1227 |
| Möhrchen 1:1 | 0.9740 | 0.9890 | 0.9827 | 0.9981 |

| | | | | |
|---|---|---|---|---|
| Möhrchen 1:2 | 0.5038 | 0.4923 | 0.4900 | 0.4968 |
| Möhrchen 1:4 | 0.2505 | 0.2550 | 0.2570 | 0.2528 |
| Möhrchen 1:8 | 0.1153 | 0.1197 | 0.1311 | 0.1220 |

**Table 39: Results of plastic contact test no. 1 after 24 hours. The results of four replicates for every dilution stage and a negative control are shown. Every neonate that is able to swim gets counted**

| HT_24hK-K_1 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 0 | 1 | 0 |
| Tiddler 1:2 | 0 | 0 | 2 | 3 |
| Tiddler 1:4 | 3 | 2 | 2 | 3 |
| Tiddler 1:8 | 3 | 4 | 4 | 4 |
| Möhrchen 1:1 | 1 | 2 | 3 | 3 |
| Möhrchen 1:2 | 4 | 4 | 4 | 3 |
| Möhrchen 1:4 | 4 | 4 | 4 | 2 |
| Möhrchen 1:8 | 4 | 4 | 4 | 4 |

**Table 40: Results of plastic contact test no. 1 after 48 hours**

| HT_48hK-K_1 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 0 | 0 | 0 |
| Tiddler 1:4 | 0 | 0 | 0 | 0 |
| Tiddler 1:8 | 0 | 1 | 0 | 1 |
| Möhrchen 1:1 | 0 | 0 | 0 | 1 |
| Möhrchen 1:2 | 1 | 1 | 2 | 1 |
| Möhrchen 1:4 | 3 | 2 | 2 | 2 |
| Möhrchen 1:8 | 3 | 3 | 4 | 2 |

**Table 41: Measurements of plastic bait weights for plastic contact test no. 2 in gram**

| HT_K-K_2 | 1 [g] | 2 [g] | 3 [g] | 4 [g] |
|---|---|---|---|---|
| Tiddler 1:1 | 1.0091 | 0.9993 | 0.9957 | 0.9997 |
| Tiddler 1:2 | 0.5032 | 0.5051 | 0.4977 | 0.5054 |
| Tiddler 1:4 | 0.2510 | 0.2538 | 0.2532 | 0.2537 |
| Tiddler 1:8 | 0.1240 | 0.1278 | 0.1275 | 0.1264 |
| Möhrchen 1:1 | 1.0054 | 0.9988 | 0.9995 | 1.0026 |
| Möhrchen 1:2 | 0.5017 | 0.5055 | 0.5071 | 0.5037 |
| Möhrchen 1:4 | 0.2574 | 0.2499 | 0.2573 | 0.2500 |
| Möhrchen 1:8 | 0.1245 | 0.1248 | 0.1296 | 0.1225 |

**Table 42: Results of plastic contact test no. 2 after 24 hours**

| HT_24hK-K_2 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 4 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 0 | 2 | 1 |
| Tiddler 1:4 | 2 | 2 | 3 | 2 |
| Tiddler 1:8 | 4 | 5 | 5 | 5 |
| Möhrchen 1:1 | 2 | 3 | 2 | 2 |
| Möhrchen 1:2 | 3 | 2 | 3 | 3 |
| Möhrchen 1:4 | 3 | 4 | 4 | 2 |
| Möhrchen 1:8 | 4 | 3 | 4 | 4 |

**Table 43: Results of plastic contact test no. 2 after 48 hours**

| HT_48hK-K_2 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 4 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 0 | 0 | 0 |
| Tiddler 1:4 | 0 | 0 | 0 | 0 |
| Tiddler 1:8 | 3 | 2 | 1 | 2 |
| Möhrchen 1:1 | 0 | 1 | 2 | 1 |
| Möhrchen 1:2 | 2 | 1 | 3 | 1 |
| Möhrchen 1:4 | 1 | 1 | 2 | 3 |
| Möhrchen 1:8 | 2 | 3 | 3 | 3 |

