# Sublethal and lethal effects of pharmaceuticals and agricultural chemicals on the reproduction of freshwater crayfish

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Kiel

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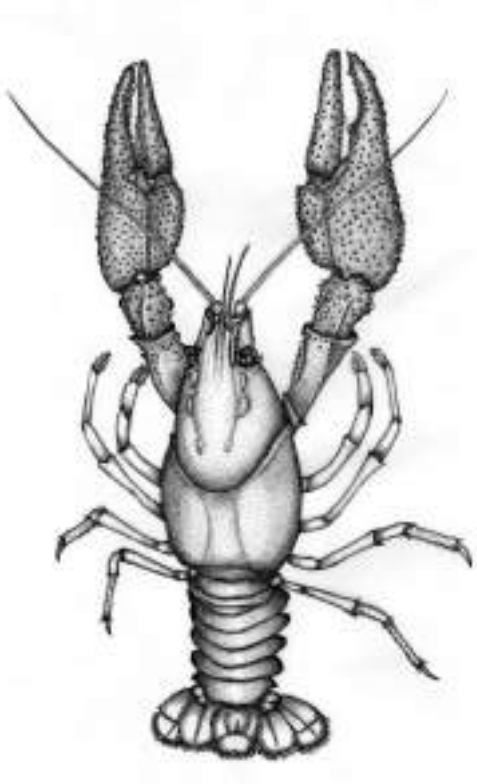

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# Zusammenfassung

„Unter Oecologie verstehen wir die gesammte Wissenschaft von den Beziehungen des Organismus zur umgebenden Aussenwelt, wohin wir im weiteren Sinne alle „Existenz-Bedingungen“ rechnen können. Diese sind theils organischer, theils anorganischer Natur; sowohl diese als jene sind, wie wir vorher gezeigt haben, von der grössten Bedeutung für die Form der Organismen, weil sie dieselbe zwingen, sich ihnen anzupassen.“

So lautet die erste Definition des Begriffes Ökologie aus dem Jahr 1866, in dem von Ernst Haeckel verfassten Werk „Generelle Morphologie der Organismen“. Noch heute, mehr als 150 Jahre später, hat diese Definition nicht an Bedeutung verloren. Im Gegenteil, die Forschung der letzten Jahrzehnte befasst sich zunehmend mit einem ganzheitlichen biologischen Ansatz.

Der Einbezug anthropogener Einflüsse nimmt dabei eine immer wichtigere Rolle ein. Vor allem die Betrachtung negativer Effekte aufgrund eingetragener Chemikalien in Oberflächengewässer auf Organismen und somit auf das gesamte Ökosystem gewinnt dabei stetig an Bedeutung. Daher wird dieser Aspekt auch in der vorliegenden Dissertation aufgegriffen. Um das Verständnis von Auswirkungen chemischer Belastungen zu erweitern, wurde in dieser Arbeit untersucht, ob und wie bestimmte Stoffe die Reproduktion von Flusskrebsen – eine Gruppe, die für das Ökosystem aufgrund ihrer Ernährungsweise und ihres Einflusses auf die Gewässerstruktur einen besonderen Stellenwert innehat – beeinflussen.

Zu diesem Zweck wurde zunächst in Laboruntersuchungen der Einfluss zweier Chemikalien auf die Gonadenreifung und die Embryonalentwicklung der einheimischen, gefährdeten Edelkrebse (*Astacus astacus*) und der nicht endemischen, sich parthenogenetisch vermehrenden Marmorkrebse (*Procambarus virginalis*) untersucht. Die Stoffe Diclofenac und Terbuthylazin wurden hierfür aufgrund ihres hohen Vorkommens in Oberflächengewässern sowie ihrer bekannten Toxizität ausgewählt. Diclofenac ist ein Arzneimittel, das bei Schmerzen und Entzündungen sowohl human- als auch veterinärmedizinische Anwendung findet, wohingegen Terbuthylazin ein Vorauflauf-Herbizid darstellt, welches vornehmlich im Sorghum-, Zitrus-, Mais-, Wein- und Apfelanbau verwendet wird. Die Ergebnisse zeigen eine Beeinträchtigung von Edelkrebsen in allen getesteten Konzentrationen, die so gewählt wurden, dass sowohl real gemessene Konzentrationen in Oberflächengewässern als auch weit größere Dosen beider Stoffe abgedeckt wurden. Vor allem subletale Effekte, wie histopathologische Veränderungen und Größendefizite der Nachkommen, treten hier auf. Marmorkrebse hingegen zeigen gegenüber Schadstoffen eine höhere Resistenz. Während die durch Diclofenac ausgelösten Effekte auf die Embryonalentwicklung noch vergleichbar mit denen der Edelkrebse sind, zeigt sich bei den übrigen Ergebnissen, dass die Reproduktion von Marmorkrebsen erst bei Konzentrationen, die bereits ein

Vielfaches über den Stoffmengen liegen, die in Oberflächengewässern nachgewiesen werden können, beeinträchtigt wird. Die Ergebnisse der Laboruntersuchungen zeigen also, dass die Reproduktion und damit auch die Populationsdynamik von Edelkrebsen durch aktuell in den Oberflächengewässern bestehende Stoffkonzentrationen negativ beeinflusst wird. Zusätzlich konnte gezeigt werden, dass die Nutzung des Marmorkrebses als Modellorganismus für die Effektgrenzenbestimmung einzelner Stoffe nur bedingt möglich ist.

Da jedoch beide Stoffe in der Umwelt in der Regel nicht als alleinige Verunreinigung auftreten, sondern meist mit einer Reihe anderer Stoffe durch Drainagen der Landwirtschaft oder Auslässe von Kläranlagen in Oberflächengewässer eingetragen werden, wurden auch Auswirkungen real existierender Mischkontamination im Feld untersucht. Zu diesem Zweck wurden eitragende Edelkrebsweibchen verschieden stark belastetem Oberflächengewässer ausgesetzt. Die Verschmutzung, die punktuell durch einen Kläranlagenabfluss eingeleitet wird, hatte immense Auswirkungen auf die Embryonalentwicklung der Tiere. Sowohl letale, als auch subletale Effekte konnten nachgewiesen werden.

Insgesamt zeigt diese Arbeit, dass Konzentrationen von Umweltchemikalien, die momentan in Gewässern Europas und der Welt gemessen werden, die Reproduktion und damit die Arterhaltung der gefährdeten Edelkrebse negativ beeinflussen. Als Konsequenz sollte daraus folgen, dass die Kontamination von Gewässern stärker überwacht und die Einleitung chemischer Stoffe effektiver verhindert werden muss.

# Summary

"By ecology, we understand the entire science of the organism's relations to the surrounding outside world, which include all "conditions of existence" in a broader sense. These are partly organic, partly inorganic; both are, as we have previously shown, of the greatest importance for the form of organisms, because they force it to adapt to them."

This is the first definition of the term ecology from 1866, written down in the work "Generelle Morphologie der Organismen" (General Morphology of Organisms) by Ernst Haeckel. Even today, more than 150 years later, this definition has not lost its meaning. On the contrary, research has increasingly focused on a holistic biological approach in recent decades.

The inclusion of anthropogenic impacts is a growing field of study. In particular, the consideration of negative effects on organisms and, thus, on the entire ecosystem due to chemicals introduced into surface waters has become more and more important. Therefore, this aspect is focused on in this dissertation. In order to expand the understanding of the effects of chemical pollution, this thesis investigates whether and how certain substances affect the reproduction of crayfish, a group that is of particular importance for the ecosystem due to their diet and their impact on the structure of the water body.

To this end, the influence of two chemicals on the gonadal maturation and embryonic development of the endangered, native noble crayfish (*Astacus astacus*) and the non-native parthenogenetically reproducing marbled crayfish (*Procambarus virginalis*) was investigated in laboratory studies. The substances Diclofenac and Terbuthylazine were selected for this study because of their high incidence in surface water bodies and their known toxicity. Diclofenac is a drug that is used to treat pain and inflammation in both human and veterinary medicine, while Terbuthylazine is a pre-emergence herbicide used primarily in the cultivation of sorghum, citrus fruits, corn, grapes and apples. The results show the impairment of noble crayfish reproduction in all tested concentrations, which were chosen to cover both, actually measured concentrations in surface waters and much higher doses of both substances. Especially sublethal effects such as histopathological changes and size deficits of the offspring occurred. In contrast, marbled crayfish show a higher resistance to the pollutants. While the effects induced by Diclofenac are still comparable to those on noble crayfish, the other results show that the reproduction of marbled crayfish is only impaired at concentrations that are multiple times higher than the amounts of substances that can actually be detected in surface waters. Therefore, the results of the laboratory investigations show that the reproduction and also the population dynamics of noble crayfish are impaired by currently existing substance concentrations in surface waters. In

addition, it was shown that the use of marbled crayfish as model organisms for the determination of effective concentrations of individual substances is only possible to a limited extent.

Since both substances do not usually occur in the environment as single contaminants but are usually introduced into surface waters together with a number of other chemicals through agricultural drains or outlets of sewage treatment plants, the effects of real mixed contamination were also investigated. For this purpose, egg-carrying female noble crayfish were exposed to different levels of contamination in surface waters in a field study. The pollution, which was discharged through a sewage treatment plant outlet, had immense effects on the embryonic development of the animals. Both lethal and sub-lethal effects could be demonstrated.

Overall, this work shows that concentrations of environmental chemicals, currently measured in surface waters of Europe and beyond, influence the reproduction and, thus, the conservation of endangered noble crayfish. As a consequence, the contamination of water bodies should be controlled more closely, and the discharge of chemical substances should be prevented more effectively.

# 1 Overall introduction

The ecology of freshwater ecosystems is influenced by human actions in various ways: structural changes resulting in an unnatural flow regime, the intervention in flora and fauna due to overfishing and stocking of non-indigenous species and the discharging of chemically contaminated wastewater leading to a disruption of ecological networks. The aim of the European water framework directive (WFD) is the improvement of deficient structures of water bodies. The species protection law and the regulation (EU) No. 1143/2014 on the prevention and management of the introduction and spread of invasive alien species aims to protect the endemic flora and fauna. The handling of problems regarding chemicals induced to water bodies is more difficult to address. Apart from an upper limit of all toxic substances in drinking waters of 0.1 µg/L, only regulations and recommendations of usage and cleaning of water introduced in surface water bodies are available.

At least 223 chemicals are frequently detected in European freshwater bodies (Malaj et al., 2014), each of which can show different effects on the present species. The vast majority of chemicals originate from agricultural or pharmaceutical substances (Murray et al., 2010). While agricultural substances, intentionally used as fertilisers or plant protection products, occur in surface waters in peaks during the application period, pharmaceuticals are used and introduced throughout the whole year. Pharmaceuticals can represent the entire range of human and veterinarian medicine. Both chemical groups can lead to the formally intended effects, side effects and totally unexpected effects in non-target organisms.

The Impact of substances on the ecosystem is highest, when organisms that have an extraordinary influence on their surroundings are affected. Freshwater crayfish are of exceptional importance for their environment. They affect nearly every trophic level of their habitat and influence their structural environment due to their burrowing activity. Therefore, these largest invertebrates of freshwater bodies are called “keystone species“ and “ecosystem engineers“ (Weinländer and Füreder, 2016). Consequently, the habitat directive protects all native freshwater crayfish species in Europe (European Commission, 2000). Nevertheless, these species are highly endangered. Invasive species, the crayfish plague (*Aphanomyces astaci*), structural stress and chemical load cause population decline (Chucholl, 2011). Especially the influences of presumed toxic chemicals on sensitive life stages affect population recruitment and dynamics. Therefore, freshwater crayfish and their sensitive life stages during reproduction are the focus of this investigation, in combination with a selection of chemicals, which will be described in the following chapters. The native noble crayfish *Astacus astacus* is exceptionally well-suited for this study due to its preferred habitat, which is often found in surface waters influenced by wastewater plants and agriculture (Skurdal, J. and Taugbøl, T., 2002). In addition to this species, the non-endemic marbled crayfish *Procambarus virginalis* was investigated. Its reproduction cycle, which

is described in more detail in the following chapters, enables the researcher to carry out investigations on toxicological effects much safer and faster than for other freshwater crayfish. If the observed effects are comparable to those of other crayfish species, the marbled crayfish could serve as a model organism and be of great importance in future crayfish studies.

Due to their known toxic effects and the regular detection of these substances, Terbuthylazine (TBA) and Diclofenac (DCF) are of special concern in toxicological investigations. Therefore, these two chemicals were used in laboratory experiments. Additionally, a field experiment was conducted to evaluate the effects a real sewage treatment plant (STP) output can have on freshwater crayfish.

## 1.1 Organisms

### *1.1.1 Astacus astacus*

The European noble crayfish (*Astacus astacus,* Linnaeus 1758, Figure 1.1) is an arthropod of the class Crustacea, the subclass Malacostraca and the order Decapoda and is a member of the family Astacidae. Other members of this family are *Astacus leptodactylus* (Eschscholz 1823), *Austropotamobius pallipes* (Lereboulette 1858), *Austropotamobius torrentium* (Schrank 1803) and *Astacus pachypus* (Rathke 1837). *Astacus* shows three subspecies: *A. astacus*, *A. balcanicus* and *A. colchicu.* Male noble crayfish can reach a length of more than 15 cm without claws and a weight of 250 g. Female body and claw size is smaller and they reach up to 15 cm and 200 g (Hager, 2003). As in all freshwater crayfish, the body of noble crayfish consists of a cephalothorax and a pleon. Dorsal colour is dark brown, but can vary to a brighter beige; in some cases, even blue or red animals occur (Füreder, 2009).

*Figure 1.1: Adult male* Astacus astacus. *Picture: Daniel Konn-Vetterlein.*

The carapace is smooth and does not show thorns or humps, but it has lateral granular tubercles. As distinguishing features, the noble crayfish has two thorns or humps behind the cervical furrow and two postorbital ridges (Füreder and Machino, 2002). Claws of noble crayfish are bigger in male specimens. They are heavily grained and dorsally of the same colour as the carapace. The colour of the ventral side of the body and of the end of the pereiopods is red to red-brown (Füreder and Machino, 2002).

The individuals can live up to 15 years and sexual maturity is reached at a size of 60 to 70 mm for male individuals. Females reach sexual maturity at 62 to 85 mm. In outdoor populations, these sizes are reached between the third and fifth year after hatching (Skurdal, J. and Taugbøl, T., 2002). Mating takes place between September and November as temperatures decrease (Ackerfors, 1999). As in most crayfish species, male noble crayfish attach spermatophores between the second and fourth pleopods and the telson of females. During oviposition, the eggs get in contact with the spermatophores for fertilisation and are attached to the pleopods for the rest of the embryonic development until the first moulting of juvenile crayfish (Skurdal, J. and Taugbøl, T., 2002). Embryonic development will last for 1900 degree days. In Germany, this is equivalent to a hatching in May to June (Burk, 2004). Noble crayfish are omnivorous. Their main food source consists of semiaquatic vegetation, benthic invertebrates and detritus. Further, zooplankton, tadpoles and small fishes can be a food resource (Olsson et al., 2008).

The noble crayfish is commonly known as a species requiring extraordinarily clear and cold water to survive and reproduce. Therefore, its presence is regarded as an indicator of good water parameters.

Low pH-values, however, can cause a lack of calcium carbonate and pose a problem for the synthesis of chitin. Furthermore, temperatures over 25 °C are critical for the survival this species (Skurdal, J. and Taugbøl, T., 2002). The assumption about the crayfish's distribution in clear and cold water can be explained by the occurrence of the crayfish plague, which does not appear in these kind of water bodies. The oomycete, the causative agent of the crayfish plague, is a key threat to crayfish biodiversity worldwide (Svoboda et al., 2017). North American crayfish species, which have co-evolved with *A. astaci*, are considered to be chronic but largely asymptomatic carriers. They combat *A. astaci* through consistent production of prophenoloxidase, which activates a cascade resulting in melanization of hyphae that prevents their invasion into host soft tissues (Persson et al., 1987). European, Japanese and Australian freshwater crayfish species like *Astacus astacus* ,however, have been found to be highly susceptible to the crayfish plague fungus. Mortalities occur in percentages of 100 % for these species after a fungal attack (Unestam, 1969). The mycelia grow rapidly through the cuticle and reach the internal body cavity, which results in crayfish death within 6–10 days (Unestam and Weiss, 1970). Outbreaks of the crayfish plague have eradicated many populations of native crayfish all over Europe, including *Astacus astacus* populations (Kozubíková-Balcarová et al., 2014). Therefore, the crayfish plague in combination with an unnatural water flow regime caused by human interference in water structure as well as chemical loads in surface waters are considered the main reasons for the decreasing numbers of *A. astacus* populations (Svobodová et al., 2012). As a result, this species is on the IUCN Red List of Threatened Species and is also protected through the habitat directive (Edsman et al., 2015).

### *1.1.2 Procambarus virginalis*

The marbled crayfish *Procambarus virginalis* (Lyko 2017, Figure 1.2) occurred for the first time in 1995. Its origin is unknown, but this species developed most likely through breeding for aquaristic distribution (Vogt, 2018). Due to its appealing appearance and the special parthenogenetic reproduction method, the animals were popular in pet shops, and this species was the most demanded crayfish in northern America in 2015 (Faulkes, 2015). In 2010, (Martin et al.) found the origin of this species in the slough crayfish *Procambarus fallax* (Hagen, 1870), which belongs to the family of Cambaridae. The triploid set of chromosomes of marbled crayfish was caused by a fusion of a not-reduced, diploid ovum with a haploid sperm cell or the fusion of a normal ovum with two haploid spermatophores, respectively. Analyses showed that all gametes of *P. virginalis* have their origin in *P. fallax*. Therefore, *P. virginalis* is no hybrid (Vogt, 2015), but an autopolyploid, which often results in parthenogenetic reproduction just like in the example of the marbled crayfish (Martin et al., 2015). The parthenogenetic reproduction of this species is apomictic, which means that there is no reduction division of the egg cells of the marbled crayfish. Thus, the genome of the offspring of apomictic animals is identical with their mother's genome, so that there are only female animals known (Simon et al.,

2003). Thanks to their parthenogenetic reproduction, this species is able to lay eggs every 8 to 9 weeks under optimal conditions (Vogt, 2010).

Adult marbled crayfish reach lengths of 4–8 cm without claws and a weight of 1.5–15 g. Some individuals can even reach up to 12 cm and 25 g (Vogt, 2011). The lifespan of this species of two to four years is noticeably lower than for noble crayfish. First reproduction normally starts between 150 to 250 days after hatching (Vogt, 2010). The main characteristic of this species is the smooth, marbled carapace, which is the origin of its trivial name. There are several thorns behind the cervical furrow. The claws of this species, which are normally smaller than half of the carapace, also show the characteristic marbling. They are lightly grained on the dorsal side, and a clear spike is visible on the root of the two parts of the claw. They also have a pair of postorbital ridges.

These animals have lower demands on their environment than noble crayfish. The preferred temperature lays between 20 and 25 °C (Seitz et al., 2005). However, they are able to outlast temperatures below 8 and over 30 °C, but without being able to reproduce (Hunter et al., 2011).

The extraordinary reproduction strategy of these animals is advantageous for researchers. The steady supply of offspring and the genetic conformity makes them a suitable model organism (Vogt, 2010). On the downside, this fast reproduction results in uncontrollable populations in outdoor water bodies if one animal escapes or is released into nature. Additionally, this species is known as a carrier of the crayfish plague, which results in a high-risk potential for endemic crayfish species, especially in combination with the potentially high reproduction rate and the food and habitat competition. In many countries, including Germany, wild and stable populations of marbled crayfish are known to date (Chucholl et al., 2012).

*Figure 1.2: Adult* Procambarus virginalis *with eggs. Picture: Jan Laurenz.*

## 1.2 Substances

### 1.2.1 Terbuthylazine

Terbuthylazine (N2-tert-butyl-6-chloro-N4-ethyl-1,3,5-triazine-2,4diamine, Figure 1.3) is a chloro-s-triazine characterized by ethylamino and tert-butylamino side chains. It is a replacement of the herbicide Atrazine (Figure 1.3), which was banned in Germany and Italy in 1991 (Sassine et al., 2017) and in the remaining countries of the European Union in 2004 (Fingler et al., 2017) due to the widespread contamination of ground and surface waters as well as its associated endocrine disrupting activity. TBA is one of the most frequently detected pesticides in natural waters (Tasca et al., 2018). Concentrations of up to 34.0 µg/L were detected in surface waters (Herrero-Hernández et al., 2017). Not only high concentrations and persistence (average half-life of 22 days), but also the deethylated metabolite desethylterbuthylazine occurs, which has been found to be one of the most abundant polar plant protection metabolites in EU aquifers (Loos et al., 2010).

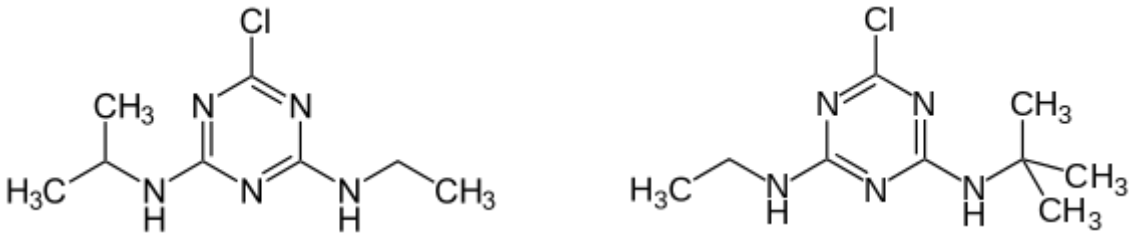


*Figure 1.3: Structural formula of Atrazine (left) and Terbuthylazine (right).*

TBA is used as a pre-emergence herbicide to inhibit the photosynthesis of the target organisms, such as annual dicotyledonous weeds or the cockspur *Echinochloa crus-galli*.

Approximately 60 % of the combined area in corn production in Europe receives TBA. It is used in more than 45 countries and remains a key weed control tool in crops such as corn, sorghum, pea, bean, lupin, grape, pome fruit, citrus and vine (Heri et al., 2008).

We can find toxicological effects of TBA a wide range of LOECs (lowest observed effective concentrations). The approval report for successor T, for instance, describes median effective concentrations for *Pseudokirchneriella subcapitata* at 12 µg/L, for *Lemna gibba* at 13.3 µg/L and for *Daphnia magna* at 19 µg/L. Bókony et al. (2020) showed effects starting at 0.3 µg/L for *Rana dalmatina*. Velisek et al. (2015) showed LOEC of TBA on common carp at 2.9 µg/L. For freshwater crayfish, there are no direct data for Terbuthylazine, but a derivate, terbuthylazine-2-hydroxy, was tested. Koutnik et al. (2017) showed effects on weight of juvenile marbled crayfish (*Procambarus virginalis*) at 75 mg/L. Stara et al. (2016) showed effects of terbuthylazine-desethyl on the histology of red swamp crayfish (*Prombarus clarkii*) from 2.9 µg/L. The log KOW (octanol/water partition coefficient) of 3.4 leads to a higher possibility of bioaccumulation over time (Barbieri et al., 2019) and,

therefore, even higher impacts of this pesticide on organisms over time and as well for animals in higher trophic levels.

Investigations that reflect the actual chemical load of surface waters are of severe importance for crayfish populations. The pollution of water bodies is composed of several different chemicals (Kienzler et al., 2014). Velisek et al. (2017) showed a higher risk of mixtures of triazine derivates on crayfish than the single components. Therefore, investigations regarding effects of chemical mixtures including triazines are also of great importance to improve the understanding of effects caused by these substances.

### 1.2.2 Diclofenac

Diclofenac (2-(2,6-dichloranilino) phenylacetic acid, Figure 1.4) is one of the most used pharmaceuticals in the world. It is a human and veterinary drug and approximately 940 tons are used worldwide per year (Zhang et al., 2008). In Europe, 179.8 tons per year were sold in 1999 (Ferrari et al., 2003), whereas most was applied in Germany with 82 tons in 2009 (Bergmann et al., 2011). The non-steroidal anti-inflammatory drug (NSAID) reduces inflammation or is applied as a pain reliever in certain conditions (Hunter et al., 2011). It decreases the production of thromboxanes and prostaglandins (Satoh et al., 2015) to reduce pain, inflammation and fever in target organisms. Similar to Terbuthylazine, Diclofenac has a high log KOW of 4.05. Therefore, the bioaccumulation over time or trophic levels is even higher than for TBA.

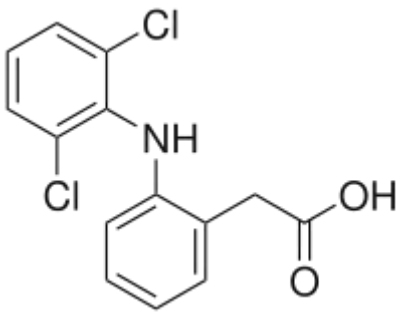


*Figure 1.4: Structural formula of Diclofenac.*

DCF is relatively stable in the environment, but similar to other NSAIDs it is sensitive to photolysis (Epold et al., 2012). Therefore, the missing UV-clarification in sewage treatment plants leads to relatively high concentrations in surface and ground waters. Bouju et al. (2016) showed that only 40 % of Diclofenac can be removed in 18 days in wastewater treatment plants. It has been found in concentrations of up to 29.8 µg/L in surface waters (Lin et al., 2008) and is, with 3.996 positively detected MECs (measured environmental concentrations), the most detected substance of the chemicals on the watch list of the database “Pharmaceuticals in the environment” of the UBA (German Environment Agency). It is considered a “contaminant of emerging concern” and was included in the previous watch list of EU Decision 2015/495 (Li et al., 2019; Lonappan et al., 2016; Sousa et al., 2018)

to gather sufficient monitoring information on surface waters (Sathishkumar et al., 2020). Further, this prevalent anti-inflammatory drug was detected in 55 countries (Dusi et al., 2019). The photolytic degradation of DCF in combination with less light, lower temperature and water chemistry is leading to higher concentrations in surface waters during winter and, therefore, over the reproduction or embryonic development time of freshwater crayfish.

The effects of DCF on prostaglandin expression can also show a wide range of effects on non-target organisms. Prostaglandin influences not only symptoms of pain and fever, but is also essential for ion-transport, oogenesis, spermatogenesis, sperm maturation and the immune defense (Rossitto et al., 2015; Rowley et al., 2005). Eades and Waring (2010) showed effects of DCF on osmoregulation of *Carcinus maenas* at concentrations of 10–100 ng/L. Median lethal concentrations ($LC_{50}$) for regularly used organisms for admission procedures like *Daphnia magna* ($LC_{50}^{48h}$ = 60.7 mg/L) and *Moina macrocopa* ($LC_{50}^{48h}$ = 142,6 mg/L) were much higher than actual concentrations in surface waters (Lee et al., 2011). Nevertheless, the same study showed that DCF influences time and ratio of hatching of *Oryzias latipes.* Even more alarming, DCF reduces reproduction success of the second generation to zero even though only the first generation was exposed to 10 mg/L DCF. Du et al. (2016) investigated the influences of DCF on the total number of broods per female and the total egg production number per female (Figure 1.5). The median effective concentration ($EC_{50}$) for these parameters were 0.94 mg/L and 0.52 mg/L. In conclusion, the concentration peak during the reproduction and embryonic development, paired with the high influence of DCF on these sensitive live stages, shows the importance of investigations dealing with these effects.

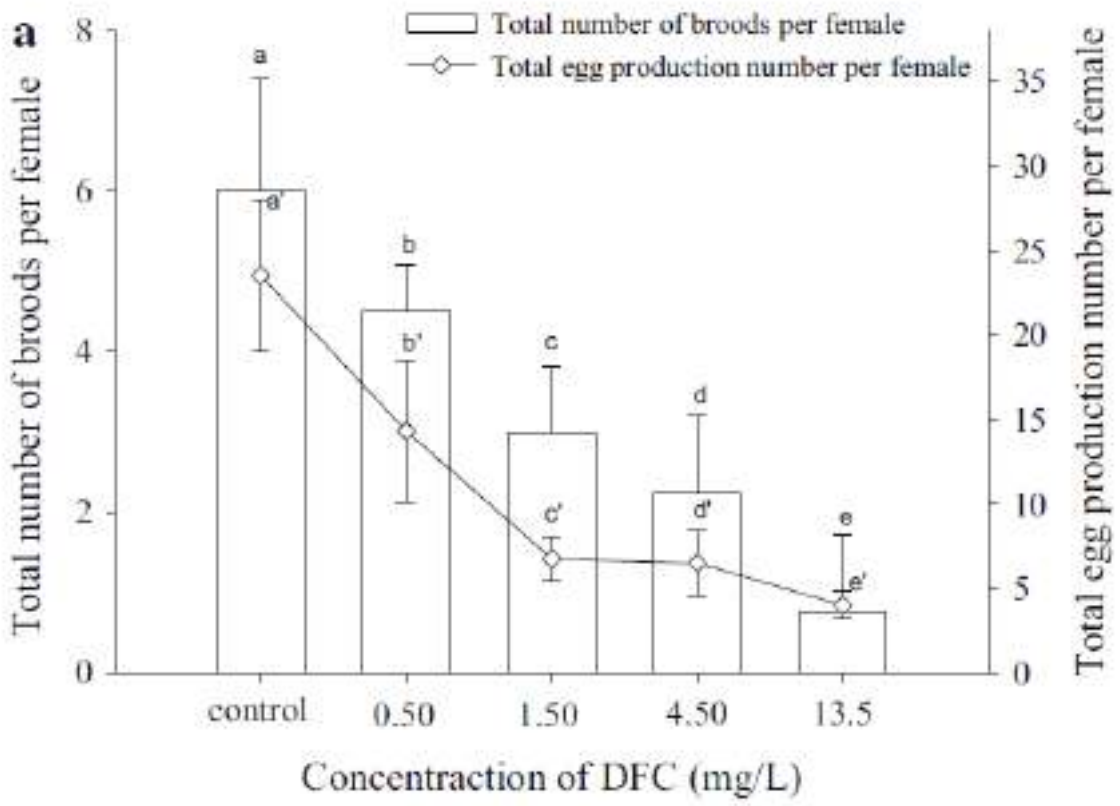


*Figure 1.5: Adverse effects of Diclofenac on the reproduction of* D. magna *with endpoints including the total number of broods per female and the total egg production number per female at 21 days. Significant differences (p\0.05) among the treatments for the total number of carapaces per adult and body length were indicated by different letters (a, b, c, d, e, f and a`, b`, c`, d`, e`, f`), respectively (changed after Du et al. (2016).*

## 1.3 Aim of study

The available literature data reveal the lack of information on influences of chemicals on the vast majority of non-target organisms. This study will help to fill some of these gaps and can initiate new approaches to questions regarding toxicology on freshwater crayfish. Therefore, two main questions are examined:

1: Are reproduction stages of freshwater crayfish influenced by the chemicals TBA and DCF or by the mixture of chemicals with in the sewage of treatment plants?

2: Are marbled crayfish suitable as model organisms in toxicological studies?

To address the first question, the whole reproduction cycle of both crayfish species *Astacus astacus* and *Procambarus virginalis* has to be investigated. Because of the complexity of the reproduction and the accompanying difficulty to detect the time and type of appearing effects on the animals, the reproduction cycle is split into two parts. The first part, described in chapter 2, covers effects on gonadal maturation of both female and male crayfish. This approach involves the exposure of mature crayfish to the two chemicals mentioned above.

The second part is designed to show effects occurring during the embryonic development of the two species in chapter 3. Therefore, the embryos were analysed while being separated from maternal animals. Investigated parameters represent both lethal and sublethal effects on the two species.

To reflect and evaluate laboratory experiment results, a field experiment analysing the effects of wastewater treatment plants was carried out. The unknown influences of mixtures of anthropogenically produced and discharged concentrations of chemicals in surface waters are investigated in the last manuscript in chapter 4. To this end, adult egg-carrying female crayfish were exposed to an area influenced by wastewater.

The inclusion of marbled crayfish in these studies is supposed to reveal possible similarities in responses to DCF and TBA concentrations in the two crayfish species. The highly invasive potential of this species excludes it from all outdoor setups. In laboratory experiments, however, these animals could improve the data in quantity and quality due to their high reproduction number and genetic conformity so that they could serve as model organisms for native freshwater crayfish species.

## 1.4 References overall introduction

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# 2 Gonadal development

## 2.1 Effects of Diclofenac and Terbuthylazine on gonadal maturation of noble crayfish

The first step of the reproduction of freshwater crayfish, even before mating, is the development of gonads. Therefore, this period is the first that was investigated in this study. The following section explains our experimental approach as well as the results and conclusion. These can help us to understand the influences of chemicals on the reproduction and on population dynamics of freshwater crayfish.

The included version of the manuscript represents the state of preparation prior to full corrections and approval of co-authors. Submission is planned for the journal: “Ecotoxicology”.

# Effects of Diclofenac and Terbuthylazine on gonadal maturation of noble crayfish

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Keywords: *noble crayfish, Diclofenac, Terbuthylazine, gonadal*

### 2.1.1 Abstract

In recent decades, the production and use of pharmaceuticals and pesticides has increased significantly. However, this does not only lead to improvements in the human quality of life. Sooner or later, large quantities are discharged into surface waters through sewage treatment plants or agricultural drains. The effects of these substances on the aquatic environment are largely unknown. Therefore, part of these effects will be investigated in this study.

Since noble crayfish are of particular importance for functioning ecosystems, we investigated the effects of two frequently detected and toxically relevant substances. In particular, we investigated the influence of Diclofenac and Terbuthylazine on the gonadal maturation and histopathology of the hepatopancreas of noble crayfish. The results show that even at the lowest tested concentrations of 40 µg/L Diclofenac and 25 µg/L Terbuthylazine, sperm production and histology of the hepatopancreas of the animals is affected. The egg production of females was reduced from concentrations of 0.16 mg/L Diclofenac and 1.6 mg/L Terbuthylazine. These results show that the reproduction and, thus, the population dynamics of noble crayfish are affected by the input of both substances.

### 2.1.2 Introduction

In recent years, there has been growing concern about the release of organic compounds of anthropogenic origin to the environment, known as emerging organic contaminants. These contaminants include a diverse group of thousands of chemical compounds, such as pharmaceuticals and personal care products, pesticides, hormones, surfactants, flame retardants, plasticizers and industrial additives, among others (García et al., 2020). Two of the most frequently detected substances are Diclofenac (DCF) and Terbuthylazine (TBA).

DCF is a non-steroidal anti-inflammatory drug (NSAID) that reduces pain, inflammation and fever (Satoh et al., 2015). It is introduced into surface waters due to human and veterinarian usage over the whole year, mostly through sewage treatment plants. Concentrations of up to 29.8 µg/L were observed in 55 countries (Dusi et al., 2019). TBA on the other hand is a chlorotriazine used worldwide as a pre-emergence herbicide in corn farming. Therefore, highest concentrations are frequently detected between September and April in concentrations up to of 34.0 µg/L in European water bodies (Herrero-Hernández et al., 2017). Their use is of emerging concern because of their persistence, toxicity and proven endocrine disruption in wildlife and humans.

It is especially important to understand that toxic effects on animals, that have a strong influence on their environment in order to be able to estimate impacts on the whole aquatic environment. One of the animal groups with a high impact on its environment are freshwater crayfish. These are the largest invertebrates of freshwater bodies and affect nearly every trophic level of their habitat and influence their structural environment. All native freshwater crayfish in Europe are regarded as “keystone species“ and “ecosystem engineers“ (Weinländer and Füreder, 2016), and are consequently protected by the habitat directive (European Commission, 2000). Nevertheless, these species are highly endangered. Besides invasive species, the crayfish plague (*Aphanomyces* ) and structural stress, chemical loads are one of the main reasons for population decline (Chucholl, 2011). In particular, the influences of presumed toxic chemicals on sensitive phases in the life cycle affect population recruitment and dynamics. One of these phases is the gonadal maturation. In this period the foundation for the next crayfish generation is built and disruptions can lead to smaller offspring numbers and to changing population dynamics. Therefore, we decided to investigate the influences of the two mentioned chemicals on the gonadal maturation of noble crayfish in a laboratory setup.

We used noble crayfish (*Astacus astacus,* Linnaeus 1758), a species native to European freshwater systems. Its reproduction cycle can be described as the standard reproduction of freshwater crayfish. Mating of animals takes place in October to November, which is directly followed by the extrusion of eggs by female crayfish. Therefore, the gonadal maturation starts in June (Ackerfors, 1999).

### 2.1.3 Material and Methods

#### 2.1.3.1 Noble crayfish

The 54 individuals of *Astacus astacus* (36 females and 18 males) were obtained from a hatchery in Oeversee (Krebszucht Oeversee, Schleswig-Holstein, Germany) in June. Their genetic origin lays in a northern German population of the Langsee. The crayfish had a carapace length between 43.6 mm and 61.0 mm and weighed between 23.5 g and 49.6 g. Individuals were separated to exclude cannibalism (especially during moulting), competition and stress. During adaption and experimental phase, they were fed with a mixture of frozen midge larvae, peas and a commercially used dry food. Temperature was adjusted to outdoor conditions (mean of 18 °C), and the light regime was L:D = 12:12 hours.

#### 2.1.3.2 Substances

All substances were used only up to concentrations of solubility to exclude the need of solvents, which could have additional effects on the animals. Concentrations were measured weekly by high performance liquid chromatography (HPLC) and adjusted when differing more than 15 % from the targeted concentrations. Groups were named according to concentrations and substances, D for DCF, T for TBA and 0 for the control.

##### 2.1.3.2.1 Terbuthylazine

TBA was obtained from Sigma Aldrich, Germany, in 98 % purity. Concentrations were chosen to cover the range from concentrations occurring in surface waters to known effective concentrations from other taxa (Cedergreen and Streibig, 2005; Schramm et al., 1998; Shehata et al., 1997) as follows: 0.025 mg/L, 0.4 mg/L, 1.6 mg/L and 6.4 mg/L.

##### 2.1.3.2.2 Diclofenac

We purchased DCF (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) in 99.5 % purity. It was applied in concentrations to cover monitored concentrations of European surface waters as well as establishing higher concentrations that could occur due to bioaccumulation as follows: 0.04 mg/L, 0.16 mg/L, 2.56 mg/L and 10.24 mg/L.

#### 2.1.3.3 Parameter monitoring

Oxygen, pH, conductivity and temperature of all experimental tanks were measured three times per week with multi-parameter probes (WTW Oxi 3310 and WTW pH 3310, Xylem Analytics Germany Sales GmbH & Co. KG, WTW Weilheim, Germany) per hand and Temperature was measured additionally with a HOBO Logger (HOBO, ONS-UA-022-64 Onset Computer Corporation, Bourne, MA, USA). Nitrate, Nitrite, Ammonium and acid binding ability was measured once every week with a photometer (DR 5000, Hach Lange GmbH, Düsseldorf, Germany) and titration.

Analyses of DCF and TBA concentrations were performed on an HPLC system (Hewlett Packard 1050) equipped with an UV-detector. The analytical separation was accomplished on a Zobrax SB-C18 column (250 × 4.6 mm; 5 µm particle size). For TBA, the chromatographic mobile phase contained acetonitrile (75 %) and water (25 %). The injection volume of the analytical solution was 10 µL. The flow rate of the mobile phase was kept at 1.0 mL/min and column temperature was kept at 30 °C. The detection wavelength was 228 nm. For the processing of samples, 5 mL were transferred to a 10 mL centrifuge tube; 2.78 g of $(NH_4)_2SO_4$ was accurately weighed and added to the sample solution and shaken for 1 min until the inorganic salt was completely dissolved. An amount of 0.46 mL acetonitrile was added to the mixture, placed in an ultrasonic cleaner (Bandelin Sonorex tk 52 Transistor, BANDELIN electronic GmbH & Co. KG, 12207 Berlin, Germany) for 9.5 min and centrifuged at 4000 rpm for 5 min until the ATPS (Aqueous Two-Phase System) was formed. The upper and lower phase were acetonitrile and inorganic salt, respectively. The analytes were extracted into the acetonitrile-rich phase. Then, 100 µL of the upper organic phase was collected and filtrated with a 0.22 µm organic membrane filter for HPLC analysis. The described method is modified from the method used by Gao et al. (2018).

For DCF, the mobile phase contained 45 % acetic acid and 55 % acetonitrile. Injection Volume was 5 µl and flow rate was 1 ml/min. Temperature was set to 30 °C and the wavelength was set to 200 nm. Then, 1 ml of the samples was filtrated with a 0.22 µm organic membrane filter for HPLC analysis. This method was changed after Hartmann et al. (2008).

As reference material for quantification calculations we dissolved three different concentrations of both chemicals in fully deionized water using an ultrasonic cleaner.

#### 2.1.3.4 Experimental setup

Six *Astacus astacus* individuals per tank (W/L/H: 40 cm/120 cm/40 cm, four females and two males) were accustomed for two weeks and were later also used for the experiments. The animals were held in nine tanks with a different concentration of DCF or TBA each in a room with controllable light and temperature regime. The separation was achieved by a grid, so that females and males could detect the presence of the opposite gender, and at the same time cannibalism was excluded. The experiment started at the beginning of the ovary maturation at the end of June, according to Ackerfors (1999) and lasted until the start of egg-laying of these individuals. From the typical time of mating in late September, barriers were removed once a day for one hour. Barriers were completely removed when mating was observed. Male animals were removed at the time that all females showed the attached spermatophores. Males were then prepared for the observation of spermatophore quantity and quality in the distal ductus deferens after Farhadi et al. (2018). To this end, 1 g of the distal ductus versus was prepared and extracted and sperm cells were counted in a "Neubauer Improved" chamber. Additionally, a part of the sample was stained with trypan blue 0.4 % (Carl Roth GmbH + Co. KG, 76231

Karlsruhe, Germany) to identify the ratio of dead and alive spermatophores in the sample. After the extrusions of eggs, these were counted and artificial breeding was started in an incubator equipped with UV-clarification, biofilter, oxygen supply and egg-moving trays to document survival of the embryos. Eggs of different concentrations were placed in a minimum of three trays and distributed over the incubator to exclude influences of placement, temperature differences or oxygen supply on the survival.

#### 2.1.3.5 Histology

For histological assessment, we fixated the hepatopancreas of the prepared noble crayfish males in pH-buffered formaldehyde (3.7 %). We stored the sample in Kristensen solution for two days to ensure complete decalcification. Samples were then embedded in LR White (LR White acrylic resin, hard, sigma Aldrich, Germany) and sections of 2 µm thickness were prepared using an ultramicrotome. Sections were stained with haematoxylin and eosin (HE) with extended exposure time, referring to usage instructions of the LR White. These tissue samples were examined under a light microscope combined with a camera system (Leica DM1000 LED, Leica ICC50 HD, Leica Application Suite Version 3.0.0, Leica Microsystems CMS GmbH, 35578 Wetzlar, Germany).

A crayfish hepatopancreas is typically formed of numerous tubules, separated by connective tissues (Abd El-Atti et al., 2019) and consists of lumen, membranes and four types of epithelial cells: resorptive lipid cells (R-cell) for nutrient intake, blister-like secretory cells (B-cell) to channel off harmful substances, fibrillar cells (F-cell) as connecting tissue and embryonic cells (E-cell). That means, changes in R-cells would indicate a higher or lower intake of nutrients, changes in B-cells would indicate a higher or lower outtake of harmful substances, whereas changes in the other two types would indicate problems in biosynthesis of the individual.

Hepatopancreas cells were examined for potential membrane damages, damages in the four different cell types as well as changes in their size and quantity. To do this, ten sections per individual were photographed and subsequently analysed by counting and measuring cells under the microscope.

#### 2.1.3.6 Statistics

We performed all statistical analyses using R version 3.2. (R Core Team, 2015). Juvenile size, survival rates and number of hepatopancreas B-cells were tested for normality and equal variances prior to analysis. Both given, a one-way ANOVA (variance analysis) and post hoc Tukey test were performed. Differences in quantity of spermatophore number, spermatophore living rate, survival rate of eggs and laid eggs between different groups were tested for normality and equal variances prior to analysis. If both were evident, a t-Test was performed. For non-parametric data, a Wilcoxon test was used. Pictures were analysed with GIMP software (version 2.8, Fa. the Gimp Team).

### 2.1.4 Results

#### 2.1.4.1 Spermatozoa

Quantity of spermatozoa cells differed between the individuals of different concentrations. Overall, the groups exposed to DCF had the lowest spermatozoa numbers, with averages of 684.3 (± 405.9) for D0.04 mg/L, 547.0 (± 397.6) for D0.16 mg/L, 420.6 (± 405.9) for D2.56 mg/L and 135.96 (± 46,0) for D10,24 mg/L. Sperm-cell number was higher for animals exposed to TBA. They had averages of 470 (± 151.9) for T0.025 mg/L, 1551.6 (± 742.3) for T0.4 mg/L, 1090.9 (± 274.1) for T1.6 mg/L and 1066.6 (± 463.8) for T6.4 mg/L. All groups showed numbers significantly lower than the control group which had an average of 2022.7 cells/µl (± 405.9) ($p \leq 0{,}0005$; t-Test; Wilcoxon-Mann-Whitney-Test) (Figure 2.1). It is worth noticing that the lowest sperm-cell numbers for animals exposed to TBA was counted in the lowest concentration.

Similar to their quantity, also the quality of the spermatozoa is influenced by the two chemicals. However, the differences in quality are not as significant as for the quantity. For Diclofenac, only the highest concentration of 10.24 mg/L resulted in a significantly higher rate of dead cells ($p = 1.4e^{-16}$). For Terbuthylazine significant differences were detected for the two highest concentrations 1.6 mg/L ($p = 0.03$) and 6.4 mg/L ($p = 0.02$). Figure 2.2 shows obvious differences between dead and alive sperm cells.

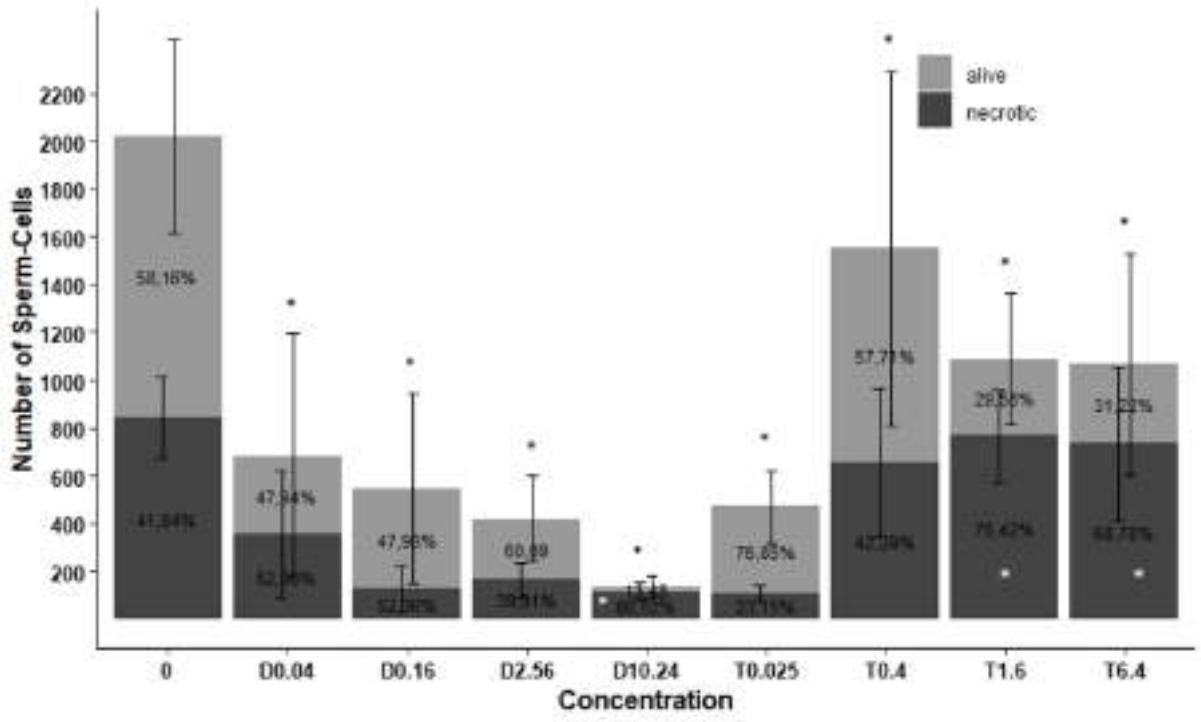


*Figure 2.1: Spermatozoa cells per µl. Dark areas represent the number of dead cells; light grey represents the living cells and the height of every bar the total average of counted cells. Stars indicate statistical differences (black: counted; white: percentage dead).*

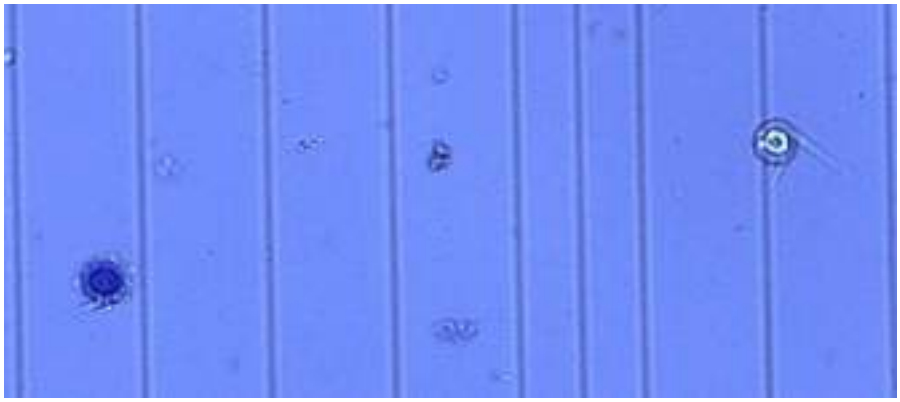

*Figure 2.2: Spermatozoa cells stained with trypan blue 0.4 %. Dead cell (left) living cell (right).*

#### 2.1.4.2 Eggs

Figure 2.3 shows the quantities and survival rates of developed oocytes of noble crayfish in relation to the concentrations of the chemicals they were exposed to. It can be seen that the presence of the substances influenced the number and the survival of the laid eggs. The control group showed an average of 241.00 eggs/female (± 35.58). The group D0.04 mg/L had 151.25 (± 89.04) eggs/female and D0.16 mg/L had 106.50 (± 29.90) eggs/female. In the group D2.56 mg/L, only two of six females laid eggs, resulting in a statistical average of 27.50 (± 28.39) eggs/female. The group D10.24 mg/L did not lay eggs at all. For TBA, average egg numbers were 219.02 (± 39.06) for T0.025 mg/L, 179.75 (± 25.66) for T0.4 mg/L, 110.21 (± 14.97) for T1.6 mg/L and 77.54 (± 46.59) for T6.4 mg/L from only three females. The statistical analyses showed differences between the three highest Diclofenac groups ($p \leq 0.002$; t-Test) and the two highest TBA groups ($p \leq 0.004$) to the control group.

Similar to the spermatozoa, the survival of the eggs is affected less by the chemicals than the number of eggs. The two highest Diclofenac groups ($p \leq 0.006$) and the highest TBA group ($p = 0.01$) showed a significantly higher mortality than the control group. This combination results again in significantly smaller numbers of hatched eggs in all Diclofenac groups and the two highest TBA groups compared to the control group ($p < 0.05$, ANOVA, Tukey).

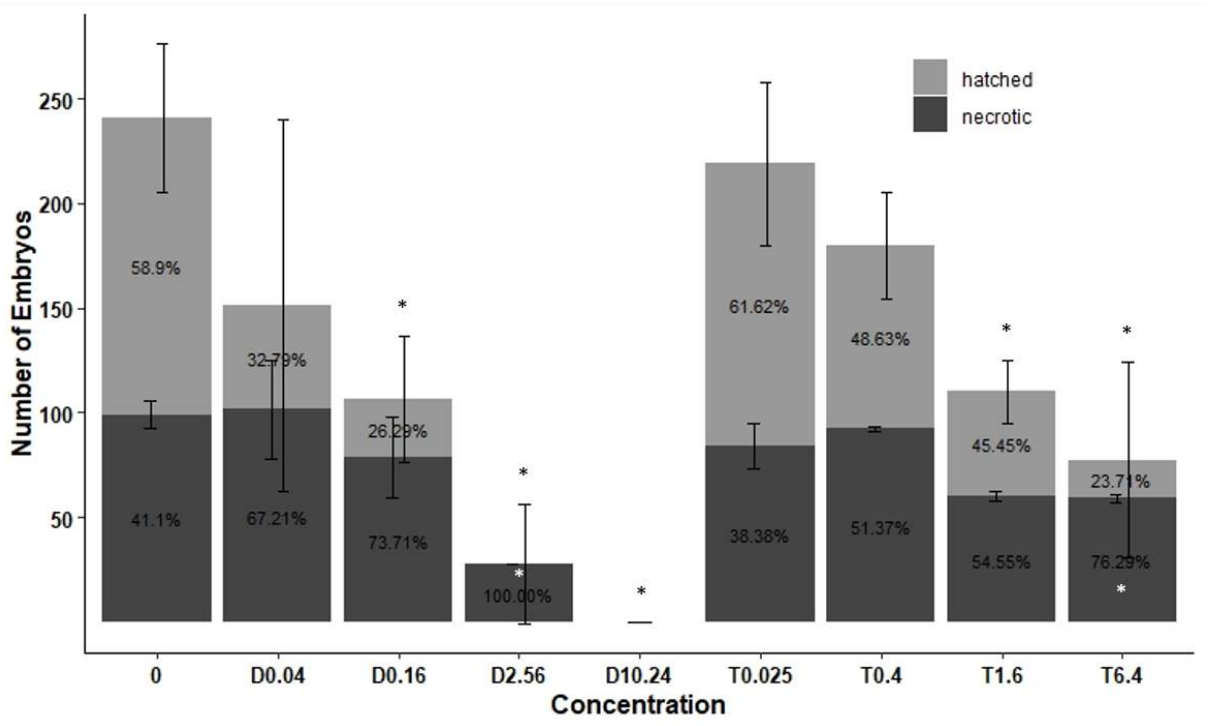


*Figure 2.3:Egg cells per µl. Dark areas represent the number of dead embryos; light grey represents the living animals and the height of every bar the total average of counted eggs. Stars indicate statistical differences (black: laid eggs; white: hatched juveniles)*

#### 2.1.4.3 Histology

The histological observations show that with increasing concentrations of both chemicals, the B-cells of the adult animals are enlarged in comparison to the control group (Figure 2.4). Additionally, in 76 % of all investigated sections of the group exposed to highest TBA concentrations, damages in the lumen membrane were found. Other sections did not show these ruptures.

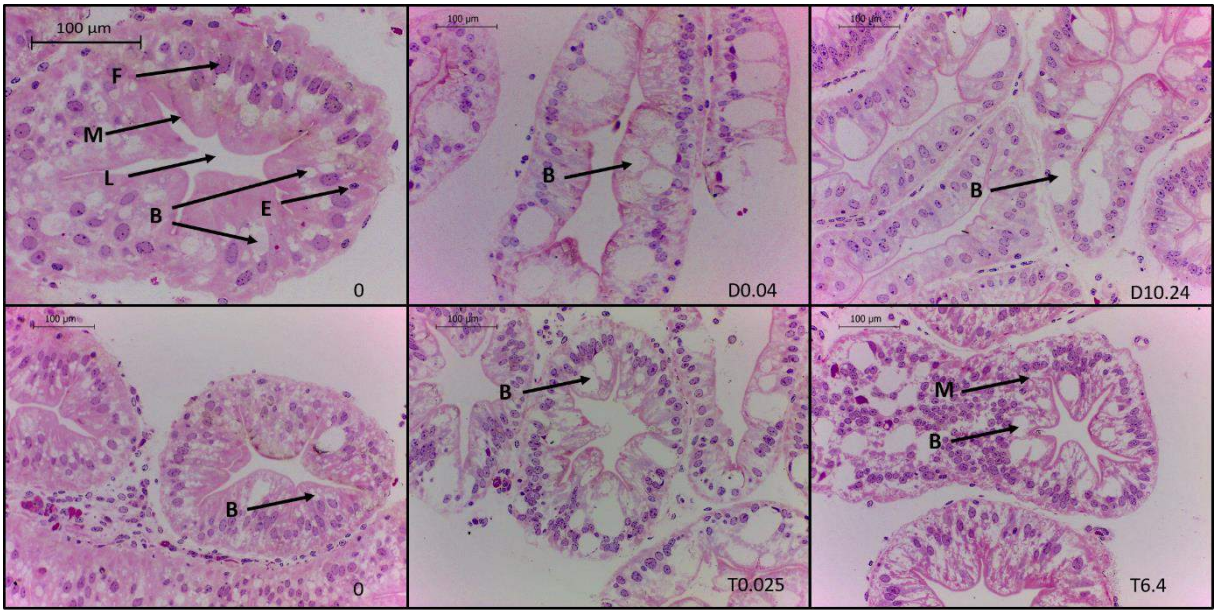


*Figure 2.4: Histological sections of hepatopancreas of noble crayfish (*Astacus astacus*) exposed to the different concentrations for 120 days. Marked areas are lumen (L), membrane (M) and four types of epithelial cells: resorptive (R) lipid cells, blister-like (B) secretory cells, fibrillar (F) cells and embryonic (E) cells; (HE stain, 100×).*

The average diameter of B-cells in the control group was 26.86 µm (± 9.90). In comparison to that, individuals exposed to lowest TBA concentration showed B-cell diameters of 39.60 µm (± 11.51), while group T0.025 mg/L showed 41.61 µm (± 11.28). Exposure to highest TBA concentrations resulted in diameters of 76.78 µm (± 25.27) and to highest Diclofenac concentrations in diameters of 73.90 µm (± 18.21) (Figure 2.5). Statistical analyses showed that all groups differed significantly from the control group ($p \leq 0{,}0001$, Tukey).