**Table 44: Measurements of plastic bait weights for plastic contact test no. 3 in gram**

| HT_K-K_3 | 1 [g] | 2 [g] | 3 [g] | 4 [g] |
|---|---|---|---|---|
| Tiddler 1:1 | 1.0105 | 0.9986 | 0.9975 | 1.0047 |
| Tiddler 1:2 | 0.5016 | 0.5111 | 0.5011 | 0.4961 |
| Tiddler 1:4 | 0.2550 | 0.2513 | 0.2529 | 0.2573 |
| Tiddler 1:8 | 0.1317 | 0.1266 | 0.1290 | 0.1223 |
| Möhrchen 1:1 | 1.0035 | 1.0018 | 1.0037 | 1.0018 |
| Möhrchen 1:2 | 0.5083 | 0.4974 | 0.4987 | 0.4960 |
| Möhrchen 1:4 | 0.2513 | 0.2517 | 0.2525 | 0.2505 |
| Möhrchen 1:8 | 0.1288 | 0.1263 | 0.1238 | 0.1259 |

**Table 45: Results of plastic contact test no. 3 after 24 hours**

| HT_24hK-K_3 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 4 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 2 | 1 | 2 |

| | | | | |
|---|---|---|---|---|
| Tiddler 1:4 | 1 | 1 | 1 | 0 |
| Tiddler 1:8 | 1 | 4 | 3 | 5 |
| Möhrchen 1:1 | 0 | 0 | 0 | 0 |
| Möhrchen 1:2 | 1 | 1 | 1 | 4 |
| Möhrchen 1:4 | 2 | 1 | 1 | 2 |
| Möhrchen 1:8 | 1 | 3 | 3 | 4 |

**Table 46: Results of plastic contact test no. 3 after 48 hours**

| HT_48hK-K_3 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 4 | 5 | 4 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 0 | 0 | 0 |
| Tiddler 1:4 | 0 | 0 | 0 | 0 |
| Tiddler 1:8 | 0 | 0 | 1 | 1 |
| Möhrchen 1:1 | 0 | 0 | 0 | 0 |
| Möhrchen 1:2 | 1 | 1 | 1 | 1 |
| Möhrchen 1:4 | 2 | 1 | 0 | 1 |
| Möhrchen 1:8 | 2 | 2 | 3 | 2 |

**Table 47: Measurements of plastic bait weights for plastic contact test no. 4 in gram**

| HT_K-K_4 | 1 [g] | 2 [g] | 3 [g] | 4 [g] |
|---|---|---|---|---|
| Tiddler 1:1 | 0.9985 | 1.0166 | 1.0008 | 0.9911 |
| Tiddler 1:2 | 0.4887 | 0.5034 | 0.4912 | 0.4903 |
| Tiddler 1:4 | 0.2457 | 0.2459 | 0.2461 | 0.2404 |
| Tiddler 1:8 | 0.1283 | 0.1156 | 0.1293 | 0.1312 |
| Möhrchen 1:1 | 0.9969 | 1.0183 | 0.9911 | 0.9962 |
| Möhrchen 1:2 | 0.4950 | 0.5043 | 0.4971 | 0.4976 |
| Möhrchen 1:4 | 0.2465 | 0.2476 | 0.2593 | 0.2590 |
| Möhrchen 1:8 | 0.1232 | 0.1172 | 0.1324 | 0.1353 |

**Table 48: Results of plastic contact test no. 4 after 24 hours**

| HT_24hK-K_4 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 0 | 0 | 4 | 2 |
| Tiddler 1:4 | 2 | 5 | 1 | 2 |
| Tiddler 1:8 | 5 | 5 | 5 | 5 |
| Möhrchen 1:1 | 3 | 4 | 2 | 3 |
| Möhrchen 1:2 | 0 | 1 | 3 | 2 |
| Möhrchen 1:4 | 5 | 4 | 5 | 5 |
| Möhrchen 1:8 | 5 | 5 | 5 | 5 |

**Table 49: Results of plastic contact test no. 4 after 48 hours**

| HT_48hK-K_4 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| negative control | 5 | 5 | 5 | 5 |
| Tiddler 1:1 | 0 | 0 | 0 | 0 |
| Tiddler 1:2 | 1 | 0 | 0 | 0 |
| Tiddler 1:4 | 0 | 0 | 0 | 0 |
| Tiddler 1:8 | 1 | 1 | 0 | 1 |
| Möhrchen 1:1 | 2 | 1 | 1 | 0 |
| Möhrchen 1:2 | 1 | 0 | 1 | 1 |
| Möhrchen 1:4 | 3 | 2 | 1 | 3 |
| Möhrchen 1:8 | 2 | 1 | 1 | 2 |