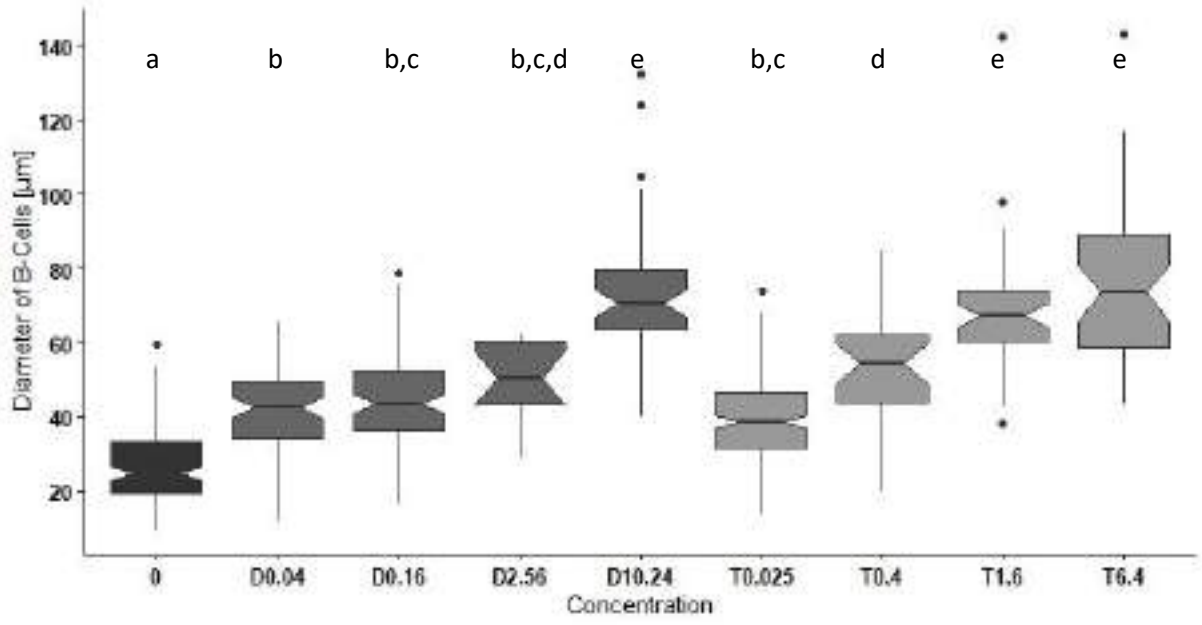


*Figure 2.5: Diameter of hepatopancreas B-Cells of male adult noble crayfish exposed to different concentrations of the two chemicals DCF and TBA. Letters represent significant differences calculated via Tukey (p≤0,05)*

### 2.1.5 Discussion

Results of this study reveal effects of DCF und TBA on gonadal development of noble crayfish. All parameters investigated were affected by the two chemicals even in lowest concentrations used in this study.

As one of the main factors influencing reproductive efficiency (Wickins and O'C Lee, 2003), sperm quality in male crustaceans is affected by the presence of environmental pollutants (Lewis and Ford, 2012). Referring to (Harlıoğlu et al., 2018), we assessed this parameter by investigating sperm-cell number and ratio of dead and alive sperm cells in male distal ductus deferens. Literature shows that these factors are influenced by environmental chemicals. Canosa et al. (2019) showed, for instance that Glyphosate can imbalance the male reproductive function of the estuarine crab *Neohelice granulata* at concentrations of 1 mg/L by producing abnormal spermatophores and a reduction in sperm count. Hence, they conclude the possibility of a reduction in brood production and larvae recruitment that takes place in the natural environment. The present results of spermatophore analyses lead to the same conclusion, insofar that the quantity and quality of sperms is significantly reduced even at the lowest used concentrations of 0.025 mg/L TBA and 0.04 mg/L DCF. Therefore, this study reveals that TBC and DCF present in surface waters through sewage treatment plants or by agriculture are a severe threat to noble crayfish populations.

But not only the male reproduction efficiency of noble crayfish is affected. The egg production and survival of embryos is also affected by the two chemicals, when exposed during gonadal development. With an average of 241 eggs per female the control group is consistent with the range of egg numbers for noble crayfish of 70 to 250 eggs reported in the literature, depending on maternal size (Skurdal et al., 2011). Even though the two chemicals do not affect the gonadal development of the females as much as for males, effects are still alarming. Especially the combination of smaller egg numbers with the smaller survival rate of embryos consequently lead to a much lower individual count of the offspring of influenced animals than for the control group, as is visible in the number of survived embryos until hatching. Groups exposed to DCF all show significantly smaller offspring numbers compared to the control group; the same applies to the two highest TBA groups. Meyer et al. (2007) revealed that spawning probability as well as juvenile and adult mortality are the most important parameters for the survival of crayfish populations. They also estimated the median time to extinction of native crayfish populations to be 80 years for a set of parameters derived from field estimates. This shows how important a stable recruitment of noble crayfish will be in the future to ensure the survival of this species.

The histology of the hepatopancreas shows effects even at lowest concentrations of DCF and TBA in the form of an increase in the number and diameter of B-cells compared to the control. In addition, we found damage to membrane structures similar to Chaufan et al. (2006) for high TBA concentrations.

Their study revealed disorganisation in hepatopancreas tubules as well as increased diameters and numbers of B-cells in hepatopancreas cells after feeding crabs (*Chasmagnathus granulatus*) with Hexachlorobenzen-contaminated *Chlorella* for three days.

Therefore, an increase in the size and number of B-cells is a sublethal effect of exposure to at least TBA and Hexachlorobenzen as examples for agricultural control chemicals. Silveyra et al. (2018) tested the influences of Atrazine (which was substituted by TBA and is similar in form and shape to TBA) on vitellogenesis, steroid levels and lipid peroxidation in female red swamp crayfish *Procambarus clarkii*. They found that Atrazine-exposed crayfish had a lower expression of vitellogenin in the ovary and hepatopancreas as well as smaller oocytes and reduced vitellogenin content in the ovary. Similar to what is observed in this study for DCF and TBA, the Atrazine leads to a lowered reproduction of the crayfish. Not only Terbuthylazine, but also its degradation products are of great interest due to their toxicity. Koutnik et al. (2017) showed effects of terbuthylazine-2-hydroxy-exposure in concentrations of down to 75 µg/L on early life stages of marbled crayfish. Influenced parameters were: embryo weight, ontogenetic development, the antioxidant system, oxidative stress and pathological changes in the hepatopancreas. Therefore, influences on the hepatopancreas are not only caused by TBA but also by its degradation products.

Only few data are available for effects of DCF on hepatopancreas tissue. Geetha et al. (2018) report significant changes in nucleation, differentiation, and hepatocytes in a histopathological study on a *Pangasius* sp. hepatopancreas sample starting from 6 mg/L DCF.

Neuparth et al. (2014) showed that Simvastatin, a lipid-lowering medication, severely impacted growth, reproduction and gonad maturation of *G. locusta*, concomitantly to changes at the histological level. Among all analyzed endpoints, reproduction was particularly sensitive to Simvastatin with significant impact at 320 ng/L. Du et al. (2016) showed a significant lower brood number of *Daphnia magna* starting from 0.5 mg/L DCF. The danger of pharmaceuticals and especially DCF on reproduction is, hence proven by several publications including the present one.

Overall, the data of the present study show that both environmentally relevant substances, DCF and TBA, pose a high risk for the reproduction of crayfish. Not included in the evaluation were mixed effects with other chemicals or the bioaccumulation of the substances, which could further increase the effects. It is, thus, of particular importance to further expand the data available on these issues and at the same time minimize inputs of pollutants into surface waters as far as possible.

### 2.1.6 References Effects of Diclofenac and Terbuthylazine on gonadal maturation of noble crayfish

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## 2.2 Effects of Diclofenac and Terbuthylazine on gonadal maturation of marbled crayfish

# Effects of Diclofenac and Terbuthylazine on gonadal maturation of marbled crayfish

### 2.2.1 Introduction

In addition to the manuscript above, we used marbled crayfish (*Procambarus virginalis,* Lyko 2017) for a similar investigation. The aim was to test its suitability as a model organism for the estimation of effective concentrations of TBA and DCF, as its gonadal maturation is significantly different from other crayfish species due to their parthenogenetic reproduction.

### 2.2.2 Material and methods

#### 2.2.2.1 Marbled crayfish

All investigated female *Procambarus virginalis* were obtained from the research group of Frank Lenich in Regensburg. Animals were caught manually via diving. Similar to the experimental setup of the noble crayfish, three marbled crayfish were kept in 20 L-tanks for two weeks to adjust to the laboratory conditions and were separated to exclude cannibalism, competition and stress. Due to different optimal reproduction conditions, the temperature was set at 24 °C and the light regime was L:D = 8:16 hours. On account of low availability, we were only able to use 35 individuals. Therefore, the Investigations were conducted with one concentration less in comparison to the noble crayfish setup.

Substances and parameter monitoring were for the same as in the noble crayfish study. The experimental setup was adjusted to the requirements of marbled crayfish, which is described in the following. Histological examinations were not carried out due to insufficient data (see below).

#### 2.2.2.2 Experimental setup

The investigated marbled crayfish were kept in 14 tanks with 18 litres volume each. The tanks were placed in climate chambers to assure constant temperature and light regimes and were equipped with one circulator 650 pump (AQUAEL Deutschland GmbH, 40699 Erkrath, Germany) with aeration each. Two or three animals were kept per tank and treatment and were separated by partitions. The parthenogenetic reproduction of these animals allowed us to start the experiment at any given date of the year. In this case, the experiment started in July and was conducted until all individuals of the control group extruded eggs at least once. After the extrusion, eggs were counted and artificial

breeding started in an incubator as in the manuscript above on noble crayfish. All juveniles were measured via photography with a scale and the software ”GIMP“ (version 2.10.18, The GIMP-Team), and mortality was observed and analysed.

### 2.2.3 Results

#### 2.2.3.1 Number of eggs and survival of embryos

As shown in Figure 2.6, the number of laid eggs and the survival of eggs are quite similar regarding the different concentrations of the two chemicals. The large standard deviations of all groups and the low egg production of the control are noteworthy. There were no significant differences in numbers of eggs or hatched individuals.

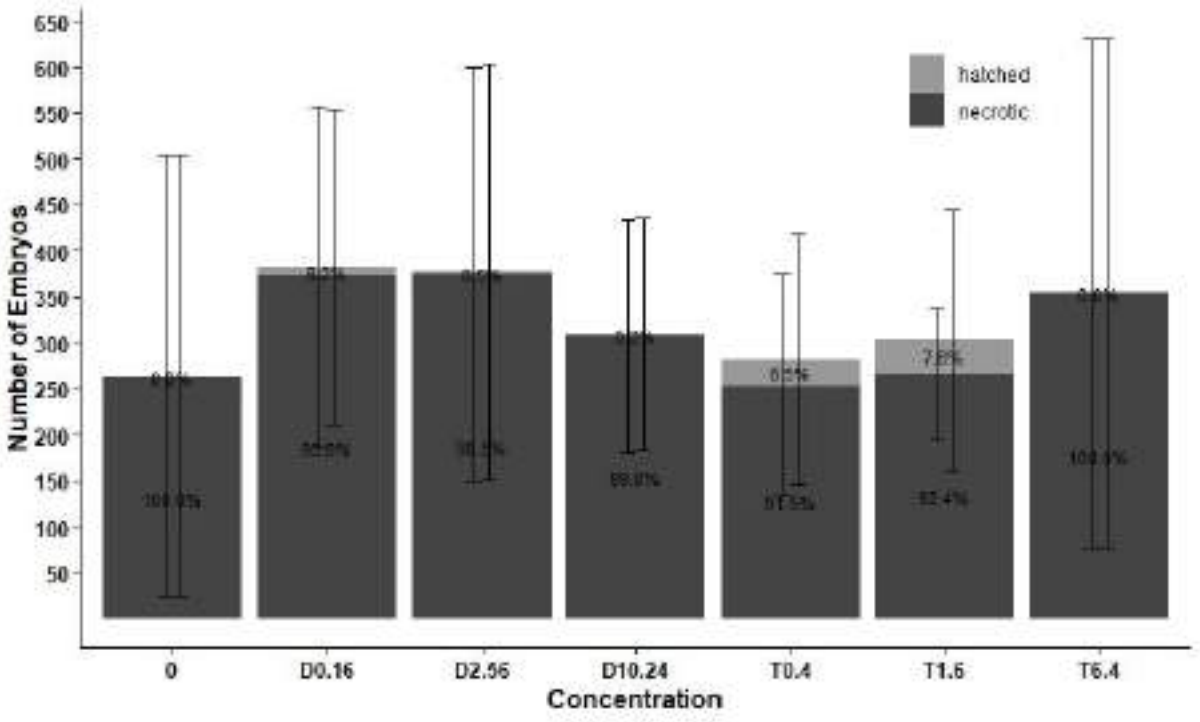


*Figure 2.6: Egg cells per µl. Dark areas represent the number of dead embryos; light grey represents the living animals and the height of every bar the total average of counted eggs.*

#### 2.2.3.2 Size of juveniles

The size of the hatched juveniles has only been measured for five concentration groups (Figure 2.7). In the other two group no embryos survived until hatching. Significant differences were found only for the DCF concentration of 0.16 mg/L, which showed a lower size in comparison with the groups exposed to 2.56 mg/L DCF and 0.4 and 1.6 mg/L TBA.

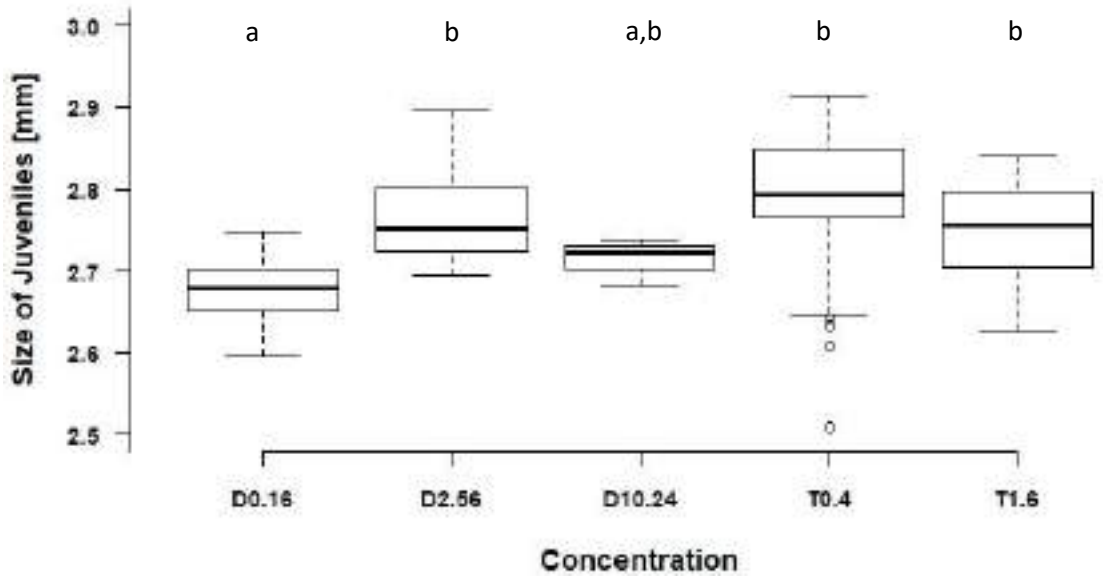


*Figure 2.7: Hatching size of marbled crayfish after parental exposure to different chemical concentrations. Letters indicate significant differences.*

### 2.2.4 Conclusion

Contrary to noble crayfish, gonadal development of marbled crayfish seems not to be negatively influenced by the two tested chemicals. Neither the number of laid eggs and survival of these, nor the size of hatched juveniles showed clear evidence of any negative effects. Even though there are significant differences in the size of juveniles exposed to 0.16 mg/L DCF in comparison to three other groups, the fact that the lowest DCF concentration results in the lowest body size of all observed groups shows that the reasons for these differences are not to be found in chemical concentrations. Most likely, maternal size and individual fitness caused this effect. Also, the very limited number of hatched juveniles impairs the reliability of this data.

Overall, it can be assumed, that the shorter gonadal development and the missing impact on spermatozoa production lead to a higher resistance of the gonadal development of marbled crayfish to environmental pollution.

# 3 Embryonic development

After the illumination of effects on gonadal development and mating, observations on effects of the chosen chemicals on the embryonic development are needed to evaluate the influences on the whole reproduction cycle and, therefore, the impacts on population dynamics due to reproduction disruptions. The following manuscripts show the impact of TBA and DCF on embryos of both species *Astacus astacus* and *Procambarus virginalis*. Both of these manuscripts are submitted to peer-reviewed journals (Terbuthylazine; Water, Air, & Soil Pollution; Diclofenac: International Aquatic Research).

## 3.1 Noble crayfish are more sensitive to Terbuthylazine than parthenogenetic marbled crayfish

The included version of the manuscript represents the initial submission of this study to the journal: "Water, Air, & Soil Pollution" prior to any changes during review process. Submission date: 20.07.2020

# Noble crayfish are more sensitive to Terbuthylazine than parthenogenetic marbled crayfish

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Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jan Laurenz and Lena Lietz. The first draft of the manuscript was written by Jan Laurenz and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Short title: Terbuthylazine and crayfish embryonic development

Keywords: *marbled crayfish, noble crayfish, juveniles, Terbuthylazine, embryonic*

# Noble crayfish are more sensitive to Terbuthylazine than parthenogenetic marbled crayfish

## 3.1.1 Abstract

We investigated the sensitivity of two freshwater crayfish species (*Astacus astacus* and *Procambarus virginalis*) during embryonic development to chronic exposure to the herbicide Terbuthylazine under laboratory conditions. The assessed parameters included time of embryonic development, survival rate, hatching weight and histopathology of hepatopancreas. $LC_{50}$ (median lethal concentration) and $ED_{50}$ (median effective concentration) were estimated. We were able to determine effects of TBA for every investigated parameter. For noble crayfish the $LC_{50}$ value after 45 days was 0.11 mg/L and the histology of the hepatopancreas showed effects starting from 0.025 mg/L. Other parameters revealed effects starting at concentrations of 1.6 mg/L for weight and 6.4 mg /L for embryonic development time and hatching rate. Marbled crayfish only showed effects concerning the hatching rate and survival rate at concentrations without a clear dose effects curve. As a conclusion, our data shows the risk of TBA in existing concentrations in freshwater ecosystems to non-target organisms and also the need of toxicological studies on directly affected species in addition to the use of model organisms.

## 3.1.2 Introduction

Terbuthylazine is a chlorotriazine used worldwide as a pre-emergence herbicide in corn farming. This leads to peaks of TBA concentrations in natural water bodies during March and April. Its use is of emerging concern because of its persistence, toxicity and proven endocrine disruption in wildlife and humans (Tasca et al., 2019). It is also one of the most frequently detected pesticides in natural waters (Dolaptsoglou et al., 2007). Concentrations of more than 34.0 µg/L were detected in surface waters in Spain (Herrero-Hernández et al., 2017). Despite its prevalence in European ecosystems, little is known about the effects of this pollutant on most freshwater invertebrate species.

Freshwater crayfish affect nearly every trophic level of their habitat and influence their structural environment due to their burrowing activity. Therefore, these largest invertebrates of freshwater bodies are called “keystone species“ and “ecosystem engineers“ (Weinländer and Füreder, 2016). Consequently, the habitat directive protects all native freshwater crayfish species in Europe (European Commission, 2000). Nevertheless, these species are highly endangered. Invasive species, the crayfish plague (*Aphanomyces astaci*), structural stress and chemical loads cause population decline (Chucholl, 2011). The influences in particular of presumed toxic chemicals on sensitive life stages affect population recruitment and dynamics. One highly sensitive life stage is the development of crayfish embryos (Khan and Nugegoda, 2007). During this period, between November and June (Ackerfors,

1999), the embryonic individual is exposed to the environment and its possibly harmful substances without the possibility of active avoidance. Because of its usage as a pre-emergence herbicide, the highest concentrations of TBA are detected at the beginning of spring and therefore in this sensitive development period of freshwater crayfish (Lorente et al., 2015).

For this reason, we examined the effects of TBA on the embryonic development of two freshwater crayfish species. We used marbled crayfish (*Procambarus virginalis,* Lyko 2017) to test its suitability as a model organism. One female can produce up to 700 eggs every 8–9 weeks. The offspring is genetically identical due to the species' parthenogenetic reproduction strategy (Chucholl and Pfeiffer, 2010; Vogt et al., 2004), thus providing a predictable and continuous supply of clonal eggs and making this species a suitable model organism for higher invertebrates with a longer embryonic development (Hossain et al., 2018; Vogt, 2018). The second organism we studied was the native noble crayfish (*Astacus astacus,* Linnaeus 1758), as it is especially suitable for our study due to its natural habitats (lower sections of streams, lakes etc.), which are often influenced by agricultural drainage and sewage (Skurdal, J. and Taugbøl, T., 2002).

Parameters that can indicate lethal and sublethal effects of herbicides on the reproduction of freshwater crayfish are embryonic development, survival rate, hatching weight and the histopathology of the hepatopancreas of the juveniles (Velisek et al., 2013). Changes in the first three parameters directly influence the development of populations. At the same time, the hepatopancreas is the site of nutrient absorption, digestion, synthesis and secretion of digestive enzymes and reserve storage in decapods (Calvo et al., 2011; Johnston et al., 1998; Xiao et al., 2014). It is formed of numerous tubules separated by connective tissues (Abd El-Atti et al., 2019) and consists of a lumen, membranes and four types of epithelial cells: resorptive lipid cells (R-cell) for nutrient intake, blister-like secretory cells (B-cell) to channel off harmful substances, fibrillar cells (F-cell) as connecting tissue and embryonic cells (E-cell). This means, changes in R-cells would indicate a higher or lower intake of nutrients, changes in B-cells would indicate a higher or lower outtake of harmful substances, whereas changes in the other two types would indicate problems in the biosynthesis of the individual. For this reason, hepatopancreas tissue is used for monitoring the health of crayfish and can indicate diseases and exposure to harmful substances (Velisek et al., 2017; Xiao et al., 2014). We hypothesize that TBA exposure influences the aforementioned parameters in both species of freshwater crayfish, *A. astacus* and *P. virginalis*, and that the marbled crayfish embryos and noble crayfish embryos are affected by the herbicide in a similar way.

### 3.1.3 Material & methods

#### 3.1.3.1 Chemicals

Terbuthylazine was obtained from Sigma Aldrich, Germany, in 98 % purity. Concentrations were chosen to cover the range from concentrations occurring in surface waters to known effective concentrations from other taxa (Cedergreen and Streibig, 2005; Schramm et al., 1998; Shehata et al., 1997) as follows: 0.0 mg/L; 0.025 mg/L; 0.1 mg/L; 0.4 mg/L; 1.6 mg/L; 6.4 mg/L and 12.8 mg/L. Due to low solubility (5 mg/L in 20°C) TBA was dissolved in Dimethylsulfoxid (DMSO). To exclude effects of DMSO a control group with this solvent was included.

#### 3.1.3.2 Origin of brood stock and eggs

Noble crayfish (15 females and six males) were obtained from a hatchery in Schleswig-Holstein (Krebszucht Oeversee, Germany) in November and transferred to the facilities of Kiel University. Three groups of five females and two males were kept in three recirculating 600 L tanks throughout the reproduction period. Temperature was set at 8°C and a light regime of L:D = 10:14 was provided. Females were checked for eggs daily. Eggs were separated from females after a two week period at 4 °C in winter, and artificial breeding was started in an incubator equipped with UV clarification, biofilter, oxygen supply and egg-moving trays.

Marbled crayfish were bred in facilities of Kiel University; animals were obtained beforehand through the aquatic trade and kept individually in 12 separate 25 L aquaria in aerated tap water. The ambient temperature was 23°C, the light regime was L:D = 10:14 hours. All animals were fed frozen midge larvae and peas ad libitum. Under these conditions, *P. virginalis* produced parthenogenetic eggs every 8–9 weeks in our laboratory. These eggs were separated 72 hours after laying.

#### 3.1.3.3 Experimental design, setup and data collection

Separated eggs were transferred directly to twelve 12-well multititer plates (Greiner bio-one, Kremsmünster, Austria) with one single egg per well; all eggs were randomly assigned to the wells. Stock solutions were made weekly for each concentration with double the concentration of final TBA. Testing solutions were then prepared daily by diluting stock solution with a mixture of 30 % tap water and 70 % VE Water, which we autoclaved. Each well was filled with 1.5 mL test-solution.

*Table 3.1: Concentrations, trials, designations and numbers of replicates per species.*

| Species | Trial [days] | Designation | No. per concentration |
|---|---|---|---|
| *P. virginalis* | 0–15 | PT | 36 |
| *A. astacus* | 0–45 | AT | 12 |
| *A. astacus* | 0–15 | A1 | 12 |
| *A. astacus* | 15–30 | A2 | 12 |
| *A. astacus* | 30–45 | A3 | 12 |

All multititer plates were placed on a laboratory shaker (Dual-Action shaker K 2, Edmund Bühler GmbH, 72411 Bodelshausen, Germany) that provided 60 movements per minute to ensure constant supply of oxygen and simulate parental movement of the abdomen. Experimental solutions were exchanged daily.

The transparent membrane of crayfish eggs enables us to examine the status of the embryonic development under a stereomicroscope (Alwes and Scholtz, 2006). Using this method, we recorded developmental stages and mortality three times per week. We converted the developmental stages described by (Sandeman and Sandeman, 1991) noble crayfish) and (Alwes and Scholtz, 2006), marbled crayfish) into percentages to allow direct comparability of the embryonic development between species. Due to their different reproduction strategies, the development time of marbled crayfish (650 degree days) is three times shorter than the development time of noble crayfish (1900 degree days) (Kozák, 2015; Skurdal, J. and Taugbøl, T., 2002). Therefore, we exposed noble crayfish for the duration of their complete embryonic development in one trial, and in another investigation, we exposed the embryos in the first, second and third phase of their development, resulting in equal exposure times of 15 days for each treatment (Table 3.1). Survival was also observed in the time after the exposure until the first moult. After this, the study was terminated. At this time, the fresh weight of the moulted animals was measured (Sartorius R160P-*D1 R160P Balance, Sartorius AG 37079 Goettingen, Germany).

#### 3.1.3.4 Histology

After 45 days of exposure three noble crayfish juveniles from each concentration (24 in total) were fixated in Formaldehyde (3.7 %) and decalcified for two weeks in Kristensen solution. After washing in Phosphate Buffered Saline (PBS) three times for 15 minutes, the animals were brought into an ethanol series (2 x 50 %, 70 %, 90 %, 2 x 99 %, 30 min. each) and bedded in LR White in gelatin capsules as described in Table 3.2 (LR White acrylic resin, hard, Sigma Aldrich, Germany).

*Table 3.2: Proportion of solutions, times and temperature for embedding juvenile crayfish in LR White.*

| LR-White: Ethanol | Time [h] | Temperature [°C] |
|---|---|---|
| 1:2 | 2 | 20 |
| 1:1 | 2 | 20 |
| 2:1 | 2 | 20 |
| 1:0 | 2 | 20 |
| 1:0 | 2 | 20 |
| 1:0 | 12 | 20 |
| 1:0 | 48 | 60 |

Sections of 2 µm were made using an ultramicrotome. Sections were stained with haematoxylin and eosin (HE) with an extended exposure time due to acrylic embedding in accordance with LR White instructions for use. Tissues of hepatopancreas were examined under a light microscope combined with a camera system (Leica DM1000 LED, Leica ICC50 HD, Leica Application Suite Version 3.0.0, Leica Microsystems CMS GmbH, D-35578 Wetzlar, Germany). The examination of hepatopancreas cells included the observation of membrane damage, damage in the four different cell types and changes in size and number of the four different cell types per hepatopancreas compartment. For this procedure, 10 sections per individual were photographed and subsequently analysed by counting and measuring cells.

#### 3.1.3.5 Statistical methods

We performed all statistical analyses using R (R Core Team, 2015). The weight of the juveniles and number of B-cells per compartment were tested for normality and equal variances prior to analysis. Afterwards, a one-way ANOVA was performed and subsequently a Tukey post-hoc test. For non-parametric data, a Kruskal-Wallis test was used. The $LC_{50}$-values were control corrected using Abbott's correction first and then estimated utilizing the trimmed Spearman-Karber method. Survival rates were analysed using the Kaplan-Meier survival analysis of Gehan Breslow and the groups were compared via the Holm-Sidak method. The embryonic development was analysed via linear regressions. Due to good correlation values (> 0.8) the linear regressions were compared with an ANCOVA (analysis of covariances). Photographs were analysed in GIMP (version 2.8, Gimp Team).

### 3.1.4 Results

#### 3.1.4.1 Embryonic development time

The development time of group PT embryos was not affected by any of the applied TBA concentrations. The development of group AT was affected by TBA concentrations with significant effects detectable at 6.4 mg/L and higher ($p \leq 0.028$, Figure 3.1).

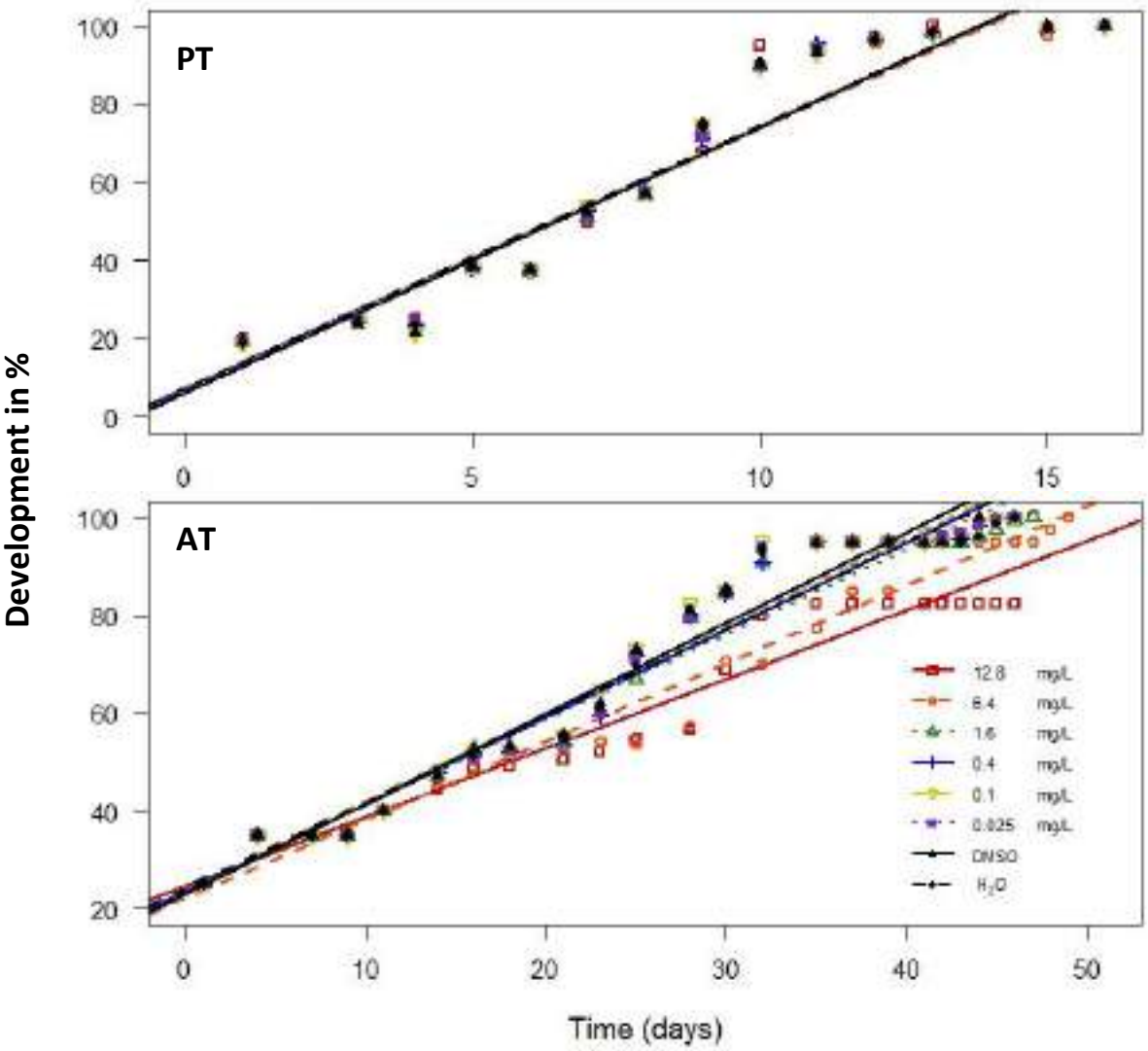


*Figure 3.1: Linear regressions of embryonic development of marbled crayfish (PT) and noble crayfish (AT) in different concentrations of TBA over Time.*

In detail, TBA did influence the last two thirds of embryonic development. There were no differences in development of group A1, but in group A2 embryos exposed to the TBA concentrations of 6.4 and 12.8 mg/L developed slower in comparison to other groups ($p \leq 0.036$). The exposure to TBA of group A3 showed that the three highest concentrations significantly slowed down embryonic development ($p \leq 0.012$, Figure 3.2).

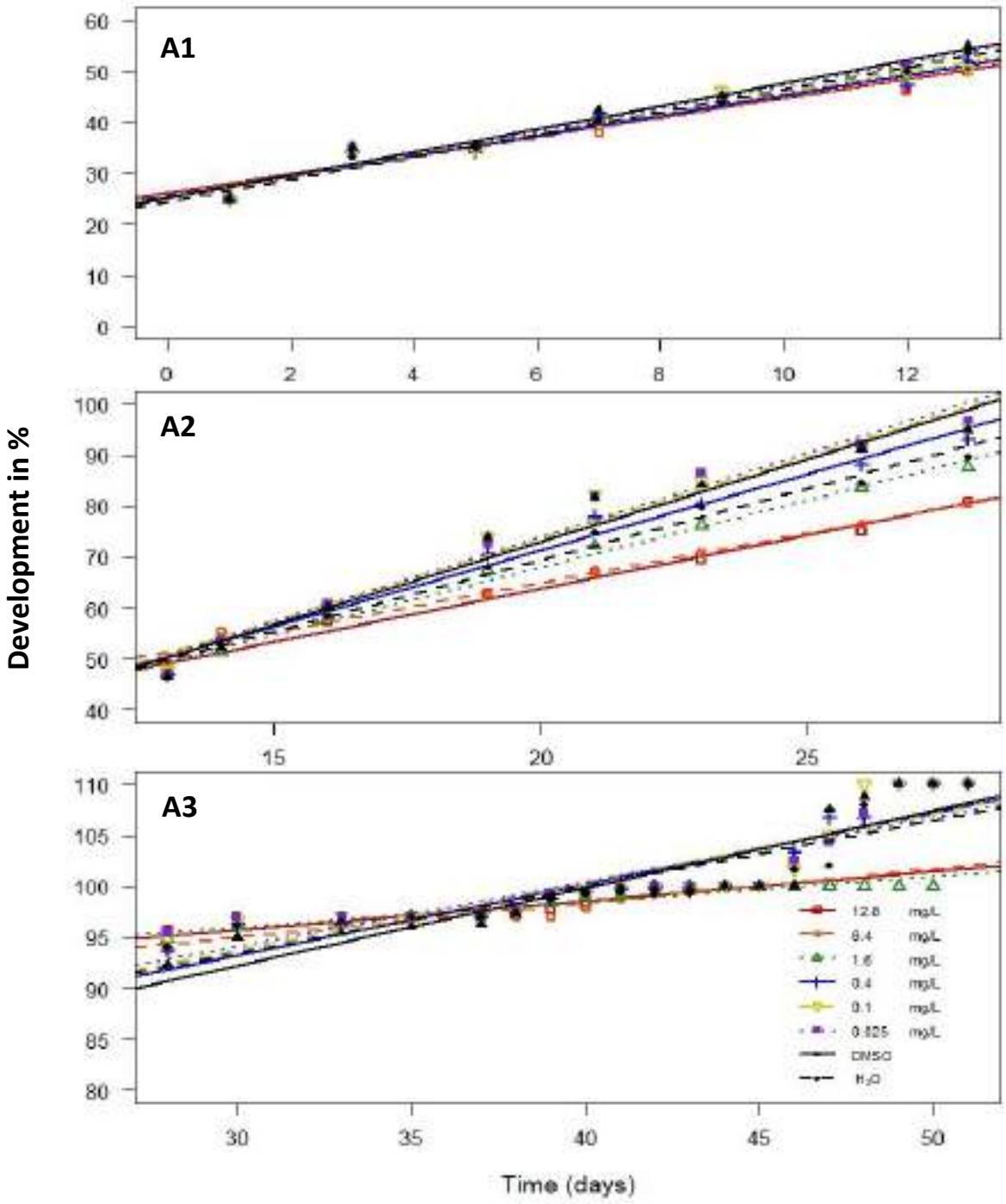


*Figure 3.2: Linear regressions of embryonic development of noble crayfish in different concentrations of TBA over time in first (A1), second (A2) and last (A3) third of development.*

#### 3.1.4.2 Weights

Animals in group PT exposed to higher concentrations tended to have a lower weight at hatching (Table 3.3). However, the ANOVA did not reveal significant differences ($p > 0.05$).

Weights of noble crayfish were only recorded for groups A1, A2 and A3 because of an overall high mortality of noble crayfish of group AT. Group A1 was strongly impacted by TBA concentrations of 1.6 mg/L upwards resulting in a decline in mean body weight of 46.36 % compared to the control groups ($p \leq 0.0434$). The influence on group A2 is limited to a concentration of 6.4 mg/L ($p \leq 0.0256$). In group A3, all individuals exposed to concentrations above 0.4 mg/L died before the first moult and no differences in hatching weight were observed for the remaining concentrations.

*Table 3.3: Average weights in mg plus standard deviation at hatching in the different treatment groups.*

| Treatment | A1 [mg±SD] | A2 [mg±SD] | A3 [mg±SD] | PT [mg±SD] |
|---|---|---|---|---|
| *0.00 [mg/L]* | 25.08 ± 1.47 | 23.53 ± 2.17 | 18.84 ± 1.8 | 3.54 ± 0.55 |
| *DMSO [mg/L]* | 24.00± 0.57 | 22.15 ± 1.09 | 19.60 ± 0.79 | 3.59 ± 0.52 |
| *0.025 [mg/L]* | 22.08 ± 4.08 | 24.34 ± 1.32 | 18.16 ± 0.88 | 3.45 ± 0.60 |
| *0.1 [mg/L]* | 22.03 ± 4.17 | 24.53 ± 0.78 | 19.78 ± 0.74 | 3.47 ± 0.51 |
| *0.4 [mg/L]* | 19.10 ± 4.30 | 23.62 ± 1.08 | 19.05 ± 0.65 | 3.50 ± 0.43 |
| *1.6 [mg/L]* | 17.35 ± 3.99 | 23.58 ± 3.69 | / | 3.15 ± 0.59 |
| *6.4 [mg/L]* | 13.45 ± 0.75 | 20.58 ± 2.15 | / | 3.14 ± 0.60 |
| *12.8 [mg/L]* | 15.65 ± 3.25 | 22.38 ± 2.60 | / | 3.11 ± 0.70 |

#### 3.1.4.3 Survival rate

The survival rates of group PT exposed to different TBA concentrations are shown in Figure 3.3. Both control groups and the lowest concentration supported the highest survival rates in a range between 60 and 66 %. The lowest survival rates were recorded at 33 % in treatments exposed to TBA concentrations of 12.8 mg/L. Statistically significant differences to control treatment $H_2O$ were found for concentrations of 0.1 mg/L, 1.6 mg/L and 12.8 mg/L ($p < 0.02$). The $LC_{50}$-value for this group was 42.38 mg/L for 15 days of exposure. Due to the absence of a correlation between dose and effect, the standard error was greater than the estimated value (± 66.00).

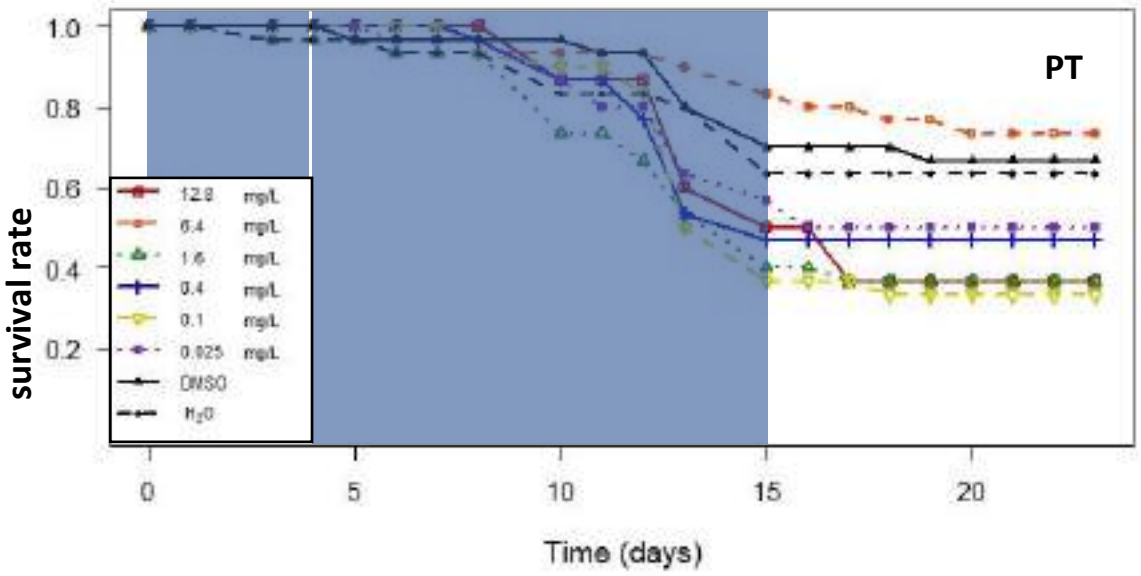


*Figure 3.3: Survival rates for marbled crayfish exposed to different concentrations of TBA during the experiment. The blue area shows the exposure time frames.*

Survival rates in group AT (Figure 3.4) were significantly lower than for marbled crayfish. Only individuals in the control group $H_2O$ (20 %) and in the lowest concentration of 0.025 mg/L (10 %) completed the first moult. The two treatments with the highest TBA concentrations were also showed significantly lower survival rates than the control groups and treatments with lower concentrations of 0.025, 0.1 and 1.6 mg/L ($p \leq 0.043$). The calculated $LC_{50}$ (45d) was 0.1110 mg/L (SD = 0.099).

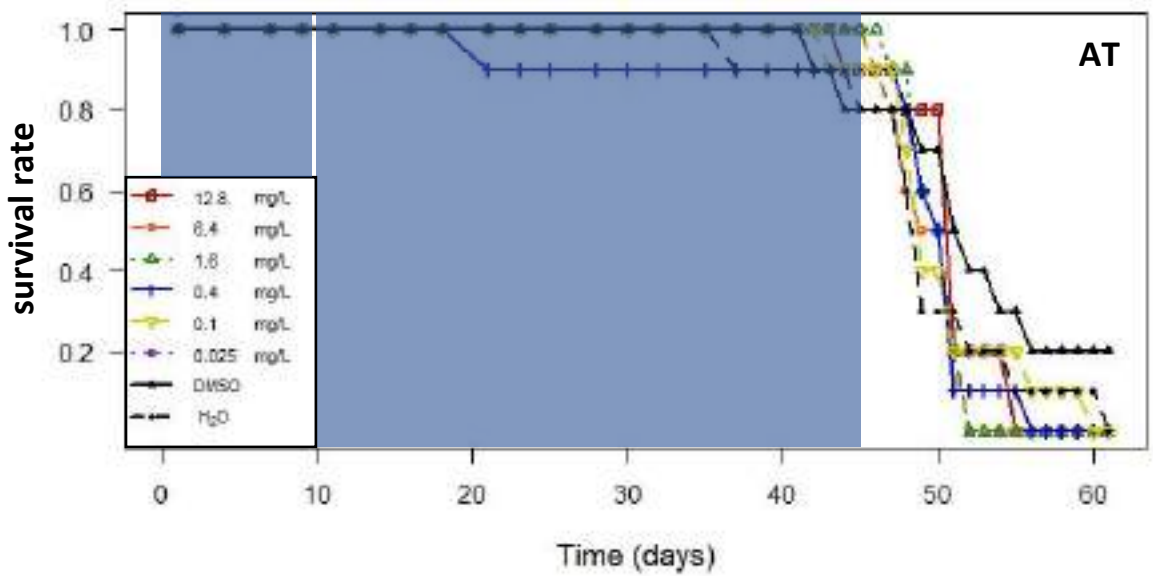


*Figure 3.4: Survival rates of noble crayfish group AT exposed to different concentrations of TBA over a period of 60 days. The blue area shows the exposure time frames*

When exposing the embryos of noble crayfish to TBA for timeslots of 15 days, higher impacts occurred in groups A1 and A3 during the entire development time. Within the first third, the survival rate dropped below 40 % for every group (Figure 3.5) with significantly lower survival rates in the three highest concentrations. If TBA exposure took place during the second period of development, only the group exposed to the highest concentration of 12.8 mg/L had a significantly lower survival rate, similar to the first third. In the final third of development, the three highest treatments showed a significant impact on the survival of noble crayfish embryos. $LC_{50}$ (15d) values (1.051 mg/L; 6.982 mg/L; 0.257 mg/L) for the three time frames showed a higher impact in the first and last part of embryonic development.

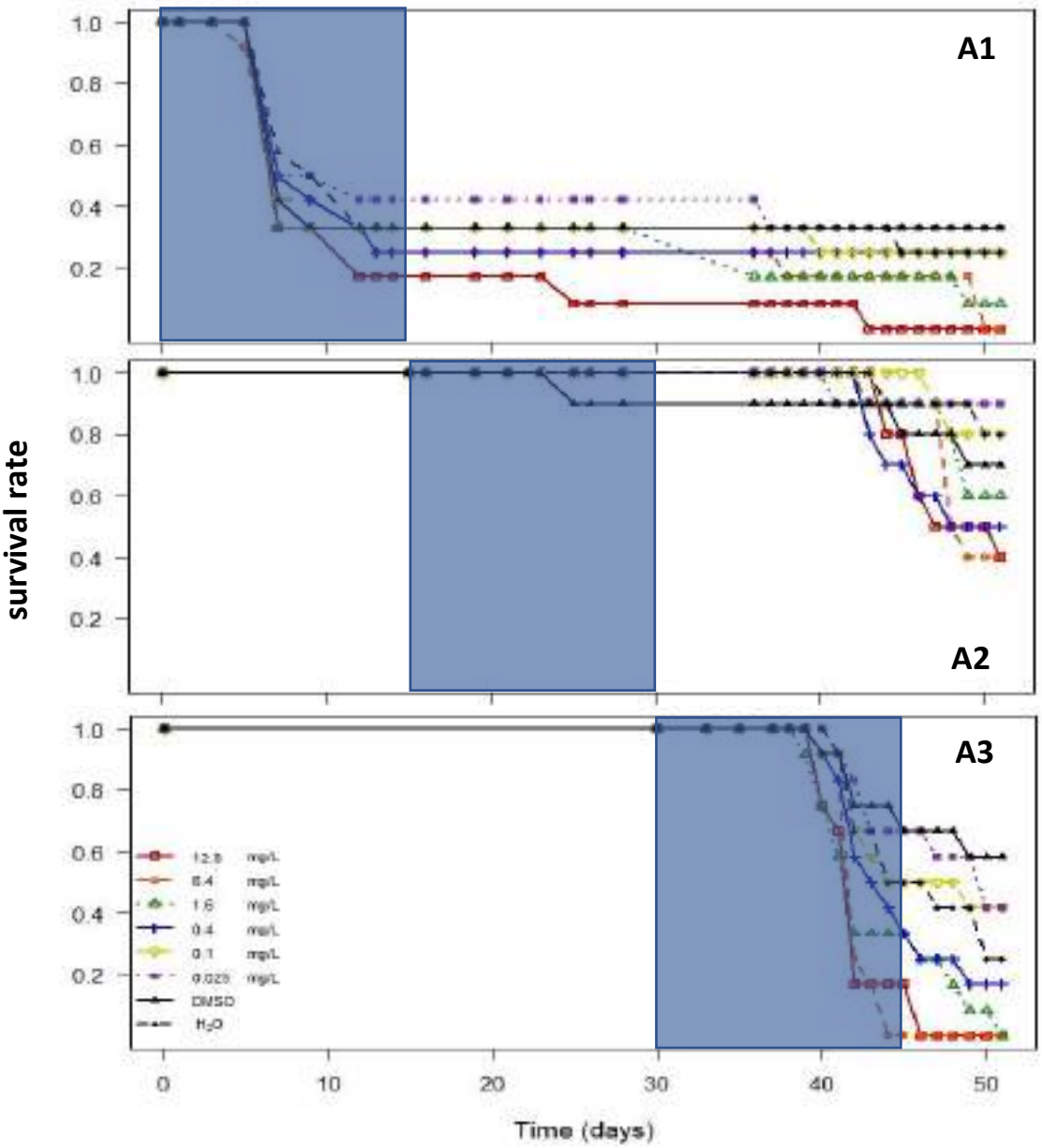


*Figure 3.5: Survival rates of noble crayfish exposed to different concentrations of TBA over time in first (A1), second (A2) and last (A3) third of development. The blue areas indicate exposure time frames.*

#### 3.1.4.4 Histology

Figure 3.6 illustrates the effects of TBA on hepatopancreas structure of *A. astacus*. The average number of B-cells per hepatopancreas cell in the control group is 24.6. All sections of all concentrations showed a higher abundance of B-cells and enlarged B-cells compared to the control group. At concentrations of 0.025 mg/l the number of B-cells (69,86) was significantly higher ($p < 2\ e^{-16}$). Even though the highest concentrations showed a number of 41.86 B-cells per section on average, the cells were about twice the size of the B-cells in the control group. Additionally, no membrane disruption occurred in the control groups, whereas we observed disruptions in groups with concentrations of 0.025 mg/L in 20 % of all sections. From concentrations of 1.6 mg/L upwards, this membrane damage was present in every section.

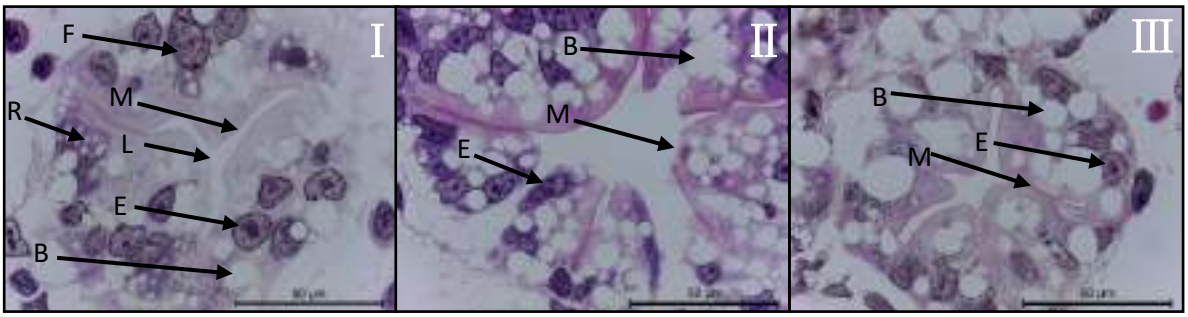


*Figure 3.6: Histological sections of hepatopancreas in juvenile noble crayfish (Astacus astacus) exposed to Terbuthylazine for 45 days. Ⅰ: control with marked lumen (L), membrane (M) and four types of epithelial cells: resorptive (R) lipid cells, blister-like (B) secretory cells, fibrillar (F) cells and embryonic (E) cells; Ⅱ: group exposed to 0.025 mg/L TBA for 45 days; Ⅲ: group exposed to 12.8 mg/L TBA for 45 days (HE stain, 200×).*

### 3.1.5 Discussion

Our results highlight clear differences in sensitivity to TBA exposure between marbled and noble crayfish embryonic development. This questions the suitability of marbled crayfish as a model when studying environmental impacts on native freshwater crayfish such as noble crayfish. Even though genetic uniformity, ease of culture, and a broad behavioral repertoire encourage the use of marbled crayfish in epigenetics and developmental biology, as well as physiological, ecotoxicological, and ethological research (Hossain et al., 2018; Vogt, 2008), the possibility of transferability of ecotoxicological effects to other species is questionable. The lower sensitivity of marbled crayfish compared to noble crayfish can on the one hand be explained by a threefold longer embryonic development and therefore a longer exposure time of the latter. Additionally, Vogt (2010) described marbled crayfish as tolerant towards broad ranges of environmental conditions for long periods of time. Considering the results of Rubach et al. (2011) demonstrating that freshwater arthropod species can be highly variable in their dynamic response to a particular stressor, it is reasonable that the low

sensitivity of marbled crayfish leads to lower effects of TBA on the organism than on noble crayfish, described as more sensitive overall (Holdich, 2002).

It is evident, however, that TBA affects/slows down the overall embryonic development time of noble crayfish, while effects are strongest if animals are exposed during the second and final third of their embryonic development. In this phase, gastrulation is complete and all compartments of the crayfish's body are present, at least in rudimentary form (Alwes and Scholtz, 2006; Sandeman and Sandeman, 1991). Consequently, TBA is likely to hinder growth and specification of the compartments once their main structures are developed. This assumption is corroborated by the work of Gutiérrez et al. (2019), who described that TBA leads to biochemical changes in the species *Scrobicularia plana*, namely in protein contents and enzymatic activity levels, since the protein and enzymatic development take part in the later embryonic development of crayfish (Alwes and Scholtz, 2006). The elongated embryonic development can be seen as a reason for the lower weights of juvenile crayfishes. Crayfish start feeding at the third juvenile stage. Up to this time the embryo gets its energy from its extensive yolk reserves (Vogt and Tolley, 2004). Lower weights of crayfish embryos caused by organic pollutants have been described previously (Velisek et al., 2017). Differences regarding the different concentrations of TBA were only present when noble crayfish embryos were exposed in the first period of their development. Here, gastrulation as well as the development of the immune system and excretory organs take place (Sandeman and Sandeman, 1991). When considering results of the histology of the hepatopancreas it is reasonable to assume that abnormalities of the hepatopancreas are connected to the lower weight of the hatched individuals. For outdoor populations late hatching and lower weight can have serious consequences, since smaller and slower growing individuals have a lower survival potential due to lower feeding success and increased mortality through predation, as shown for marine fishes (Franke and Clemmesen, 2011). For crayfish it is also known that lower body weights from, for example, starvation cannot be fully recovered (Powell and Watts, 2010).

As our data show, there is a higher influence of TBA for treatments A1 and A3 than on A2 in terms of mortality. Points in time of the highest mortalities correlate with gastrulation, biosynthesis and hatching (Alwes and Scholtz, 2006), leading to the assumption that these stages are more sensitive to the potential pollutant than other stages. The usage as pre-emergence herbicide in March and April can at the same time lead to the highest concentrations of TBA during the first period of embryonic development (Ackerfors, 1999). The combination of the resulting lower number of individuals, lower weight and later hatching eventuates in an even lower competitivity of noble crayfish against invasive crayfish species, whereas the interaction of native and invasive species is of severe conservation interest (Pacioglu et al., 2020).

The histology of the hepatopancreas shows effects even at lowest concentrations in the form of an increase in the number and diameter of B-cells compared to the control group for every concentration of TBA for noble crayfishes. In addition, we found damage to membrane structures similar to Chaufan et al.(2006) for every concentration. Their study revealed disorganisation in hepatopancreas tubules as well as increased diameters and numbers of B-cells on hepatopancreas cells after feeding crabs (*Chasmagnathus granulatus*) with hexachlorobenzen-contaminated *Chlorella* for three days. According to this result, an increase in the size and number of B-cells is a sublethal effect of exposure to at least these two agricultural control chemicals. Silveyra et al.(2018) tested the influences of atrazine (which was substituted by TBA and is nearly similar in form and shape to TBA) on vitellogenesis, steroid levels and lipid peroxidation in female red swamp crayfish *Procambarus clarki*. They found that atrazine-exposed crayfish had a lower expression of vitellogenin in the ovary and hepatopancreas as well as smaller oocytes and reduced vitellogenin content in the ovary. This shows an additional effect on the hepatopancreas caused by chlorotriazines that can lead to decreased reproduction. They also showed that atrazine caused a higher metabolic effort in terms of lactate production, presumably triggered to provide the energy needed to face the unspecific stress produced by the herbicide. This higher metabolic effort, or trade-off effect, could explain the sublethal effects pointed out in this study. Besides Terbuthylazine, the degradation products are of great interest due to their toxicity. Koutnik et al. (2017) showed that terbuthylazine-2-hydroxy-exposure in concentrations of down to 75 µg/L affected growth, ontogenetic development, the antioxidant system, and caused oxidative stress and pathological changes in the hepatopancreas of early life stages of marbled crayfish. Therefore, not only the herbicide itself is a threat to non-target organisms but also the degradation product, which prolongs harmful consequences by the usage of herbicides containing Terbuthylazine.