## 8.6 RESULTS OF GC-MS MEASUREMENT

### 8.6.1 PAHs

**Table 50: Results of PAH measurements with GC-MS method. Shown are the concentrations of samples with a dilution of 1:1. Concentrations with an unquantifiable value are summed up as 0.0 µg·L$^{-1}$**

| | Measured value [µg·L$^{-1}$] | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **NAPH** | **ACY** | **ACE** | **FLU** | **PHEN** | **ANTH** | **FLUO** |
| Leach_1_Tiddler | 85.0 | 0.9 | 1.0 | 5.1 | 1.4 | 0.4 | 0.1 |
| Leach_1_Möhrchen | 48.4 | 0.7 | 0.6 | 3.6 | 0.5 | 0.3 | 0.1 |
| Leach_2_Tiddler | 55.0 | 0.7 | 0.7 | 3.8 | 0.5 | 0.3 | 0.1 |
| Leach_2_Möhrchen | 68.2 | 0.9 | 0.9 | 5.0 | 0.6 | 0.4 | 0.1 |
| Leach_3_Tiddler | 59.0 | 0.9 | 0.9 | 5.0 | 0.6 | 0.4 | 0.1 |
| Leach_3_Möhrchen | 81.9 | 1.0 | 1.1 | 5.4 | 0.7 | 0.5 | 0.1 |
| Leach_4_Tiddler | 66.9 | 0.8 | 0.8 | 4.3 | 0.6 | 0.3 | 0.1 |
| Leach_4_Möhrchen | 51.7 | 0.8 | 0.8 | 4.4 | 0.5 | 0.3 | 0.1 |
| Leach_5_Tiddler | 107.0 | 0.9 | 0.8 | 5.0 | 0.7 | 0.4 | 0.1 |
| Leach_5_Möhrchen | 100.9 | 0.9 | 0.9 | 5.0 | 0.6 | 0.4 | 0.1 |
| | | | | | | | |
| K-K_1_Tiddler | 95.5 | 1.0 | 1.2 | 5.8 | 0.8 | 0.5 | 0.1 |
| K-K_1_Möhrchen | 55.7 | 0.7 | 0.8 | 3.8 | 0.5 | 0.3 | 0.0 |
| K-K_2_Tiddler | 67.3 | 0.9 | 0.9 | 4.4 | 0.5 | 0.4 | 0.1 |
| K-K_2_Möhrchen | 47.9 | 0.7 | 0.7 | 4.0 | 0.5 | 0.4 | 0.1 |
| K-K_3_Tiddler | 40.9 | 0.5 | 0.5 | 3.2 | 0.5 | 0.4 | 0.1 |
| K-K_3_Möhrchen | 39.8 | 0.4 | 0.5 | 2.6 | 0.4 | 0.2 | 0.0 |
| K-K_4_nc | 127.8 | 1.3 | 1.2 | 5.4 | 16.3 | 1.0 | 0.7 |
| K-K_5_nc | 121.1 | 1.2 | 1.3 | 5.3 | 1.7 | 1.0 | 0.9 |
| | | | | | | | |
| Standard_10pg/µL | 1.6 | 0.3 | 0.3 | 0.3 | 0.4 | 0.4 | 0.4 |
| Standard_100pg/µL | 5.3 | 3.0 | 2.7 | 2.9 | 3.1 | 3.7 | 3.9 |
| Standard_1000pg/µL | 36.3 | 31.4 | 26.3 | 27.3 | 29.5 | 33.7 | 32.5 |