In conclusion, TBA has an influence on the reproduction of the two freshwater crayfish species in all investigated parameters. Sublethal effects can be seen at every concentration, while their influence on future generations remains unclear. The wide range of effects of TBA on the embryonic development of freshwater crayfish shows the complexity of consequences caused by pollutants for these organisms. The length of time and type of use of TBA as a pre-emergence herbicide possibly leads to a generation of crayfish with variations in their hepatopancreas. This variation can again have influences on the respiratory activity and therefore on the overall fitness, especially of the endemic noble crayfish. The data of this study show the high risk of TBA on the non-target organisms that are crayfishes. Considering the important role of crayfishes for their habitat, the dangers posed by TBA for surface waters are highly relevant.

### 3.1.6 Declarations

Funding: This study was funded by: Deutsche Bundesstiftung Umwelt

Conflict of interest: The authors declare that they have no conflict of interest.

All applicable institutional and/or national guidelines for the care and use of animals were followed.

### 3.1.7 References Noble crayfish are more sensitive to Terbuthylazine than parthenogenetic marbled crayfish

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## 3.2 Effects of Diclofenac on the embryonic development of freshwater crayfish

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# Effects of Diclofenac on the embryonic development of freshwater crayfish

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# Effects of Diclofenac on the embryonic development of freshwater crayfish

## 3.2.1 Abstract

In recent years, there has been increasing concern about the ecotoxicological consequences of the drug Diclofenac on freshwater organisms. Influences on the largest freshwater invertebrates, the freshwater crayfish, are especially interesting in the context of cascading effects due to their important role as keystone species. In this study, we investigated lethality, influences on body weight, embryonic development, and histological changes in embryos of marbled crayfish (*Procambarus virginalis*) and noble crayfish (*Astacus astacus*) in response to their exposure to different concentrations of Diclofenac. Additionally, we proved the suitability of marbled crayfish as a model organism for endemic freshwater crayfish, when studying the effects of Diclofenac. For both species, lethal effects started at concentrations of 10.24 mg/L Diclofenac, weight was not affected, embryonic development slowed down from concentrations of 0.16 mg/L, and histological changes were visible from concentrations of 0.64 mg/L. The similarity of LOEC (Lowest Observed Effect Concentrations) between the two species shows that marbled crayfish can serve as a model for investigations regarding the effects of exposure to Diclofenac for native crayfish.

### 3.2.1.1 Highlights:

- The effects of Diclofenac can be studied on marbled crayfish as a model organism for other crayfish species.
- Lethal effects start from concentrations of 10.24 mg/L Diclofenac.
- Sublethal effects start from 0.16 mg/L Diclofenac.

## 3.2.2 Introduction

Diclofenac (DCF) is the most frequently detected drug in German surface waters. It has been measured in concentrations of up to 29.8 µg/L in 55 countries (Dusi et al., 2019). Different harmful effects have been described for non-target organisms. Concentrations as low as 1 µg/L have shown negative effects on the liver, kidney and gills of rainbow trout (*Oncorhynchus mykiss*, (Triebskorn et al., 2004), and the survival, growth and reproduction of *Daphnia magna* is reduced from concentrations of 0.4 mg/L (Du et al., (2016).

As a “keystone species“ and “ecosystem engineers“ (Weinländer and Füreder, 2016) freshwater crayfish are a central element of benthic ecosystems. During development, their sensitive embryos are directly exposed to potentially harmful chemicals dissolved in surface waters for months (Khan and Nugegoda, 2007). They rely on diffusive transport for gas and nutrition exchange during embryonic development (Reiber, 1997) and so they can be influenced by potentially harmful substances.

We therefore hypothesized that DCF has an influence on survival rate, embryonic development time, weight increase and histological effects on the hepatopancreas of freshwater crayfish embryos. The crayfish hepatopancreas is typically formed of numerous tubules separated by connective tissues (Abd El-Atti et al., 2019) and consists of lumen, membranes and four types of epithelial cells: resorptive lipid cells (R-cell) for nutrient intake, blister-like secretory cells (B-cell) to channel off harmful substances, fibrillar cells (F-cell) as connecting tissue and embryonic cells (E-cell). Consequently, changes in R-cells would indicate a higher or lower intake of nutrients, changes in B-cells would indicate a higher or lower outtake of harmful substances, whereas changes in the other two types would indicate problems in the individual’s biosynthesis.

We used the European native species *Astacus astacus*. It was once widespread in European surface waters until the crayfish plague as well as structural and chemical changes in surface waters nearly eradicated this species. The noble crayfish is very suitable for our study due to its natural habitat (lower sections of streams, lakes etc.) which is often influenced by agricultural drainages and sewage (Skurdal, J. and Taugbøl, T., 2002). We carried out the same study with marbled crayfish embryos (*Procambarus virginalis*). One female marbled crayfish can produce up to 700 eggs every 8–9 weeks. The offspring is genetically identical due to its parthenogenetic reproduction strategy (Chucholl and Pfeiffer, 2010; Vogt et al., 2004). These facts provide a predictable and continuous supply of clonal eggs, making this species a suitable model organism for higher invertebrates in the laboratory (Hossain et al., 2018; Vogt, 2018).

### 3.2.3 Material and methods

We obtained parental noble crayfish from a commercial hatchery in Schleswig-Holstein, Germany (Oversee crayfish farm). Fifteen females and six males were kept in three 600 litre aquaria during mating time at 8 °C and a light regime of L:D = 10:14. They were fed frozen midge larvae and peas ad libitum. Females were checked for eggs daily from the end of November onwards. The experiments started 72 hours after egg deposition. The reproduction strategy of marbled crayfish made it easier to obtain a large number of eggs. Twelve animals were kept separated in 25 Litre aquaria at 23 °C with a light regime of L:D = 10:14. The marbled crayfish were fed the same diet as the noble crayfish. These conditions allow us to obtain eggs from every individual marbled crayfish every 8–9 weeks. As with noble crayfish, we started experiments for marbled crayfish 72 hours after egg deposition.

For the experiments, we equally filled 10 wells of 5ml-multititer plates (Greiner bio-one, Kremsmünster, Austria) with 0.75 mL of a mixture of tap water and deionized water at a ratio of 2:1. The mixture was autoclaved and aerated beforehand to ensure an oxygen-saturated and germ-free environment for embryos. Prior to the start of the experiment, 0.75 mL of deionized water with a specific concentration of DCF was added. Subsequently, we transferred one egg per well into each well of twelve microtiter plates. Each plate contained all of the nine concentrations of DCF we used in our experiments, and additionally one zero and one solvent (Ethanol: ETH) control. The concentrations used in the experiments were chosen to cover a wide range from concentrations occurring in surface waters to concentrations known to have an effect on other animal groups (Dietrich et al., 2010; Han et al., 2006) and are shown in table 3.4.

*Table 3.4: Concentrations of DCF and numbers of replicates/embryos per species.*

| Concentration [mg/L] | 0.0 | ETH | 0.01 | 0.04 | 0.16 | 0.64 | 2.56 | 10.24 | 40.96 |
|---|---|---|---|---|---|---|---|---|---|
| *P. virginalis* [n] | 36 | 36 | 36 | 36 | 36 | 36 | 36 | 36 | 36 |
| *A. astacus* [n] A1 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| *A. astacus* [n] A2 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| *A. astacus* [n] A3 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 | 16 |

We placed the microtiter plates on a laboratory shaker (Dual–Action shaker KL 2, Edmund Bühler GmbH, 72411 Bodelshausen, Germany) with 60 movements per minute to ensure a constant supply of oxygen and simulate parental movement of the abdomen. We changed the experimental solutions daily via pipetting to maintain optimum water quality.

We recorded developmental stages and mortality three times per week. This was possible thanks to the transparent membrane of crayfish eggs that enabled us to examine the status of the living embryos' development under a binocular. We transferred the developmental stages described in (Sandeman and Sandeman, 1991), for noble crayfish and (Alwes and Scholtz, 2006), for marbled crayfish, to standardized percentages to generate direct comparability of the embryonic development of the two species.

Differences in development time of marbled crayfish (486-540 degree days (Seitz et al., 2005)) and noble crayfish (1900 degree days (Kozák, 2015; Skurdal, J. and Taugbøl, T., 2002)) were balanced by choosing exposure times of between 13 and 16 days for both species. The last period was carried out until the first moult of juvenile crayfishes. The embryonic development of marbled crayfish was fully complete by this time. For noble crayfish, we conducted the experiment for three different parts of

the embryonic development. Hereinafter, these first, second and third periods will be called A1, A2 and A3.

To avoid observer-biased data recording, the person observing did not know the respective assignments of eggs and concentrations. The experiments were terminated after the first moult of juvenile crayfish. At that time, the fresh weight of the moulted animals was measured (Sartorius Research R160 P, Sartorius GmbH, Göttingen, Germany).

#### 3.2.3.1 Chemicals

We purchased DCF (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) in 99.5 % purity. Due to its low solubility in water (5 mg/L in 20 °C) DCF was first dissolved in Ethanol (ETH). To exclude any effects of ETH on the embryos we also included a control group with this solvent.

#### 3.2.3.2 Histology

For histological assessment we fixated three noble crayfish per concentration in buffered formaldehyde (3.7 %) directly after the first moult. For decalcification, we stored the crayfish in Kristensen solution for two weeks as described in the LR white user's handbook, to ensure the complete decalcification of the exoskeleton. The samples were embedded in LR White (LR White acrylic resin, hard, Sigma-Aldrich, Germany) and hepatopancreas sections of two µm thickness were made using an ultramicrotome. The sections were stained with haematoxylin and eosin (HE) with an extended exposure time, in accordance with the LR White usage instructions. These tissue samples were examined under a light microscope combined with a camera system (Leica DM1000 LED, Leica ICC50 HD, Leica Application Suite Version 3.0.0, Leica Microsystems CMS GmbH, D-35578 Wetzlar, Germany). The examination of hepatopancreas cells included the observation of membrane damage, damage in the four different cell types, as well as changes in size and number of the four different cell types. For this procedure, 10 sections per individual were photographed and subsequently analysed by counting and measuring cells under the microscope.

#### 3.2.3.3 Statistical methods

We performed all statistical analyses using R version 3.2. (R Core Team, 2015). The weight and number of B-cells were tested for normality and equal variances prior to analysis. If these criteria were fulfilled, a one-way ANOVA (variance analysis) and post hoc Tukey test were performed. The $LC_{50}$ values (medium lethal concentrations) were first corrected following Abbott's method and then estimated utilizing the trimmed Spearman Karber method. Survival rates were analysed using the Kaplan-Meier survival analysis of Gehan Breslow and the groups were compared via the Holm-Sidak method. The embryonic development was analysed via linear regressions. Thanks to good correlation values (> 0.8) the linear regressions were compared with an ANCOVA (covariance analysis). Pictures were analysed with GIMP software (version 2.8, Fa. the Gimp Team).

### 3.2.4 Results

#### 3.2.4.1 Lethal effects

The survival rates of the marbled crayfish are shown in Figure 3.7. Overall, the survival of embryos was between 5 % and 36 %. Embryos of *P. virginalis* exposed to concentrations of 10.24 mg/L and higher showed a significantly lower survival rate than the control groups (Holm-Sidak, $p \leq 0.03$). The estimated $LC_{50}$-value over 15 days was 13.96 mg/L (SE = 3.86).

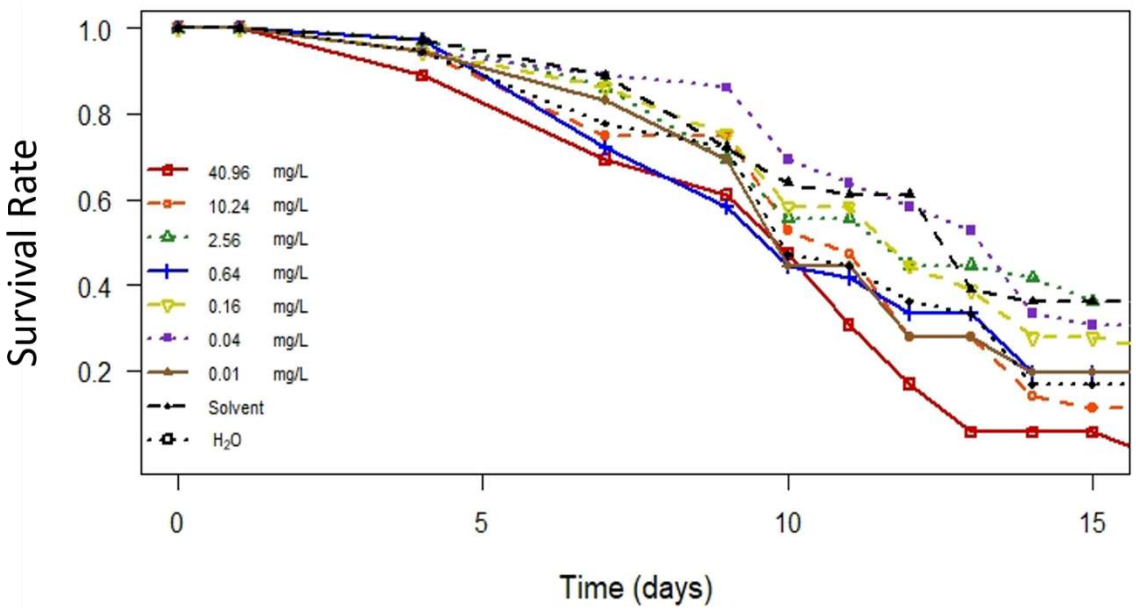


*Figure 3.7: Survival rates of marbled crayfish exposed to different concentrations of DCF over time.*

Overall, the survival of noble crayfish embryos was higher than for marbled crayfish. Similar to marbled crayfish, survival rates of noble crayfish were significantly lower for concentrations of 10.24 mg/L and higher (Holm-Sidak, $p \leq 0.007$). These differences were only observed in group A3 (Figure 3.8). During this developmental period, hatching takes place so that the effects of DCF on the survival of noble crayfish are linked to latest stage of embryonic development and hatching. An $LC_{50}$-value of 19.56 mg/L (SE = 5.22) was estimated for this group.

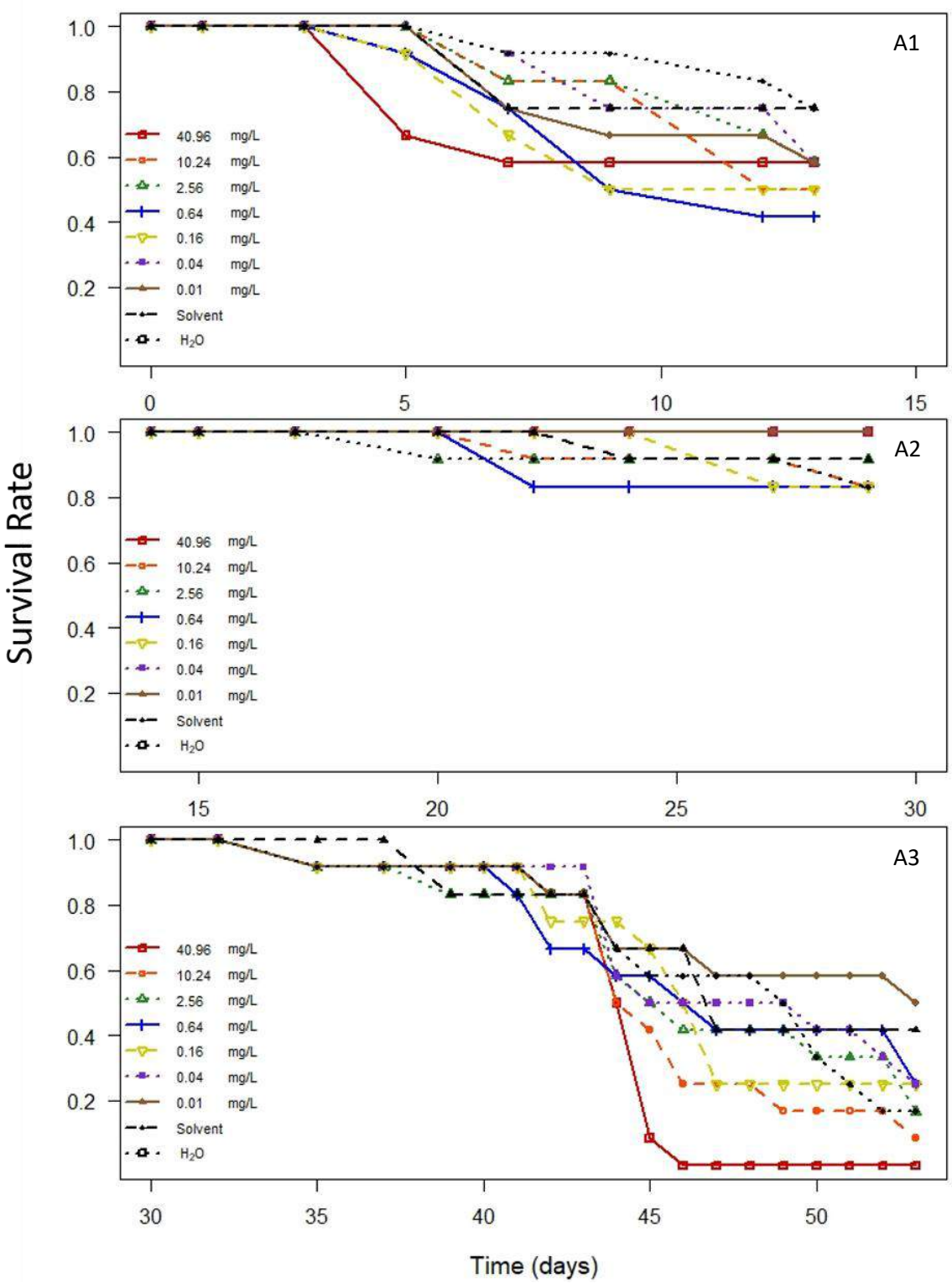


*Figure 3.8: Survival rates of noble crayfish exposed to different concentrations of DCF over time in first (A1), second (A2) and final (A3) third of development.*

#### 3.2.4.2 Sublethal effects

##### 3.2.4.2.1 A comparison of the weight

weight of both crayfish species after the first molt did not reveal any differences between the different treatments (all p>0.05).

##### 3.2.4.2.2 Embryonic development time

A comparison of the development time of the marbled crayfish revealed major differences between treatments (Figure 3.9). From concentrations of 0.16 mg/L and higher, embryonic development was slower than in the control group (ANCOVA, p = 0.047). The time until 70 % of development was completed varied between 13 days for the control groups and 18 days for the treatment group exposed to 10.24 mg/L DCF. Embryos exposed to the highest concentration did not develop further than 69 % on average.

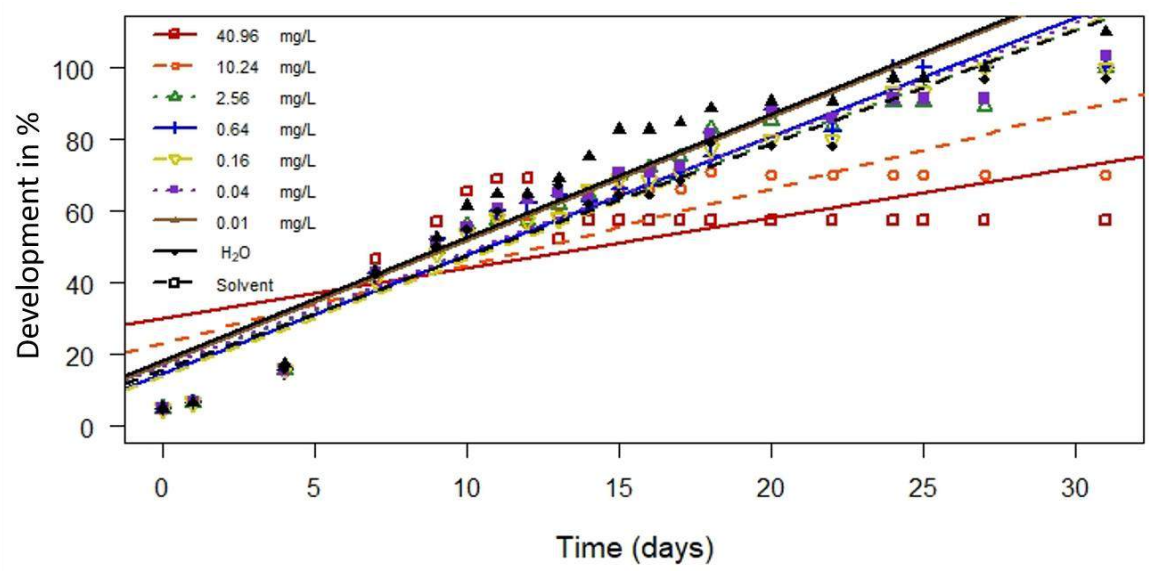


*Figure 3.9: Linear regression of embryonic development of marbled crayfish exposed to different DCF concentrations over time.*

In noble crayfish, there were no differences in development between group A1 and A2, but in group A3 the development of embryos exposed to DCF concentrations of 0.16 mg/L and higher was retarded compared to the control groups (p = 0.019) (Figure 3.10). By day 43, all embryos of the control groups had hatched. Embryos exposed to 0.16 mg/L DCF hatched after 46 days and embryos exposed to the highest concentration of DCF did not develop until hatching.

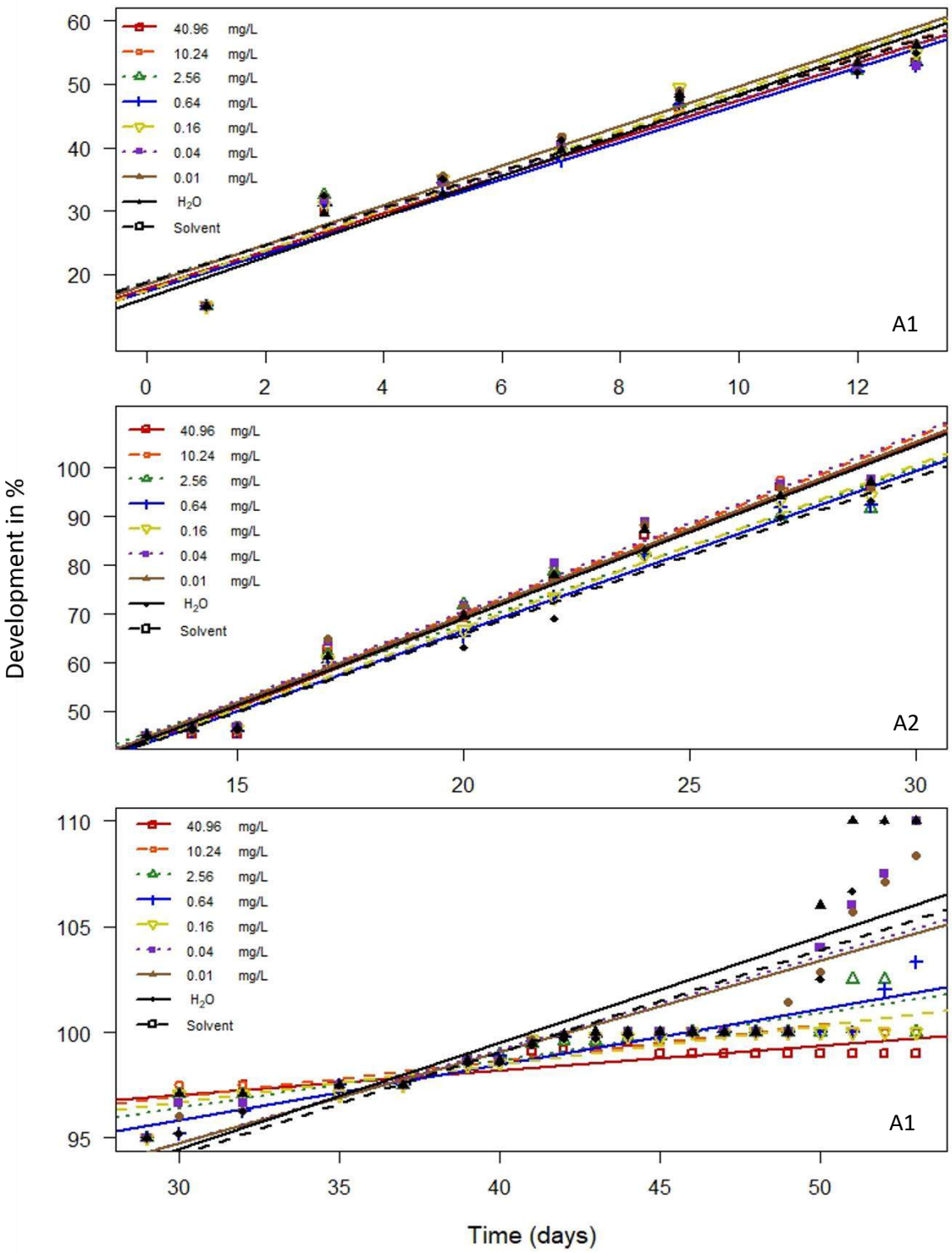


*Figure 3.10: Linear regression of embryonic development of noble crayfish exposed to different DCF concentrations over time in the first (A1), second (A2) and final(A3) third of development.*

### 3.2.4.2.3 Histology

Figure 3.11 illustrates the effects of DCF on the hepatopancreas structure of *A. astacus*. The average number of B-cells in the $H_2O$ control group is 24.6 ± 2.4 (n=30). All sections of concentrations of 2.56 mg/L and higher showed a higher number of B-cells and enlarged B-cells compared to the control groups (post hoc Tukey, p<0,001). In hepatopancreas sections of embryos exposed to concentrations of 40,96 mg/L 41.9 ± 9.8 (n=30) B-cells per section were recorded. These cells were estimated to be

30 % larger than B-cells in the control groups. Additionally, no disruption of the membrane occurred in the control groups, whereas we observed disruptions at concentrations of 0.64 mg/L DCF in 20 % of all sections. At concentrations of 1.6 mg/L and higher, this membrane damage was present in every section.

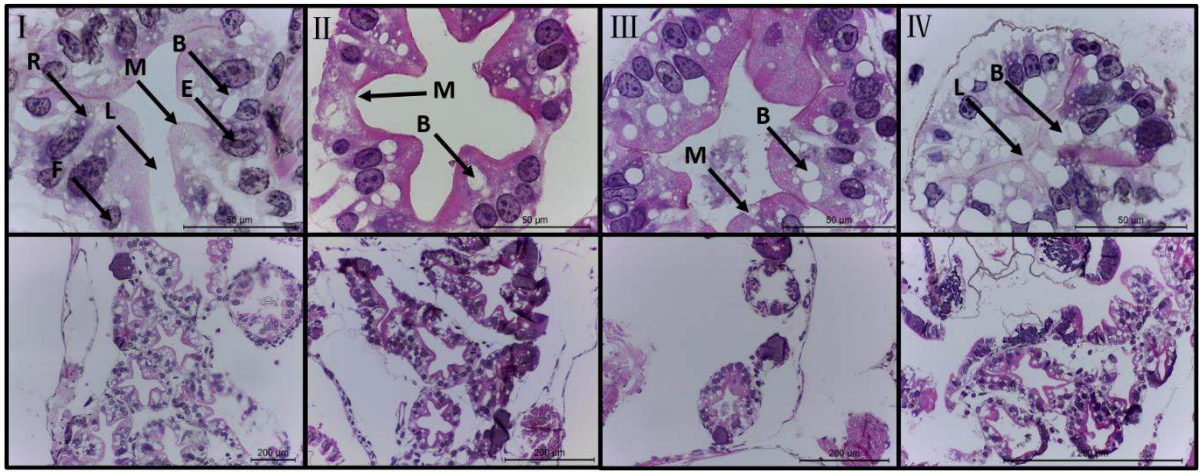


*Figure 3.11: Histological sections of the hepatopancreas in juvenile noble crayfish (Astacus astacus) exposed to DCF. Ⅰ – control showing the lumen (L), membrane (M) and four types of epithelial cells: resorptive (R) lipid cells, blister-like (B) secretory cells, fibrillary (F) cells and embryonic (E) cells; Ⅱ – group exposed to 0.64 mg/L DCF; Ⅲ – group exposed to 2.56 mg/L DCF; Ⅳ – group exposed to 40.96 mg/L DCF (HE, 200×). Top row: close up of one tubular. Bottom row: overview of hepatopancreas structure*

### 3.2.5 Discussion

The purpose of all types of non-steroidal anti-inflammatory drugs (NSAIDs) including DCF is to decrease the production of thromboxanes and prostaglandins (Satoh et al., 2015) in order to reduce pain, inflammation and fever. With 3,996 positively detected environmental concentrations on the watch list of the database “Pharmaceuticals in the environment” (UBA, German Environment Agency; Dusi et al., 2019), it is considered a “contaminant of emerging concern” and was included in the previous watch list of EU Decision 2015/495 (Sathishkumar et al., 2020). This high incidence shows the relevance of understanding the effects of this drug on non-target organisms.

Comparison of the two species

With respect to the assessed parameters, the effects of DCF on embryos of the two species were very similar. The LOEC for lethality and embryonic development were identical and $LC_{50}$-values were comparable. Therefore, we conclude that marbled crayfish can serve as a model organism for endemic crayfish concerning the effects of DCF. The suitability of marbled crayfish as a model organism for a broad range of biological disciplines is described by Vogt (2018) and Hossain et al. (2018). Both reviews claimed that marbled crayfish are organisms that can be used for studies in epigenetics and developmental biology as well as physiological, ecotoxicological and ethological research. Buřič et al. (2018) used P. fallax to assess the effects of an opioid painkiller (tramadol) and an antidepressant drug

(citalopram) on behavioural patterns. The marbled crayfish has even been used as a model for the neural and molecular mechanisms of drug addiction, (Jackson and van Staaden, 2019). This shows that marbled crayfish are already being used as model organisms. Nevertheless, not every observed effect on marbled crayfish is transferable to other species. Marbled crayfish can show a lower sensitivity to environmental factors, especially during embryonic development. The short embryonic development of marbled crayfish (Vogt and Tolley, 2004) leads to a shorter exposition time during this vulnerable period. Additionally, Vogt (2010) described marbled crayfish as tolerant towards a wide range of environmental conditions for long periods of time. Considering the results of Rubach et al. (2011), who demonstrated that freshwater arthropod species can be highly variable in their dynamic response to a particular stressor, it is reasonable that the suitability of the marbled crayfish as a toxicological model organism is dependent on the chemical compound used. Nevertheless, effects are transferable to other species, but the dose at which an effect occurs might differ.

#### 3.2.5.1 Lethality of Diclofenac

There have been few investigations into lethal concentrations of DCF for crustaceans and, to our knowledge, none for crayfish. Data are only available for the water flea *Daphnia magna* ($LC_{50}$ = 56.6 mg/L–94.1 mg/L (Quinn et al., 2011; Ra et al., 2008), the mysid *Sirella armata* ($LC_{50}$ = 0.01 mg/L–2.91 mg/L (Pérez et al., 2015)) and the copepod *Tisbe battagliai* ($LC_{50}$ = 15.8 mg/L (Schmidt et al., 2011)). These results are within the range of the observed values for crayfish in this study. It is notable that the commonly used organism for risk assessments, *Daphnia magna,* shows the highest $LC_{50}$ of all four organisms and therefore shows a very optimistic estimation of the hazardous effects of DCF. However, there are plenty of examples of the toxicity of DCF on fish. For example, the zebra fish *Danio rerio* showed an $LC_{50}$-value of 5.3 mg/L (van den Brandhof and Montforts, 2010). Zhang et al. (2020) showed for embryos of this species that Diclofenac led to the inhibition of spontaneous muscle contractions and a decreased hatching rate of zebrafish embryos at a concentration of 24.1 µg/L. The deviation in these lethal concentrations compared to crustaceans, despite the larger body volume, can be explained by bioaccumulation in tissue. Several authors have reported that DCF can accumulate in fish, even though reported bioconcentration factors differ greatly between species (Brown et al., 2007; Cuklev et al., 2011; Fick et al., 2010; Schwaiger et al., 2004).Transferred to freshwater crayfish, DCF would most likely accumulate in muscle and hepatopancreas tissue and therefore could show even more drastic effects over time. The toxicity of DCF is additionally dependent on pH. At lower pH the mortality increases (Alsop and Wilson, 2019). Nevertheless, lethal concentrations of DCF are multiple times higher than the highest monitored DCF concentration worldwide (Sousa et al., 2018). Therefore, indirect effects of DCF on fitness are more likely to have an impact on population dynamics.

#### 3.2.5.2 Sublethal effects of Diclofenac

Mohd Zanuri et al. (2017) showed effects of DCF on sperm activity of important components of the marine benthos in concentrations lower than 0.1 µg/L. In addition to the effects of DCF, mixtures with other chemicals introduced to surface waters can increase the negative effects of this analgesic (Gonzalez-Rey et al., 2014; Prokkola et al., 2015). Sublethal effects of DCF on freshwater crayfish were observed from concentrations of 0.16 mg/l and higher. These low effective concentrations support the statement made by Fent et al. (2006) that DCF seems to be the compound having the highest acute toxicity within the class of NSAIDs. Even though the weight of hatched crayfish did not decrease when exposed to DCF, the increased developmental time of the crayfish due to DCF exposure can have a negative effect on population dynamics. For natural populations, late hatching can have massive influences as later hatching leads to a later start of feeding and therefore slower growth of the respective cohort. These individuals have a lower survival potential due to lower feeding success and increased mortality through predation as shown for marine fish (Franke and Clemmesen, 2011). The sublethal effects described in this study are only one example of the expectable effects of DCF. For example, Gonzalez-Rey and Bebianno (2014) showed that concentrations as low as 0.25 µg/L can lead to biomarker responses in muscles. These or other unknown effects could also be present in crayfish muscle tissue.

##### 3.2.5.2.1 Histology

The hepatopancreas in decapods is the site of nutrient absorption, digestion, synthesis and secretion of digestive enzymes and reserve storage (Calvo et al., 2011; Johnston et al., 1998; Xiao et al., 2014). For this reason, the tissue is used for monitoring the health of crayfish and can indicate diseases and exposure to harmful substances (Velisek et al., 2017; Xiao et al., 2014). Changes in B-cells indicate a higher or lower outtake of harmful substances. The observed changes in B-cells can therefore be explained by a greater need to lead off the chemicals. The damaged membranes, on the other hand, can interrupt this mechanism. The overload of this system can therefore explain other sublethal and lethal effects of DCF on these animals. When the extrusion of harmful substances is disrupted due to hepatopancreatic damage, the harmful effects on development, growth and survival can occur unimpeded.

There are currently no data in literature describing the effects of DCF on the hepatopancreas of crayfish. Nevertheless, influences on the organ by human medications have been reported before: Wren and Gagnon (2013) showed membrane damage and size changes of cells in the hepatopancreas of *Orconectes virilis* exposed to platinum group metals commonly used for industrial and biomedical purposes at 5 mg/L after ten days. They also showed a high bioaccumulation in hepatopancreas tissue with 81.68 mg/g in a concentration of 1 mg/L platinum group metals. Marenkov et al. (2016) showed

influences of the drug Albuvir on the hepatopancreas and weight of marbled crayfish at 0.01 % solution. The results of the investigation of the hepatopancreas show the wide range of sublethal effects on freshwater crayfish and leads to the assumption that other effects are possible and should be investigated in future studies.

### 3.2.6 Conclusion

This study shows that marbled crayfish can be used as model organisms for investigations concerning the effects of DCF on noble crayfish. Furthermore, we were able to show that the non-steroidal anti-inflammatory drug has a negative influence on the embryos of freshwater crayfish. Though the reported effective concentrations are unlikely to be found in surface waters, the mixture of DCF with other introduced chemicals might reduce the effective doses of the pharmaceutical. Additionally, the source of DCF is continuous due to sewage treatment plants, so that the exposure time is endless. Therefore, effects on population dynamics are possible and should be investigated in the future.

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### 3.2.8 Competing interests

The author(s) declare that they have no competing interests

### 3.2.9 Authors' contributions

JL and KL conceived and designed the experiments. JL performed the experiments. JL analysed the data. JL, KL and HB wrote the manuscript; all authors provided editorial advice.

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# 4 Influences under outdoor conditions

Studies conducted under laboratory conditions can generate reproduceable results without the influence of uncalculatable factors. Therefore, the previously described manuscripts can show the exact influences of the two tested chemicals. Under outdoor conditions, water parameters and present chemicals are constantly changing so that these laboratory experiments cannot represent exact actual influences of the two chemicals. For this reason, we conducted a field study to determine the effects of chemical intrusion of a surface water stream.

## 4.1 Freshwater crayfish in field experiments: design and efficiency of three novel enclosures

To examine the influences of actual inputs under real conditions to embryonic freshwater crayfish, a suiting method for outdoor experimental setups had to be established. For this reason, a small study was conducted to test three different experimental enclosures and their practicability in field experiments. The study is described in the following manuscript. This manuscript is submitted to the "International Journal of Aquatic Research and Education".

The included version of the manuscript represents the state of preparation after full corrections and before approval of co-authors. Submission is planned for the journal: "Limnologica".

# Freshwater crayfish in field experiments: design and efficiency of three novel enclosures

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JL and TS conceived and designed the experiments. TS and JL performed the experiments. TS and JL analysed the data. JL, TS and HB wrote the manuscript; all authors provided editorial advice.

Short title: Enclosures for freshwater crayfish

Keywords: *noble crayfish, juveniles, embryonic, enclosures*

### 4.1.1 Abstract

Increasing anthropogenic influences on ecosystems such as aquatic habitats have severe consequences. Not only are they reducing aquatic biodiversity but thereby also degrading the ecosystem services we depend on. In this context, keystone species are of particular importance for the function of freshwater ecosystems. To assess the effects of pollutants and other man-made disruptions on keystone species such as freshwater crayfish, outdoor experimental setups are essential. One option is the use of enclosures to place crayfish in outdoor surface waters in order to monitor the activity and performance of the animals at any given time. However, not every type of enclosure is suitable for keeping crayfish in their natural habitat, in particular egg-carrying crayfish, without impacting on the embryogenesis and the physical integrity of adults and juveniles. Therefore, we have developed three new enclosure designs and compared their efficiency with two control groups in order to find the most suitable construction with the least impact on the experimental organisms and their offspring. In this study, we compared the performance of female noble crayfish (*Astacus astacus*) and their embryos in three different outdoor enclosures exposed in a pond and, for comparison, in a cycle aquaculture system and a hatchery. The study showed that animals in a floating construction made of wood and wire fence with a mesh size of 8 mm have a survival rate and development time of the crayfish embryos comparable to animals under optimum conditions.

### 4.1.2 Introduction

Interactions between stressors, water quality, flow velocity, habitat structure and many more factors can have a high impact on research results. In their review, Calisi and Bentley (2009) highlighted the general need for field experiments in addition to laboratory experiments, as the data from the laboratory experiments often differ from or even contradict the findings in natural complex biological systems. To estimate the effects of individual stressors in the context of environmental influences it is necessary to transfer predictions from laboratory environments to outdoor experiments. The observed effects can range from behaviour, feeding, levels of food chain and pathogenic load to environmental changes. The usage of enclosures is an established method to create controllable conditions for in situ studies concerning aquatic animals (Marchetti Maroneze et al., 2020; Nichols et al., 2019; Yin et al., 2017). However, data quality is dependent on the suitability of the experimental design. Therefore, the used methods have to be evaluated.

Especially *in situ* experiments of freshwater crayfish are of great interest due to the major influence of these animals on their habitat. Because of their omnivorous diet they have an impact on every trophic level, and as the biggest invertebrates of freshwater habitats they affect the overall benthos structure (Kettunen, M. & ten Brink P., 2006; Nyström, P.,2002). For that reason, freshwater crayfish reached the status of keystone species (Skurdal, J. and Taugbøl, T., 2002). Local or functional extinction as well as a decrease of populations of keystone species, to the point that they no longer contribute to ecosystem processes, can have dramatic impacts on ecosystem services (Kettunen, M. & ten Brink P., 2006) .

Stress factors of limnic habitats are especially dangerous during the reproduction period of crayfish and their embryonic development. This is due to the naturally higher sensitivity of embryonic and juvenile organisms (Khan and Nugegoda, 2007) and the reproduction strategy of crayfish. As these animals carry their eggs under their abdomen (pleon) for months, the embryos are directly exposed to external stressors.

Influences of environmental chemicals on freshwater crayfish are increasingly becoming the focus of laboratory experiments (Buřič et al., 2018; Sohn et al., 2018; Stara et al., 2018). Yet very little is known regarding the influences in their natural habitat or even embryonic development. To obtain significant and independent results from field experiments involving the use of any form of artificial enclosure, it is relevant to estimate the impacts of the enclosure itself on the organism.

To observe effects of stressors on crayfish and the early life stages of their offspring in contaminated waters, we designed three different outdoor enclosures. Ideal enclosures should minimize stress for the study animals, but maximize interaction with the environment and must also be easy to handle for

the observer. The designs were based on existing enclosures used in other studies involving freshwater crayfish, all of which used small mesh sizes and were placed in benthic regions of water bodies. (Albertson and Daniels, 2018; Chucholl, 2013; Jussila et al., 2011; Mueller and Bodensteiner, 2011). Jussila et al. (2011) used a wooden construction to investigate latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish population. The exact structure of these enclosures however, has not previously been described, so that the study at hand will provide first evaluated and detailed designs for future studies. Requirements for the designs used in this study were the possibility of constant water exchange and an active interchange with the environment. These characteristics made the designs suitable for experiments studying environmental parameters such as oxygen, nitrogen and other water parameter. and the impact of pollutants or pathogens on the survival, growth and development of crayfish during embryonic development in particular.

This research was conducted in order to develop an enclosure method with which the effect of the selected parameters can be estimated, while facilitating the handling and minimizing the stress exposure on the animals.

### 4.1.3 Material and Methods

#### 4.1.3.1 Study animals

We used noble crayfish females obtained from “Krebszucht Oeversee, Germany” and originated from Langensee in Schleswig-Holstein, Germany. All study specimens were age- two, had a carapace length between 38 and 47 mm and were gravid. To monitor egg development and the overall physical condition of females before the start of the experiment, the animals were kept in a recirculating aquaculture system (RAS) at the university of Kiel for 14 days under optimized conditions according to Bohl (1989).

#### 4.1.3.2 Enclosure designs

The three enclosure designs (Figure 4.1, 4.2 and 4.3) we developed were designed on the basis of information from existing investigations. From these studies, we derived mesh size, size per animals and the overall construction methods. The final structure and materials were then complemented.

**Type A** was fabricated after the model of Jussila et al. (2011). It was made of 1.8 cm strong *Paulownia* wood, which is water resistant and has a low density. This results in a floating enclosure. The sides are covered with wire fence of 8 mm mesh size and the top cover is hinged. The construction of this enclosure type took about three hours each.

**Type B** is the simplest design of the three and is similar to the design Mueller and Bodensteiner (2011) used. It is made of 8 mm mesh size wire fence and cable ties. The build time took about 15 minutes each. This type is placed on the bottom of the lake.

**Type C** is a modified version of the enclosures used in the study of Chucholl (2013). It is made of a wooden frame which is covered with nylon gauze with a 1.5 mm mesh size. The top cover mesh is removable thanks to a clip system. Construction took about 35 minutes each. This type is also placed on the bottom of the lake. All types were equipped with clay pipes for shelter which also functioned as weights to keep the enclosure in place in the pond. Additionally, the enclosures can be fixed in place using weights. All materials were obtained in a local hardware store.


*Figure 4.1: Enclosure type A: 60 x 25 x 25 cm (L x W x H), wooden top and bottom (Paulownia wood, 1.8 cm thickness), hinged top cover, sides covered with wire fence (8 mm mesh size), 2 clay pipes placed inside, construction balanced with weights to float just below the water surface.*


*Figure 4.3: Enclosure type B: 70 cm side length and 22 cm diameter, tubular enclosure made of wire fence (8 mm mesh size), 2 clay pipes placed inside, construction placed at a depth of 1.30 m.*


*Figure 4.2: Enclosure type C: 60 x 25 x 25 cm (L x W x H), wooden framework, sides covered with gauze (1.5 mm mesh size), removable top cover by means of built-in clip system, 2 clay pipes placed inside, construction placed at a depth of 1.30 m.*

#### 4.1.3.3 Experimental Design

In the field test, we compared five treatment groups, each with six adult egg-carrying *Astacus astacus (Table 4.1)*. The initial embryonic stage in all groups was observed at approximately 40 % embryonic development (Alwes and Scholtz, 2006; Sandeman and Sandeman, 1991). Starting from the end of April, the three groups (A, B, C) were kept outdoors in a semi-intensive aquaculture pond system in the Oeversee Crayfish Farm in the different enclosures, one animal per enclosure (Figure 4.1). One group (D) was kept in a CAS in a single 610 litre tank, with similar environmental parameters but under controlled conditions. These six females were fed with *Chironomidae* larvae, stonewort and peas *ad libitum*. For another trial group (E), we transferred the eggs, stripped from female crayfish, to a hatchery to eliminate the maternal influences. This hatchery, with a total volume of 150 litres, was equipped with a degassing unit, UV-clarifier, aeration system and a motor providing movement of the eggs. The CAS and hatchery groups served as controls.

Table 4.1 Summary of treatments and their parameters

| Treatment | Site | Materials | Purpose | Number Crayfish |
|---|---|---|---|---|
| A | Crayfish farm | Enclosure out of wood and wire | Experimental treatment | 6 egg-carrying females |
| B | Crayfish farm | Enclosure out of wire | Experimental treatment | 6 egg-carrying females |
| C | Crayfish farm | Enclosure out of wood and gauze | Experimental treatment | 6 egg-carrying females |
| D | Laboratory | CAS | control | 6 egg-carrying females |
| E | Laboratory | Hatchery | control | 6 egg-carrying females |

Overall, six enclosures per type and 18 in total were used. To establish the best method that provides ideal conditions to keep the animals in outdoor experiments, we compared the embryonic development rate, the weight of hatched crayfish and the survival of hatchlings. To track the development, 2–3 eggs per individual and week were collected and the developmental stage was assessed according to (Alwes and Scholtz, 2006) and converted into percentages of embryonic development. The survival rate of the embryos was calculated by counting the number of eggs carried per animal at the start of the experiment and the number of resulting hatchlings at the end. Eggs

attached to animals were counted twice directly on the animals. This had to be done fast but also the number of the two counting should not differ. Otherwise a third counting would have to be made. This however did only happen twice. One time for the control and one time for enclosure C. Separated eggs are not capable of surviving due to the lack of movement and the resulting oxygen deficit. The study was terminated when all embryos hatched. The weight of 12 randomly selected juveniles per adult was measured immediately after hatching in all treatment groups was completed. To avoid any influence of the exact location in the pond on the results, we chose six evenly spread locations, which were each stocked with each type of enclosure. Continuous data loggers for temperature and light (HOBO Pendant temp/light (Onset Computer Corporation, Bourne, USA)) were installed at each of the six locations and one oxygen logger (HOBO Dissolved Oxygen Logger (Onset Computer Corporation, Bourne, USA)) was installed in the centre of the locations. The temperature and light regime in the control groups in the hatchery and the CAS was adjusted to that given in the outdoor experiment. Oxygen in the controls and pH in all treatment groups were measured with multi-parameter probes (WTW Oxi 3310 and WTW pH 3310, Xylem Analytics Germany Sales GmbH & Co. KG, WTW Weilheim, Germany) and nitrite, nitrate and ammonium concentration with a photometer (DR 5000 Hach Lange GmbH, Düsseldorf Germany). All parameters were measured once a week.

#### 4.1.3.4 Statistical analysis

All statistical analyses were performed using SigmaPlot 13 (Systat Software, Inc., San Jose, USA). Data on survival, temperature, embryonic development and weight were tested for normality and equal variances prior to analysis via the Levene and Shapiro-Wilk test. If both were given, a one-way ANOVA was performed for weight and temperature. For non-parametric data, a Kruskal-Wallis test was used. The embryonic development was analysed via linear regressions. Due to good correlation values ($R^2 > 0,9$) the linear regressions were compared with an ANCOVA (Analysis of covariances). The correlation of embryo survival and maternal carapace size was tested with Spearman Rank Order Correlation.

#### 4.1.3.5 Authorization

The official authorization for this study with live animals was obtained by the “Ministerium für Energiewende, Landwirtschaft, Umwelt, Natur und Digitalisierung Schleswig-Holsteins“ (MELUND).

### 4.1.4 Results

#### 4.1.4.1 Adult animals

Hatching time took from 33 days (group E) to 36 Days (group C). Within the experiment, five out of thirty adult crayfish died in their treatments. Three of them were held in enclosure type B and two were held in the CAS as control group. This leads to a total mortality of 50 % for group B and 33.33 %

for group D. Additionally, one female from group A and one from group C escaped during the experiment due to material failures. Six of these seven eliminated females died or escaped within the first five days, so we replaced them immediately.

#### 4.1.4.2 Embryonic Development

The embryos' survival rates in all treatments were above 50 %. The different treatments do not show any significant differences, but very large standard deviations of up to 28.8 % (Figure 4.4).

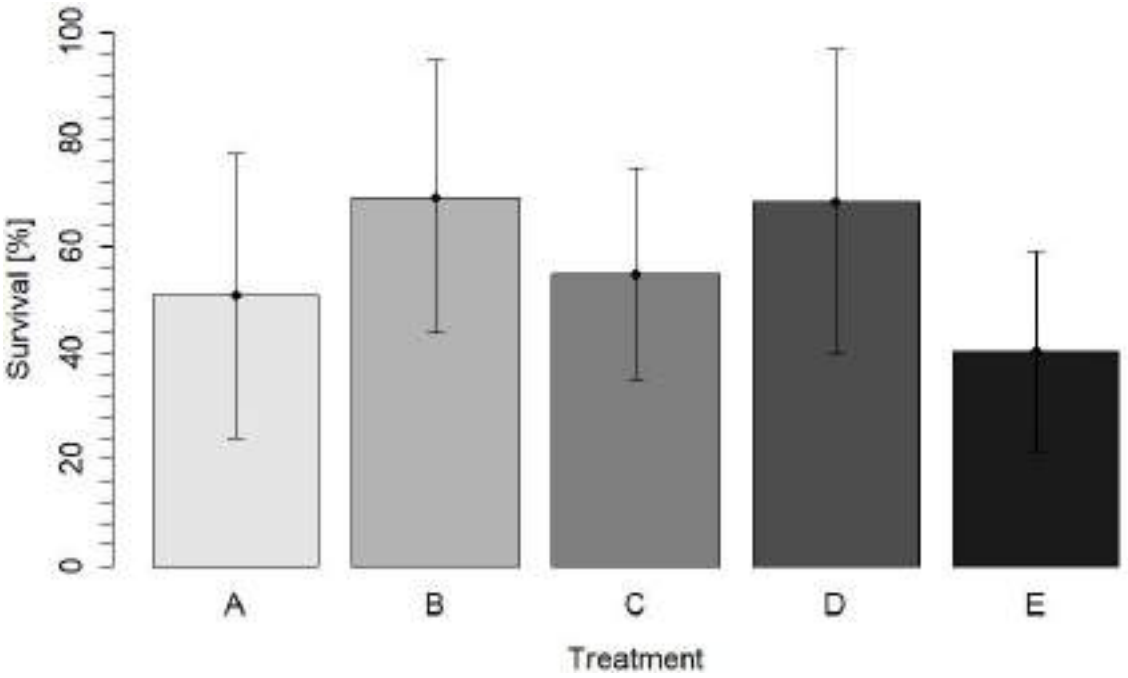


*Figure 4.4: Survival rates of A. astacus embryos in different enclosures (A, B, C) and control groups (D = CAS, E = hatchery); mean ± SD*

To verify that the observed differences in the embryonic survival rates can be treated irrespectively of the body size of the maternal crayfish we tested the maternal body size and the survival rate of the related embryos for correlation. No significant correlation ($p = 0.888$, adjusted $R^2 = -0.03496$) was found, thus, it was assumed that the survival rate is mainly affected by different treatments.

At the start of the experiment, all embryos were at a developmental stage of 40 % and were observed for five weeks of their development until hatching. The fastest development took 33 days (hatchery) and the slowest 36 days (enclosure type C). During this time, the crayfish embryos diverged in their pace of development. Linear regression analysis of embryonic development (Figure 4.5) showed a significantly faster development of embryos in enclosure type A compared to the other enclosures ($p < 0.05$), but not to the control group in the hatchery (E) ($p = 0.633$).

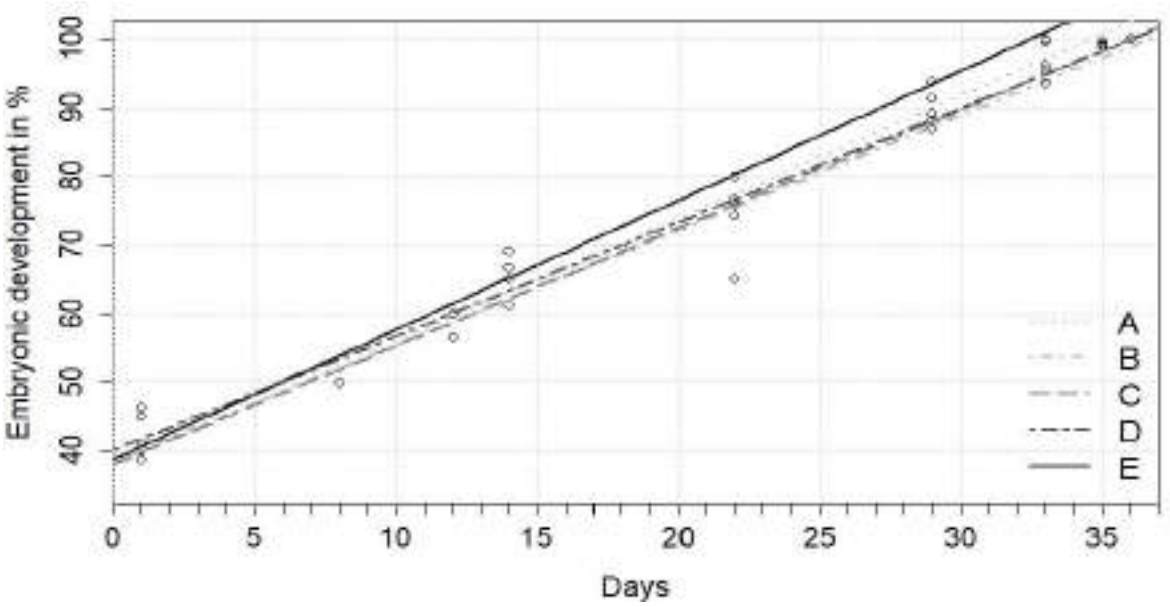


*Figure 4.5: Comparison of embryonic development [%] in the different enclosures (A, B, C) and the control groups (D, E).*

The treatment group in enclosure A showed similar degree days to the control groups (group D and E), whereas the enclosure group C showed significantly higher degree days than both control groups ($p < 0.007$) and group B had significantly higher degree days than control D ($p = 0.019$). Comparing only the enclosure treatments, group C had significantly higher degree days than A ($p = 0.013$)(Table 4.1).

*Table 4.1: Indication of the average water temperature, mean development days and degree days of embryos in enclosure A (A), enclosure B (B), enclosure C (C), the CAS (D) and the hatchery (E), n = 6 per treatment*

| Group | A | B | C | D | E |
|---|---|---|---|---|---|
| temperature (± SD) | 19.23 (± 3.73) | 18.94 (± 3.60) | 19.14 (± 3.63) | 17.91 (± 3.08) | 19.71 (± 1.70) |
| development days (± SD) | 34.00 (± 1.31) | 35.50 (± 1.04) | 36.00 (± 0.0) | 35.67 (± 0.44) | 33.00 (± 0.0) |
| degree days (± SD) | 653 (± 27.24) | 672 (± 21.18) | 689 (± 0) | 638 (± 8.44) | 650 (± 0) |
| n (hatched juveniles) | 460 | 453 | 420 | 547 | 373 |

The median weights of hatched crayfish varied from 22.1 mg ± SD 3.2 in enclosure C to 24.4 mg ± 0.19 in the hatchery (E) (Figure 4.6). The statistical analysis confirmed a higher weight of the hatchery control (E) than all other groups except for group A ($p = < 0.001$). Juveniles in enclosure A in turn showed a significantly higher weight than individuals in enclosure C ($p = 0.017$).