| | Measured value [µg·L$^{-1}$] | | | | | | |
|---|---|---|---|---|---|---|---|
| **Sample** | **PYR** | **B[a]A** | **CHR** | **B[b]F** | **B[k]F** | **IND** | **B[ghi]P** |
| Leach_1_Tiddler | 0.1 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 |
| Leach_1_Möhrchen | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leach_2_Tiddler | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leach_2_Möhrchen | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leach_3_Tiddler | 0.1 | 0.1 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 |
| Leach_3_Möhrchen | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leach_4_Tiddler | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leach_4_Möhrchen | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leach_5_Tiddler | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Leach_5_Möhrchen | 0.0 | 0.1 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 |
| | | | | | | | |
| K-K_1_Tiddler | 0.1 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 |
| K-K_1_Möhrchen | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| K-K_2_Tiddler | 0.1 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 |
| K-K_2_Möhrchen | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| K-K_3_Tiddler | 0.1 | 0.3 | 0.2 | 0.1 | 0.9 | 0.0 | 0.1 |
| K-K_3_Möhrchen | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| K-K_4_nc | 0.1 | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 |
| K-K_5_nc | 0.1 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | | | | |
| Standard_10pg/µL | 0.2 | 0.3 | 0.2 | 0.3 | 0.3 | 0.2 | 0.2 |
| Standard_100pg/µL | 2.9 | 3.3 | 2.0 | 3.4 | 3.5 | 2.5 | 3.4 |
| Standard 1000pg/µL | 26.4 | 33.5 | 28.9 | 41.9 | 43.6 | 42.5 | 47.3 |

### 8.6.2 PHTHALATES

**Table 51: Results of PHTH measurements with GC-MS method. Shown are the concentrations of samples with a dilution of 1:1. Concentrations with an unquantifiable value are summed up as 0.0 µg·L$^{-1}$**

| | Measured value [µg·L$^{-1}$] | | | | | |
|---|---|---|---|---|---|---|
| **Sample** | **DBP** | **BBP** | **DEHP** | **DNOP** | **DINP** | **DIDP** |
| Leach_1_Tiddler | 6.4 | 2.1 | 82.3 | 12.0 | 94.1 | 10.3 |
| Leach_1_Möhrchen | 10.2 | 31.5 | 33.5 | 16.9 | 25.7 | 10.0 |
| Leach_2_Tiddler | 52.9 | 115.3 | 82.2 | 64.2 | 49.2 | 43.7 |
| Leach_2_Möhrchen | 6.0 | 12.4 | 14.6 | 3.6 | 6.7 | 1.9 |
| Leach_3_Tiddler | 18.4 | 68.1 | 49.4 | 24.9 | 81.7 | 17.3 |
| Leach_3_Möhrchen | 5.5 | 8.7 | 13.5 | 3.6 | 9.4 | 1.9 |
| Leach_4_Tiddler | 12.4 | 49.2 | 34.6 | 15.4 | 57.1 | 11.2 |
| Leach_4_Möhrchen | 6.9 | 3.6 | 12.8 | 10.8 | 11.9 | 5.7 |
| | | | | | | |
| K-K_1_Tiddler | 2.0 | 0.9 | 8.9 | 5.4 | 18.6 | 5.7 |
| K-K_1_Möhrchen | 6.0 | 13.7 | 13.3 | 10.8 | 18.1 | 5.9 |
| K-K_2_Tiddler | 125.0 | 76.9 | 60.4 | 57.6 | 41.8 | 39.3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| K-K_2_Möhrchen | 7.7 | 23.9 | 18.8 | 8.3 | 11.7 | 7.6 |
| K-K_3_Tiddler | 3.6 | 4.3 | 18.4 | 8.3 | 75.2 | 4.7 |
| K-K_3_Möhrchen | 4.8 | 5.5 | 16.8 | 6.4 | 21.7 | 3.4 |
| K-K_4_nc | 8.3 | 0.0 | 1.2 | 0.1 | 12.4 | 1.3 |
| K-K_5_nc | 10.9 | 0.0 | 1.2 | 0.1 | 1.5 | 6.7 |
| | | | | | | |
| Standard_10pg/µL | 4.0 | 0.3 | 0.4 | 0.3 | 1.5 | 0.6 |
| Standard_100pg/µL | 20.2 | 3.8 | 3.1 | 2.3 | 3.2 | 2.1 |
| Standard_1000pg/µL | 191.4 | 46.4 | 39.4 | 32.6 | 20.4 | 18.3 |
| Standard_10000pg/µL | 1878.2 | 541.6 | 386.8 | 388.7 | 255.3 | 293.4 |