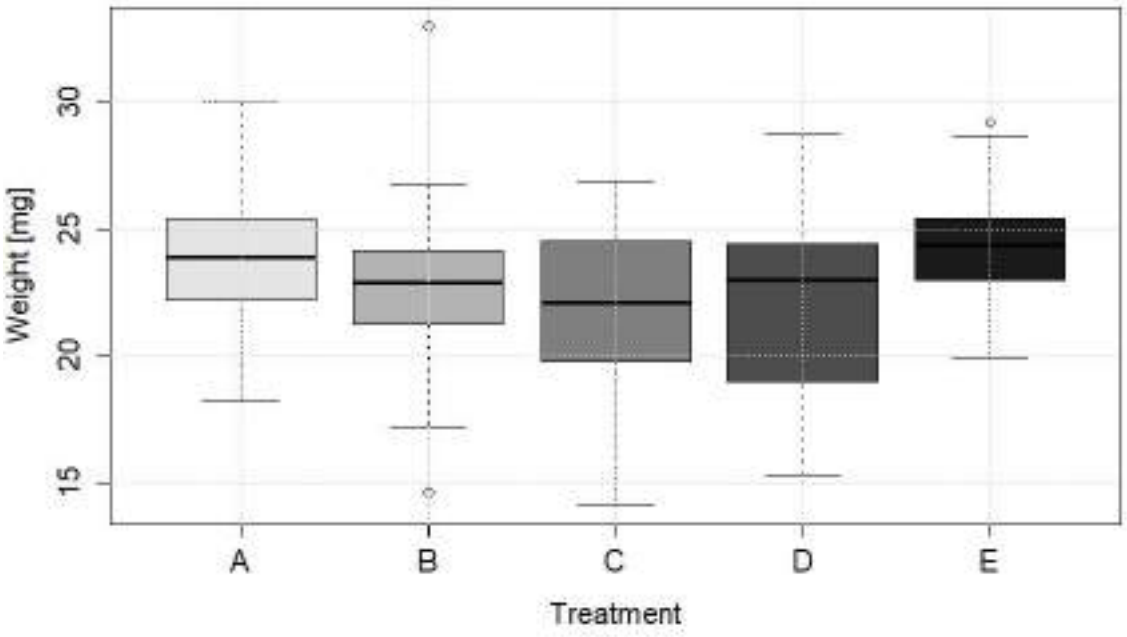


*Figure 4.6: Comparison of treatment groups in boxplots, indicating the weights [mg] of the A. astacus juveniles after first moult. The lines in the boxes indicate median values, the lines above and below the boxes indicate standard deviations, and dots represent outliers.*

### 4.1.5 Discussion

We detected that, of the three compared enclosures, a floating enclosure with a wide mesh size produces good results in terms of the embryo's survival, development and weight gain. Literature shows that enclosures in past studies were often quite varied in their measurements, mesh size and crayfish biomass content (Albertson and Daniels, 2018; Chucholl, 2013; Jussila et al., 2011; Mueller and Bodensteiner, 2011)

Fifty percent of the adult animals placed in enclosure B died within the first week of the experiment and had to be replaced. Even though no statistical test was performed, as the number of animals was too low, it leads to the assumption that enclosure design B might increase the stress level in the maternal animals, therefore resulting in a higher maternal mortality rate and consequently in fewer juveniles.

(Cukerzis et al., 1979) stated that one *A. astacus* female can produce 10–15 young crayfish under natural conditions, while a breeding experiment obtained 40 juveniles per female. Later (Pursiainen et al., 1983) reported a mean output of 60–80 stage 2 juveniles per noble crayfish female

in culture and (Mackevicienė et al., 1997) obtained mean offspring numbers of 22.4 to 70. In this study, one female produced an average of 66.55 stage 2 juveniles (A: 71.16; B: 75.50; C: 53.00) and 70.19 in the control groups (D: 78; E: 62). Therefore, this study's data provided very convincing results, as the reproduction of the tested animals was similar described in previous studies (Kozák et al., 2007; Policar et al., 2004; Policar et al., 2006).

The pace of embryonic development between the experimental groups started to differ eight days into the experiment, which corresponded with the time when the outdoor water temperature started rising. The speed of the embryonic development was fastest in enclosure A. According to (Westin and Gydemo, 1986) a sufficient increase of the water temperature can even reduce the developmental time in *A. Astacus* by half. This suggests that one reason for the faster development was the overall higher temperature in the floating design A. Nevertheless, the degree days in this enclosure type were the lowest, which leads to the assumption that the temperature is not the only reason for faster development. Most likely, it is a combination of the higher temperature and better flow conditions, which enhance the supply of oxygen and nutrients. Hypoxia can cause problems especially in the later embryonic stages (Reiber, 1997).

The overall weights of the hatched juveniles are comparable to the literature data as (Kanta, 2007) showed, with weights of 0.018 to 0.025 g. The measured higher weights of the juveniles in the hatchery control group and in enclosure A are based on the faster embryonic development in these groups. The earlier hatching results in more energy from the egg yolk being left and not being used up for cellular respiration and therefore can be used for biomass production (Pandian, 1970)(Policar et al., 2004).

#### 4.1.5.1 Enclosure quality and handling

Type A

The design of these enclosures supported the attempt to expose the crayfish to as little stress as possible and the best development conditions in terms of temperature and water circulation. The large lid and floating construction allowed us to take samples directly in the water and made the work fast and precise. The wire fence stayed permeable over the whole experiment. It is indispensable to use water-resistant glue for the wooden panels to ensure durability.

Type B

This design was more durable than the first one, but treatment and sampling were more complicated. The enclosure's openings on the long side made it difficult to reach the animals and had to be reclosed with zip-ties every time after sampling. This resulted in a longer handling time and therefore led to more stress for the maternal crayfish.

Type C

The design of enclosure type C was as easy to use as type A. The weak point of this design was the small mesh size of the gauze, which already overgrew with algae in the first week of use. This led to an accumulation of sludge inside the enclosure. In addition, the gauze of one enclosure tore apart during the experiment resulting in the escape of one animal.

Not only the applied measurable parameters indicate the advantage of enclosure type A, but its quality and handling also proved to be the best of the three tested designs. The large hinged cover and the option of not having to lift the enclosure out of the water for sampling reduced the handling time and made it less stressful for animals and handlers. The large mesh size prevented the enclosure from clogging with algae. In contrast, enclosure B was harder to handle because of the cover on the long side and design C was clogged with algae within one week as well as its gauze being quite prone to damage. Furthermore, the disadvantage of enclosure A's non-water-resistant glued wood panels can be easily resolved by using solid wood panels. The main aim of the enclosures devised in this study is their suitability for experiments with adult and embryonic crayfish without substantial adverse effects from the accommodation itself. This research tool enables the gathering of data that are even more reliable against the background of predictable influences from the enclosure method.

Almost every significant aspect considered in this experiment shows that enclosure A is the most suitable design type for outdoor experiments (Tab. 4.2) due to the floating design and therefore higher temperatures and better flow regime for the animals. Therefore, it can be recommended for use in future experimental setups.

*Table 4.2: Summary of all statistically significant differences found between treatments in this study $p \leq 0.05$*

| Compared treatments | Temperature | Survival rate embryos | Embryonic development | Weight | Degree days |
|---|---|---|---|---|---|
| A vs. B | A = higher | / | A = faster | / | / |
| A vs. C | / | / | A = faster | A = heavier | A = lower |
| A vs. D | A = higher | / | A = faster | / | / |
| A vs. E | A = higher | / | / | / | / |

### 4.1.6 References Freshwater crayfish in field experiments: design and efficiency of three novel enclosures

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## 4.2 Sewage treatment plants affect embryonic development of *Astacus astacus* (Linnaeus 1758)

After the successful development of the experimental setup, the actual study to observe effects of chemicals introduced to surface waters was conducted. Here, sewage treatment plants can serve as a good example of a selective entry of chemicals. They contain substances from various origins like pharmaceuticals, plant protection products or industrial waste like heavy metals. The following manuscript shows the effects of wastewater, originating from a sewage treatment plant, on noble crayfish embryos under realistic outdoor conditions as an example of effects caused by human wastewater introductions into the habitat of the animals.

The included version of the manuscript represents the state of preparation prior to full corrections and approval of co-authors. Submission is planned for the journal: “Ecotoxicology”.

# Sewage treatment plants affect embryonic development of *Astacus astacus* (Linnaeus 1758)

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JL and KL conceived and designed the experiments. JL performed the experiments. JL analysed the data. JL, KL and HB wrote the manuscript; all authors provided editorial advice.

Short title: Sewage treatment plants for freshwater crayfish

Keywords: *noble crayfish, juveniles, embryonic, enclosures, sewage treatment plants*

### 4.2.1 Abstract

Pharmaceutics and agriculturally used chemicals are suspected and partially proven to have negative effects on aquatic organisms. Many of these compounds are not removed completely in sewage treatment plants. The effects on organisms or populations in the influenced areas are widely unknown. To address this problem, we conducted an in vitro experiment by exposing female egg-carrying crayfish to a river section of the Eider with influence of a sewage treatment plant. We monitored the effects of the wastewater residues in four groups with different distances to the entry. The results show that the composition of at least 26 chemicals, including high concentrations of Carbamazepin, Diclofenac and Glyphosate, have an influence on embryonic survival and hatching weight of the animals. The effective dose of the mixture is, therefore, lower than effects known by single compound investigations. Therefore, the results of this study demonstrate the need of an optimisation of sewage treatment plants to protect the endangered species *Astacus astacus* and, therefore, the aquatic biotic communities and provide representative data, which help to estimate the impacts of different pollution intensities on freshwater crayfish populations and, thus, on the whole ecosystem.

### 4.2.2 Introduction

In recent years, there has been growing concern about the release of organic compounds of anthropogenic origin, known as emerging organic contaminants, to the environment. These contaminants include a diverse group of thousands of chemical compounds, such as pharmaceuticals and personal care products, pesticides, hormones, surfactants, flame retardants, plasticizers and industrial additives, among others (García et al., 2020). A majority of the substances is designed to have effects on biological structures and is, therefore, active in their environment for a relative long period. As studies have shown, conventional sewage treatment plants (STPs) are inefficient in the removal of many biological active compounds (Bouju et al., 2016; Cacace et al., 2019; Corno et al., 2019; Manaia et al., 2018).

In most toxicological studies, only one single potentially harmful substance or a mixture of only few chemicals is investigated. It is almost impossible to artificially produce actually occurring mixtures of substances observed in surface waters under laboratory conditions. This is especially crucial as mixtures of contaminants of emerging concern may lead to more bioaccumulation and stronger effects than expected from only a single contaminant (Ding et al., 2016). Therefore, the only possibility to respond to this problem, is to investigate effects under outdoor conditions in areas influenced by STP and to carry out studies in the actual influenced habitats.

The greatest relevance on the habitat are effects on organisms with a high influence on their habitat. The absence or presence of some animals influences several trophic levels and their structural

environment. One example for these “keystone species” or “ecosystem engineers” are freshwater crayfish due to their omnivorous diet and their building of hollows in their structural environment (Weinländer and Füreder, 2016). At the same time, freshwater crayfish are known to be sensitive towards water pollution (Haddaway et al., 2015; Kocour Kroupová et al., 2018). Early live stages, like embryonic development, are even more sensitive to external influences (Khan and Nugegoda, 2007). However, the reproduction strategy of most crayfish species leads to a potentially long exposure time of embryos to potentially harmful substances. Female noble crayfish, for example, carry their eggs outside their body under their abdomen for a period up to nine months (Ackerfors, 1999). This relatively long timespan may lead to a high impact of pollutants on these early developmental stages. Hence, the investigation of influences of pollutants on these live stages are key to understanding the influences of STP on, for example, crayfish populations.

To this end, we exposed egg-carrying female noble crayfish in a river to surface waters with different concentrations of chemicals, originating from an STP. This procedure was intended to answer the following questions:

i.: Do chemicals that origin from STPs affect the survival of noble crayfish embryos?

ii.: Is the embryonic development of noble crayfish influenced by these chemicals, and

iii.: if yes, which concentrations show measurable effects?

Due to dilution within a relatively short distance, the chosen study site can represent different types of chemically loaded surface waters and still be comparable to other environmental parameters. This is important to estimate influences of different intensities of pollution on freshwater crayfish reproduction under realistic conditions.

### 4.2.3 Material and methods

#### 4.2.3.1 Study site

The study was conducted in the federal state of Schleswig-Holstein, Germany, in the Eider. This river lays in a valley with a catchment area of 135 $km^2$ upstream from the study site. During the last 150 years, the upper Eider river has strongly been influenced by human activities, such as river regulation, drainage, mowing of macrophytes each summer and mowing and/or grazing of the adjacent fen grasslands (Vogt et al., 2007). In the direct study area (54°15'06.5"N 10°03'57.9"E) an influx of the local STP is present (Figure 4.7). This gave us the opportunity to compare embryos exposed to water contaminated with the output of this plant with embryos placed upstream and, therefore, without the influences of this plant. In this area, the river is approximately 7 m broad and between 0.8 and 1.5 m deep. The direct surrounding is dominated by pasture land. Effects of STP in this area can be especially relevant because of an autochthone genetic structure of noble crayfish found in this study site revealed

by (Schrimpf et al., 2014). Therefore, a loss of this population could lead to a loss of this special genetic structure.


*Figure 4.7: Overview of the study area. Map extracted from OpenStreetMap Deutschland (2020).*

#### 4.2.3.2 Experimental design

To investigate influences of STP on the embryonic development and survival of freshwater crayfish, we exposed four groups (A-D) of ten egg-carrying noble crayfish to different locations with different chemical loads in the study site area. Group A was exposed to the area at a radius of 3 m meters around the inlet of the treatment plant. Group B was placed at a distance of 10 m and group C at a distance of 15 m. Group D served as control group and was exposed in an area of 10 m upstream of the inlet to exclude influences of the chemical residues (Figure 4.8).

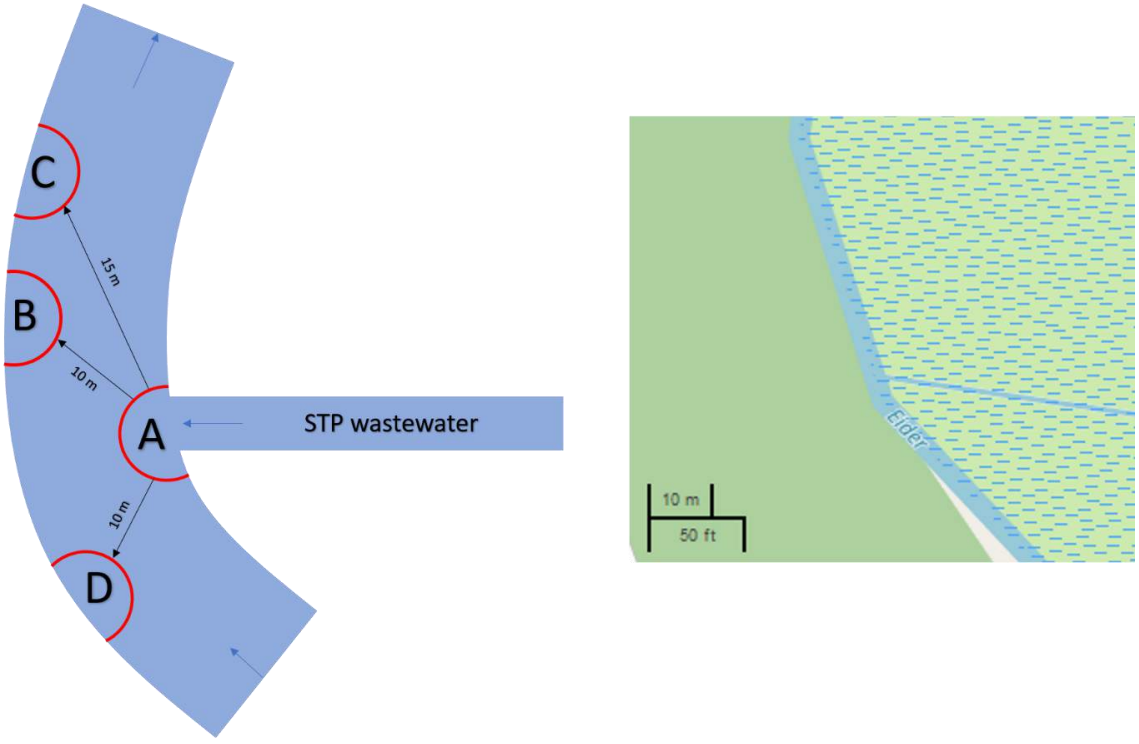


*Figure 4.8: Sketch (left) and overview (right) of the study site (OpenStreetMap Deutschland, 2020).*

The animals were held in enclosures, whose suitability was evaluated in a former study (Laurenz et al., chapter 5.1, Figure 4.9), with two animals separated in one enclosure so that five enclosures each were exposed to the different concentrations.

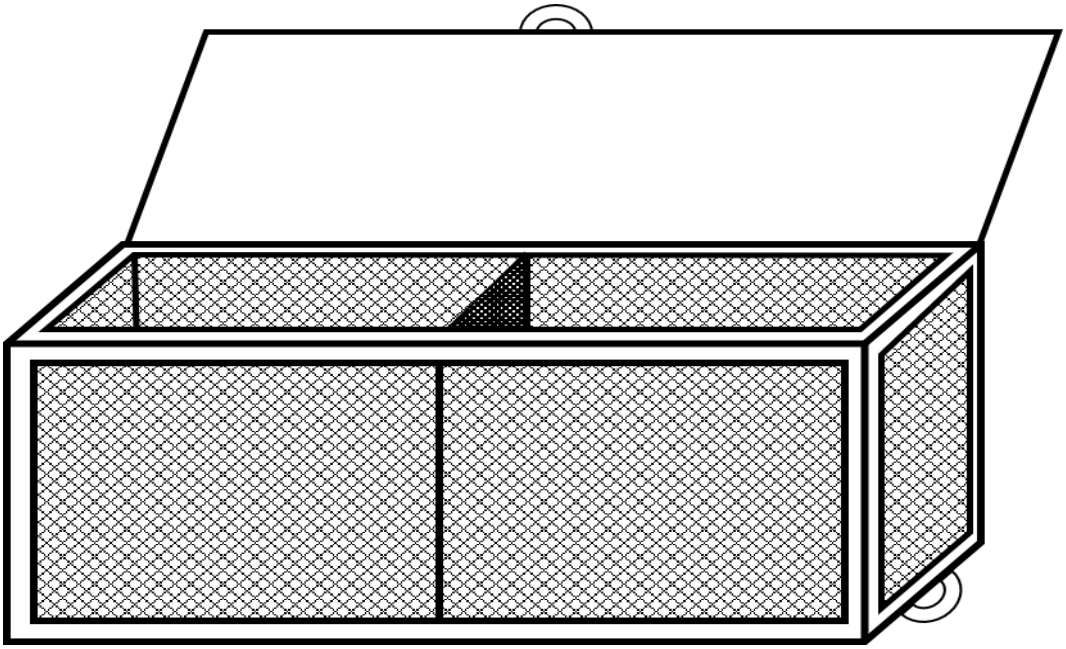

*Figure 4.9: Construction of enclosures L/W/H = 60/25/25 cm; wooden top and bottom (Douglas fir, 2 cm thickness), hinged top cover, sides covered with wire fence (8 mm mesh size), two clay pipes placed inside, construction balanced with weights to float just underneath water surface.*

The investigation started in late March 2020 at development stages of embryos of approximately 40 %. All adult females were measured and the numbers of eggs were counted twice on the animals to ensure corrects numbers. The counting of eggs was proceeded and documented for every pleopod, but had to be finished in less than 10 minutes to reduce stress for the animals and the impact of stress on embryos. Due to individual enclosing, the animals were recognizable over the whole study. Therefore, survival rate of embryos could be attributed to every single female.

The study area and the enclosures were inspected every day. Furthermore, once per week one egg per female was taken to document the development stages of embryos under a binocular microscope at 40 x magnification after the work of (Alwes and Scholtz, 2006). At the dates of hatching, successfully hatched juveniles were removed from adult females, counted and measured. Measurement was performed by photographing juvenile crayfish with a scale under a binocular (Leica DM1000 LED, Leica S8APO, Leica Application Suite Version 3.0.0, Leica Microsystems CMS GmbH, D-35578 Wetzlar, Germany) and calculating the length with GIMP (Version 2.10.20, Fa. The Gimp Team).

Six random juveniles per group were subsequently prepared for histological observations of the hepatopancreas. The whole animals were fixated in buffered formaldehyde (3.7 %). We stored the

juveniles in Kristensen solution for two days to ensure the complete decalcification of the exoskeleton. The samples were dehydrated with an ethanol series and then embedded in LR White (LR White acrylic resin, hard, sigma Aldrich, Germany). We produced sections of 2 µm thickness, using an ultramicrotome. These were stained with haematoxylin and eosin (HE) with extended exposure time, referring to usage instructions of the LR White. Animal sections and especially hepatopancreas cells were examined under a light microscope combined with the same camera system as described before. The examination included the observation of membrane damages, damages in different cell types as well as changes in size and numbers of B-Cells. For this procedure, ten sections per individual were photographed and subsequently analysed by counting and measuring cells under the microscope.

A crayfish hepatopancreas is typically formed of numerous tubules separated by connective tissues (Abd El-Atti et al., 2019) and consists of lumen, membranes and four types of epithelial cells: resorptive lipid cells (R-cell) for nutrient intake, blister-like secretory cells (B-cell) to derive harmful substances, fibrillar cells (F-cell) as connecting tissue and embryonic cells (E-cell). That means, changes in R-cells would indicate a higher or lower intake of nutrients, changes in B-cells would indicate a higher or lower outtake of harmful substances, whereas changes in the other two types would indicate problems in biosynthesis of the individual.

#### 4.2.3.3 Animals

The egg-carrying female noble crayfish (*Astacus astacus)* were obtained from a hatchery in Schleswig-Holstein (Krebszucht Oeversee, Germany). They were hatched and raised in a semi-intensive aquaculture system so that external influences were minimized. The animals were three years old and showed carapace lengths of 40.29 cm to 61.58 cm with an average of 52.14 ± 4.27. They carried between 67 and 436 eggs, with an average of 166.6 ± 70.85. The animals were gathered the day before the experiment started and were kept in 600 L tanks in the recirculating aquaculture system (RAS) of the limnological department of Kiel University.

#### 4.2.3.4 Water parameters

To be able to draw conclusions of observed effects on human influences, a comprehensive data acquisition of nutrient content, biotic and abiotic parameters and substantial load is necessary. Therefore, we equipped enclosures of every group with loggers collecting data every 10 minutes for oxygen, temperature, light intensity and pH, whereas conductivity was measured every 15 minutes (HOBO, Oxigen:ONS-U26-001, temp. and lux: ONS-UA-022-64, pH: ONS-MX2501, conductivity: ONS-U24-002-C, Onset Computer Corporation, Bourne, MA, USA). Three plant protection products and thirty-three pharmaceutical products were monitored at three time points during the experiment (first day, after 30 days and on the last day) by an external lab (AGROLAB, Agrar und Umwelt GmbH, Kiel,

Germany). Nitrate, nitrite, ammonium and acid binding capacity was also measured at the three time points with a photometer (DR 5000, Hach Lange GmbH, Düsseldorf, Germany).

#### 4.2.3.5 Statistical methods

All statistical analyses were performed using R version 3.2. (R Core Team, 2015). Differences in survival and juvenile size between different groups were tested for normality and equal variances prior to analysis. If both were evident, a t-Test was performed. For non-parametric data, a Wilcoxon test was used. The embryonic development was analysed via linear regressions. Due to good correlation values (> 0.8), the linear regressions were compared with an ANCOVA (analysis of covariances). Pictures were analysed in GIMP 2.8 (version 2.8, Fa. the Gimp Team). Influences of maternal size on survival, size of juveniles and number of laid eggs was also tested with correlation values of regressions.

### 4.2.4 Results

#### 4.2.4.1 Water parameters

Environmental parameters of the four locations were very similar as described in Table 4.3. The only difference can be observed in the temperature of the locations. The average temperature of location A is 1 °C higher than of the other groups (Figure 4.10). This higher temperature is caused by the warm water of the brook emerging from the STP. The shallow depth of only up to 20 cm causes a rapid heating of the water. Due to the differences in mean temperature, parameters were not analyzed over time but over degree days. Thereby, influences caused by different temperatures could be excluded.

*Table 4.3: Environmental parameters measured using photometer, titration or HOBO Logger.*

| Parameter/group | A ± SD | B ± SD | C ± SD | D ± SD |
|---|---|---|---|---|
| Nitrite [mg/L] | 0.17 ± 0.02 | 0.11 ± 0.03 | 0.13 ± 0.03 | 0.13 ± 0.01 |
| Nitrate [mg/L] | 4.43 ± 0.36 | 2.80 ± 0.21 | 3.25 ± 0.42 | 2.88 ± 0.18 |
| Ammonium [mg/L] | 0.55 ± 0.08 | 0.24 ± 0.29 | 0.24 ± 0.06 | 0.18 ± 0.02 |
| Acid binding capacity | 5.35 ± 0.07 | 5.17 ± 0.02 | 5.18 ± 0.06 | 5.15 ± 0.04 |
| Conductivity | 597.00 ± 203.52 | 401.00 ± 63.00 | 422.00 ± 62.20 | 441.00 ± 64.20 |
| pH | 7.79 ± 0.17 | 7.86 ± 0.26 | 7.55 ± 0.29 | 7.82 ± 0.34 |
| Oxygen | 7.53 ± 3.38 | 7.25 ± 2.78 | 8.38 ± 1.79 | 8.45 ± 1.48 |
| Temperature | 13.03 ± 2.67 | 12.10 ± 2.46 | 12.04 ± 2.46 | 12.00 ± 2.46 |

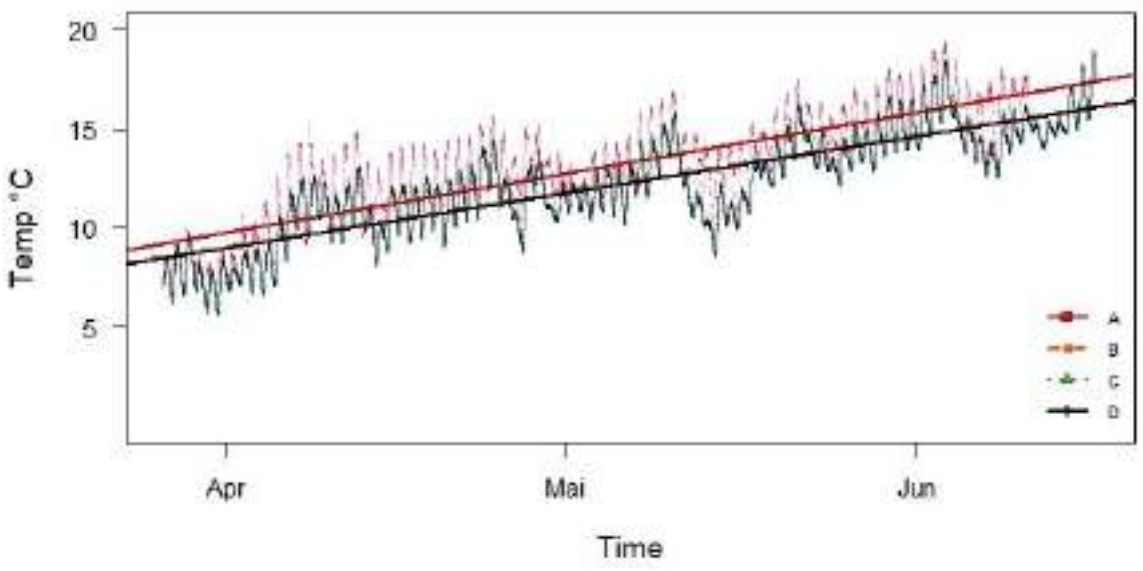


*Figure 4.10: Temperatures measured with HOBO loggers inside of enclosures group B, C and D are identical and, thus, only one line is visible.*

#### 4.2.4.2 Chemical load

The analysis of chemical loads in the study area showed that 25 of the 36 tested chemicals were detectable (Table 4.4). Most of these were traceable only in the direct area of the STP input. Highest concentrations were found for the pharmaceuticals Carbamazepin (antiepileptics), Diclofenac (analgetica), Metformin (biguanide), 4-Acetamidoantipyrin (metabolite of metamizole, analgetica), 4-Aminoantipyrin (derivate of Pyralozone, analgetica) and 4-Formylaminoantipyrin (metabolite of Aminophenazone, analgetica). For plant protection products, AMPA (metabolite of Glyphosate) and Glyphosate were detected in highest concentrations at the end of the experiment with 1.5 µg/L AMPA and 5.8 µg/L Glyphosate.

*Table 4.4:Averages of monitored pharmaceuticals and plant protection products. Results of single measurements are given in supplemental material*

| Detected chemical [µg/l] /group | A | B | C | D |
|---|---|---|---|---|
| Acetylsulfamethoxazol | 0.021 ± 0.021 | - | - | - |
| Bezafibrat | 0.117 ± 0.056 | - | - | - |
| Carbamazepin | 2.767 ± 0.249 | <0.03 (+) ± 0 | <0.03 (+) ± 0 | <0.03 (+) ± 0 |
| Diclofenac | 2.767 ± 0.125 | 0.02 ± 0.02 | 0.02 ± 0.02 | 0.013 ± 0.019 |
| Fenofibrat | <0.03 (+) ± 0 | - | - | - |
| Fenofibrinsäure | <0.03 (+) ± 0 | - | - | - |
| Ibuprofen | <0.30 (+) ± 0 | - | - | - |
| Indometacin | <0.03 (+) ± 0 | - | - | - |
| Ketoprofen | <0.05 (+) ± 0 | - | - | - |
| Lidocain | 0.17 ± 0.005 | - | - | - |
| Metformin | 1.443 ± 0.575 | <0.05 (+) ± 0 | 0.045 ± 0.045 | 0.045 ± 0.045 |
| Naproxen | 0.18 ± 0.071 | - | - | - |
| Paracetamol | 0.034 ± 0.025 | - | - | - |
| Phenazon | 0.167 ± 0.119 | - | - | - |
| Primidon | 0.560 ± 0.399 | - | - | - |
| Tris-2-Chlorethylphosphat | <0.50 ± 0.170 | <0.10 ± 0 | <0.10 ± 0 | <0.10 ± 0 |
| 10-Hydroxy-10,11-dihydrocarbamazepin | 0.80 ± 0.572 | 0.03 ± 0 | 0.015 ± 0.015 | 0.01 ± 0.014 |
| 4-Acetamidoantipyrin | 1.50 ± 0.356 | 0.06 ± 0.02 | 0.075 ± 0.01 | 0.07 ± 0.016 |
| 4-Aminoantipyrin | 1.397 ± 0.458 | <0.03 (+) ± 0 | 0.04 ± 0 | 0.02 ± 0.02 |
| 4-Dimethylaminoantipyrin | <0.03 (+) ± 0 | - | - | - |
| 4-Formylaminoantipyrin | 10.667 ± 0.943 | 0.23 ± 0.11 | 0.19 ± 0.155 | 0.163 ± 0.133 |
| AMPA | 0.91 ± 0.429 | 0.06 ± 0.02 | 0.055 ± 0.02 | 0.043 ± 0.019 |
| Glyphosate | 2.007 ± 2.682 | <0.03 (+) ± 0 | <0.03 (+) ± 0 | <0.03 (+) ± 0 |
| Terbuthylazine | <0.03 (+) ± 0 | <0.03 (+) ± 0 | <0.03 (+) ± 0 | <0.03 (+) ± 0 |

#### 4.2.4.3 Survival

With 80.81 % ± 10.15 embryonic mortality until hatching, animals exposed to the direct area of the STP (group A) showed a significantly higher mortality compared to groups B and D ($p \leq 0.049$, Figure 4.11). The other locations did not differ significantly in their mortality. Mortality of the control group (D) was 61.79 % ± 24.09 on average. The regression of maternal size with embryonic mortality showed an $R^2$-value of -0.0068, which shows that mortality is independent of maternal body size. However, the number of initially laid eggs correlated with maternal size with a value of $R^2$ 0.499, as expected.

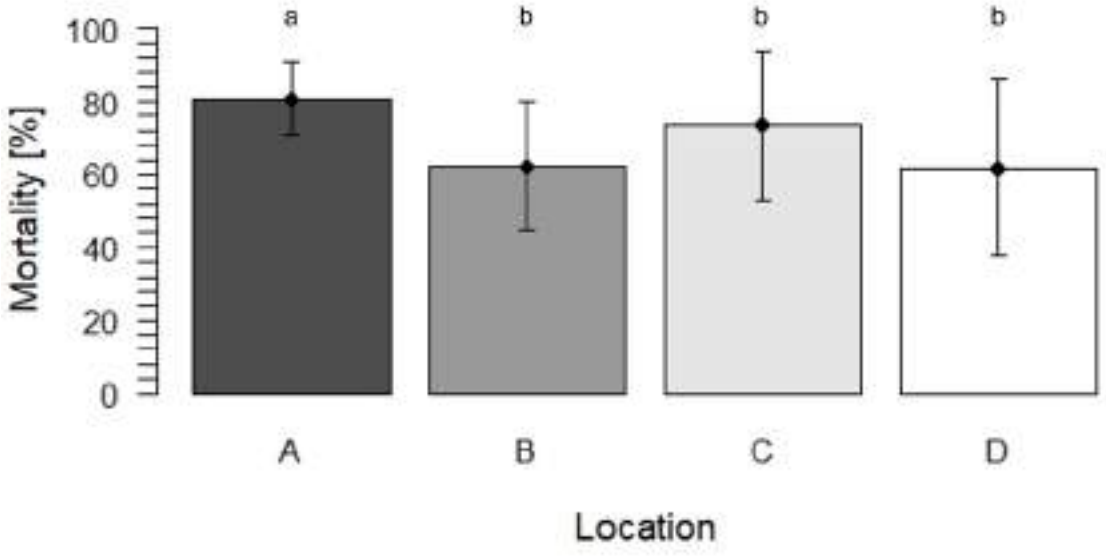


*Figure 4.11: Mortality in % and standard deviation of the four exposure groups. Letters show significant differences after t-Test*

#### 4.2.4.4 Embryonic development

Embryonic development did not differ between the four locations, taking the different temperatures into account (Figure 4.12). However, the embryos developed similarly in regard to degree days, but not to actual dates. For this specific area, this results in a hatching of juveniles of the first group six days earlier than of the other groups.

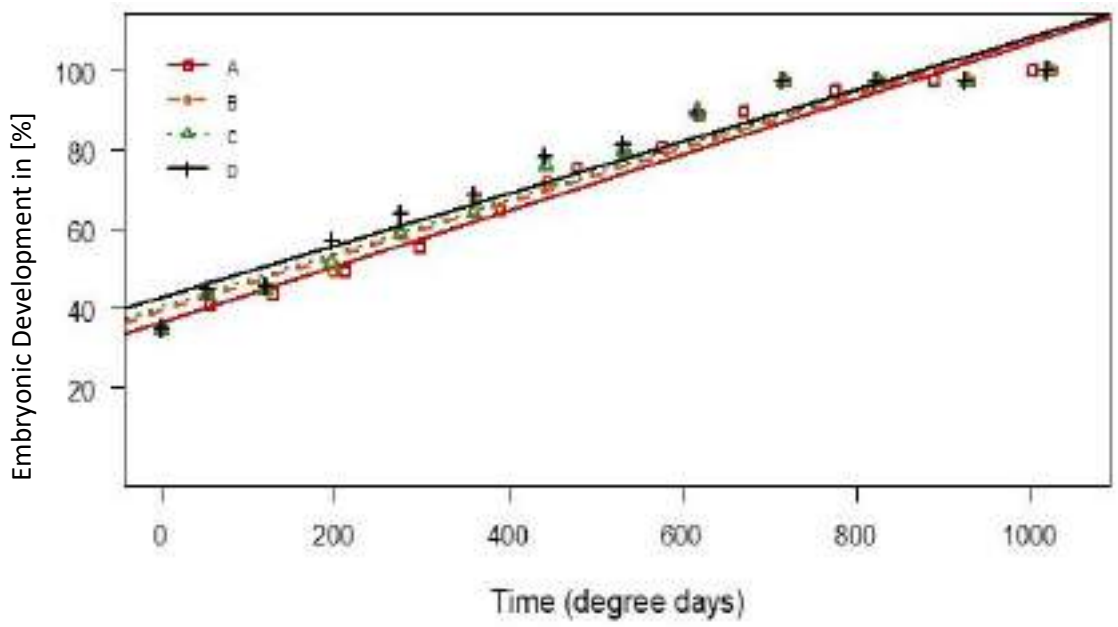


*Figure 4.12: Embryonic development of the four groups with linear regression*

#### 4.2.4.5 Size of juveniles

Carapace sizes of freshly hatched juveniles showed a clear correlation to the location and therefore present chemicals (Figure 4.13). Juveniles developing in group A showed an average length of 4.23 mm ± 0.21 and were, thus, significantly smaller than the animals developing in all other locations ($p \leq 0{,}0004$). At the same time, the juveniles that hatched in control group D had an average length of 4.56 mm ± 0.19 and were significantly larger than all other groups ($p \leq 9.4e^{-05}$). Only the two groups exposed to low concentrations of the wastewater were similar to each other (B = 4.38 ± 0.23, C = 4.40 ± 0.19).

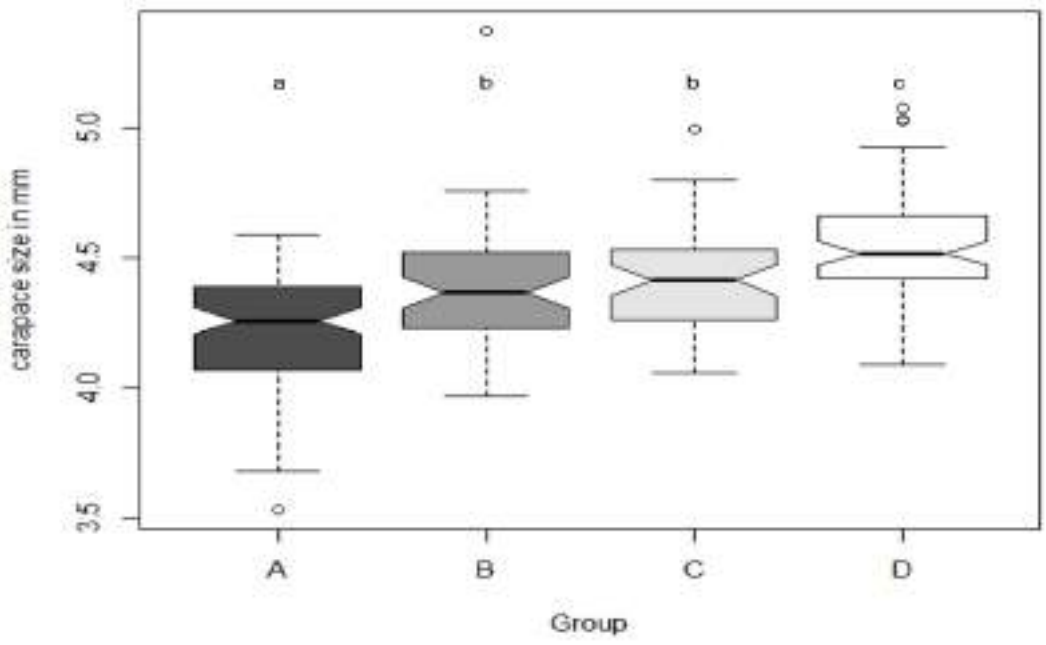


*Figure 4.13: Average size for the different exposure locations of the groups. Letters indicate significant differences (t-Test).*

#### 4.2.4.6 Histology

Figure 4.14 shows the impact of the different placements of enclosures in the study site on the hepatopancreas cells. For groups B and C, there are no differences in B-cell sizes or numbers per cell compared to the control. For hepatopancreas cells of group A (the group exposed directly to the influences of the STP with highest measured concentrations of chemicals), however, we found damages in every section and for nearly all observed cells. Membranes were disrupted resulting in the formation of abnormal lumen, and necrotic cells were found in every section. Number of B-cells, which normally are connected to the lumen were significantly lower ($p < 0.0001$) for this group compared to all others. B-cell numbers per group were: A: 21.32 (SD = 3.14); B: 44.25 (SD = 6.20); C: 45.18 (SD = 5.54); D: 44.58 (SD = 5.67).

Because of the many damages and indistinct borders of the B-Cells, the sizes of B-cells were not compared.

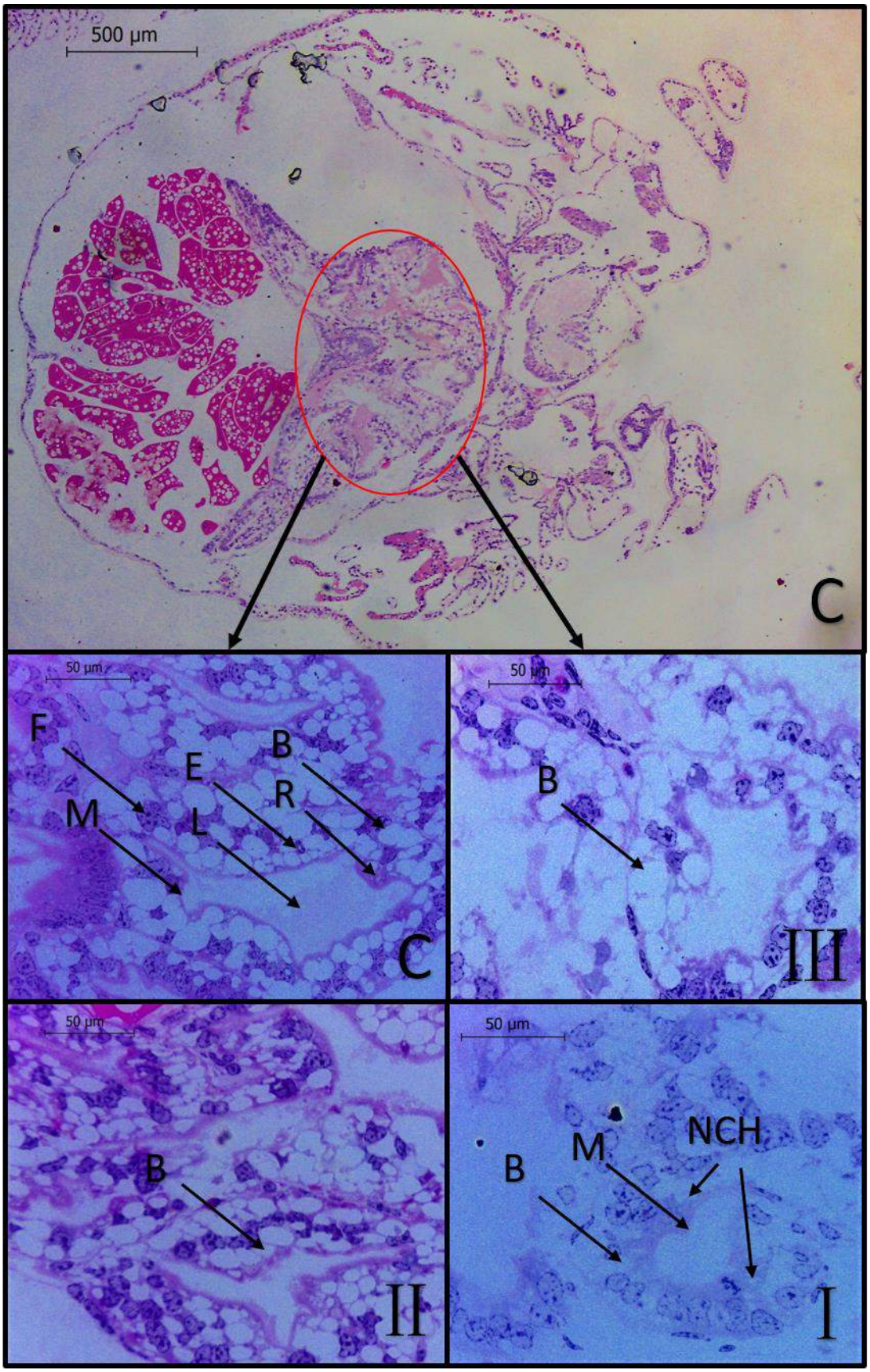


*Figure 4.14: Transversal histological sections of noble crayfish (*Astacus astacus*) exposed to the different environmental influences during embryonic development. Marked areas are lumen (L), membrane (M) and four types of epithelial cells: resorptive (R) lipid cells, blister-like (B) cells, NCH: Necrotic cells of hepatopancreas. C = Control; Ⅰ = Group A; Ⅱ = Group B; Ⅲ = Group C*

### 4.2.5 Discussion

#### 4.2.5.1 Chemical loads

Even though STPs are one of the major sources of chemicals influx into aquatic environments (Gagné et al., 2006), little is known about effects of the diverse and constantly changing chemical load on aquatic organisms. In our example, 25 out of 36 tested substances were detected in the water of the Eider. Many of these substances, originating from human and veterinarian pharmacy and agriculture, are known to have toxic effects on non-target organisms. On the one hand, Paracetamol and Metformin show toxicological effects on the standard organism *Daphnia magna* starting from 6.5 mg/L and 64 mg/L, respectively, and, therefore, at higher concentrations than detected in the Eider (Cleuvers, 2003; Jungkon Kim et al., 2010). On the other hand, Carbamazepine shows effects on *Daphnia magna* starting at concentrations of 0.5 µg/L (Dietrich et al., 2010) and on the green crab (*Carcinus maenas*) at concentrations of 1 µg/L (Aguirre-Martínez et al., 2013) ), which represent lower concentrations than measured in this study. Diclofenac, one of the chemicals with the highest monitored concentrations, is described by Fent et al. (2006) as the compound having the highest acute toxicity within the class of NSAIDs. Especially the effects of DCF mixtures with other chemicals introduced to surface waters can increase the negative effects of this analgetic (Gonzalez-Rey et al., 2014; Prokkola et al., 2015). Sublethal effects of DCF on freshwater crayfish were observed from concentrations of 0.16 mg/l and higher with condemned embryonic development in marbled and noble crayfish (Laurenz et al., chapter 3.2). Glyphosate is one of the better studied chemicals regarding toxic effects. Canosa et al. (2019) showed that Glyphosate can imbalance the male reproductive function of the estuarine crab *Neohelice granulata* at concentrations of 1 mg/L. Banaee et al. (2020) demonstrated additionally that effects of Glyphosate are increasing when mixed with other pesticides like Chlorpyrifos. Therefore, lowest effective observed concentrations could be even lower than the 40 mg/L observed by Avigliano et al. (2014). The higher toxicity of mixtures is verified by countless observations (Müller et al. 2020; Oliver et al. 2020).

These effects of mixtures make it even more important to understand the impact of contamination caused by human input into surface waters on non-target organisms.

#### 4.2.5.2 Effects on freshwater crayfish

##### 4.2.5.2.1 Survival

At 61.8 %, the mortality of the control group (D) is comparable to known hatching rates of noble crayfish (54.6 %) as shown by Kouba et al. (2010). However, the mortality of group A, which was exposed directly to the influences of the STP, is higher than uninfluenced survival rates found in literature (Policar et al., 2004; Policar et al., 2006; Reynolds et al., 1992).

The monitored concentrations of chemicals are very limited to a small location at the entry of the STP. This enabled the implementation of this study in a comparatively small area and, therefore, the effects on the crayfish population are limited to that small range. Nevertheless, concentrations of pharmaceuticals and agricultural substances are not always limited to small areas and can also affect whole streams and large lake habitats (Kandie et al., 2020; Maasz et al., 2019; Picó et al., 2020; Wang et al., 2020). While the monitored compounds and concentrations differ for every location, influences of the compositions of residues of the human products for health and agriculture are very likely.

Survival rate of the juveniles is one, if not the most important factor for stabilisation of populations. This means that a decrease of juvenile survival of 30.85 %, as shown in this study, can lead to a reduction of population size of the endangered species (Edsman et al., 2015).

#### 4.2.5.2.2 Development

The development and time until hatching did not differ between groups regarding degree days. The hatching took place in June for all groups, which fits the observations of Ackerfors (1999). However, when comparing development of groups regarding days, the group directly exposed to the influences of the STP shows a shorter development time, so that the juveniles hatched six days earlier. This is due to the higher water temperature of the stream delivering the STP water into the river. A shorter development time can have positive and negative effects at the same time. The earlier hatch can lead to an advantage of the animals in lower food competition and, thus, faster growth (Franke and Clemmesen, 2011). Temperatures, that are too high can lead to a faster development and consequently to higher rates of abnormal development and higher mortality of juveniles (Jin et al., 2019). In this study, no significant differences in development or malformations were detectable, and the temperature of all groups was below 20 °C and, thus, in the optimal range for noble crayfish reproduction (Policar et al., 2004; Westin and Gydemo, 1986). Therefore, we conclude that the higher temperature leads to an early hatching of juvenile crayfish, but the chemical load does not affect the hatching time or development.

#### 4.2.5.2.3 Juvenile size

Effects of the locations on the offspring are visible in the size of juveniles. The overall sizes are higher than described in literature, where the very few data showed illustrations with stage 1 juveniles of about 1 to 1.5 mm carapace size (Kawai and Kouba, 2020). The differences in sizes are correlated to a higher load in chemicals of the locations. Smaller body sizes caused by toxic influences are described in the literature. Mac Loughlin et al. (2016), for instance, showed that Atrazine can influence weight gain of *Cherax quadricarinatus* at concentrations of 2.5 mg/L and Avigliano et al. (2014) as well as Frontera et al. (2011) showed a decrease of weight gain due to Glyphosate for the same species starting from 40 mg/L and 22.5 mg/L, respectively. Velisek et al. (2015) showed that terbuthylazine-2-

hydroxy can lead to a decrease in weight gain for *Procambarus virginals* when exposed during first life stages including embryonic development at a concentration of 75 µg/L of. These early life stages are known to be more sensitive to chemical impacts than advanced juveniles (Barki and Karplus, 2004; Jones, 1990). This fact in addition to the higher toxicity of mixtures of chemicals can explain the high sensitivity of the noble crayfish embryos in terms of growth observed in this study. The smaller size can lead to a disadvantage in competition for food and habitat and, therefore, on survival of the endangered species. Additionally, the smaller body size causes a higher feeding pressure on the juvenile crayfish, especially through other invertebrates (Zimmermann J.K.M., 2009).

#### 4.2.5.2.4 Histology

Histology of the hepatopancreas is serving as a parameter to measure sublethal toxicological effects in several publications. The organ is known to be analogous to the mammalian liver, which is also susceptible to chemicals such as pesticides. Saravana Bhavan (2000) showed effects of the pesticide Endosulfan on the prawn *Macrobrachium malcolmsonii*. Damages reported in this study were increasing number of R-cells, formation of abnormal lumen, necrotic cells of the hepatopancreas separated from basal laminae and thickened basal laminae. Especially the necrotic cells and abnormal lumen were very similar to the damages found in this study. However, we did not detect differences in number of R-cells or thickness of basal lamina. Chaufan et al. (2006) found epithelial disorganisation in hepatopancreas tubules of *Chasmagnathus granulatus*. In addition, diameters and numbers of B-cells increased after feeding the crayfish Hexachlorobenzene-contaminated *Chlorella* for three days, similar to the effects of the STP output.

Koutnik et al. showed in 2017 pathological changes in hepatopancreas of early life stages of marbled crayfish through chronic terbuthylazine-2-hydroxy exposure in concentrations of up 75 µg/L. In particular crayfish exposed to higher concentrations showed an alteration of the tubular system including disintegration of tubular epithelium with complete loss of structure in some places of the hepatopancreas. These losses of structure can also be found in this study for embryos exposed to the direct influence of the STPs.

Additional effects of the exposure of crustaceans to pesticides can be interstitial sinus haemocytic infiltration, melanisation and coagulation in the thickened basal laminae, necrotic tubules containing tissue debris, and haemocytes that can constitute a wall around the thickened basal laminae of the tubules (Negro et al., 2011). However, these were not observed in the present study.

The similarities of the effects in this study to effects of studies examining single chemical influences (in much higher concentrations than any chemical measured in this study) show that the mixture of substances most probably represents a risk that is multiple times higher for freshwater crayfish.

Therefore, the limit values of chemicals in surface waters should be defined with regard to an inclusion of possible mixture effects.

### 4.2.6 Conclusion

The initially asked questions can be answered as follows:

I: Yes, chemicals that origin from STPs affect the survival of noble crayfish embryos.
ii: Yes, the embryonic development of noble crayfish is influenced by these chemicals, and
iii.: concentrations of first occurring effects are as measured in group C of this study.

The results of this study reveal the effects of chemicals originating from an STP on freshwater crayfish. The effects decrease with increasing distance to the chemical input on a relatively small area. Therefore, there will probably be no significant influences on the local crayfish population. But the data are more important to estimate the danger of various intensities of chemical load on freshwater crayfish. Therefore, the chosen study site was an excellent opportunity to investigate influences of different naturally occurring pollutions on these organisms. As a consequence, the results should be considered for the estimation of limit values. Furthermore, if monitoring data of surface waters show similar concentrations, this study can help to understand and predict the impacts on freshwater crayfish populations and, thus, on the whole ecosystem.

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# 5 Overall discussion and perspective

## 5.1 Discussion and connection of chapters 2, 3 and 4

The present studies show that the influences of chemicals originating from human sources can affect the reproduction and the fitness of freshwater crayfish.

At first, we want to try to answer the first question asked in the introduction:

1: Are reproduction stages of freshwater crayfish influenced by the chemicals TBA and DCF or by the mixture of chemicals in sewage treatment plants?

As the above manuscripts demonstrate, influences are present in all periods of the freshwater crayfish' reproduction. The gonadal development of noble crayfish was affected resulting in a smaller number of next generation crayfish (see chapter 2). For noble crayfish, concentrations of 0.16 mg/L DCF and 1.6 mg/L TBA resulted in smaller egg numbers and all investigated concentrations, resulted in smaller numbers of spermatozoa. For marbled crayfish on the other hand, these effects were not detectable at all.

The study on embryonic development presented in chapter 3 shows that embryos of noble crayfish have a lower survival rate starting from concentrations of 0.1 mg/L TBA and 10.24 mg/L DCF. In addition, sublethal effects are observed starting from concentrations of 0.025 mg/L TBA and 0.04 mg/L DCF. Equivalent results for experiments involving DCF were detected for marbled crayfish, but a higher sensitivity for noble crayfish compared to marbled crayfish was found when exposed to TBA.

Linking the results about the gonadal development (chapter 2) with the results of the embryonic development (chapter 3) under the influence of the two chemicals reveals that survival of crayfish during both periods of reproduction is affected at concentrations multiple times higher than to be expected in surface waters (0.035 mg/L for TBA and 0.029 mg/L for DCF, (Herrero-Hernández et al., 2017; Dusi et al. 2019). Nevertheless, we have to take into account, whether the influences on both reproduction periods may accumulate and could result in negative effects on the reproduction of crayfish even at lower concentrations. There are trends in our data indicating that the lowest concentrations of the used chemicals result in lower numbers of eggs after exposure of parental animals during gonadal development for noble crayfish. These effects are also visible but not statistically significant for embryonic development. If these two trends are summed up, survival might be significantly lower for animals exposed to TBA and DCF over time, i. e. over several reproduction cycles.

In order that the effects can accumulate, animals would have to be exposed during both periods of the reproduction cycle. However, for TBA, exposure times of several months would be unrealistic. TBA is a pre-emergence herbicide in corn farming. This leads to peaks of TBA concentrations in natural water bodies during March and April (Tasca et al., 2018). Therefore, an exposure to this chemical over at least four months of gonadal development plus at least 45 days of embryonic development is unlikely. Consequently, accumulating effects of the two periods in one reproduction cycle are implausible. However, it is possible that one generation of one population is affected during both periods of reproduction in different reproduction cycles. For example, if a generation is weakened during their embryonic development resulting in a smaller number of individuals and is additionally exposed to TBA while developing oocytes and spermatozoa, this generation could produce an even smaller number of offspring. Overall, this would lead to a decreasing trend of population offspring numbers.

In contrast to TBA, a permanent exposure over the whole year is the general case for DCF due to its year-round use. It is the most frequently detected drug in German surface waters. It has been perennially detected in surface waters in concentrations of up to 29.8 µg/L in 55 countries (Dusi et al., 2019). Therefore, aquatic animals can be exposed to DCF and other drugs throughout their whole life. Number and survival of eggs were affected by concentrations of 160 µg/L after four months exposure during gonadal development, which is less than six times the measured concentration of DCF in surface waters. As a result, direct effects on the number of freshwater crayfish are more likely to be found for DCF than for TBA, but for both chemicals these direct effects are unlikely in terms of a single chemical influence on the animals. More important are the accumulating effects over several years and the sublethal effects found in the studies of chapters 2 and 3.

Considering that the two sublethal effects spermatozoa production and the histology of the hepatopancreas are affected by concentrations of the two chemicals found in surface waters of Europe, it has to be assumed that these are a threat to the endangered noble crayfish. Sperm quality is one of the main factors affecting reproductive efficiency in male crustaceans (Wickins and O'C Lee, 2003). This parameter is affected by the presence of environmental pollutants (Lewis and Ford, 2012). Canosa et al. (2019) showed that the herbicide Glyphosate can imbalance the male reproductive function of the estuarine crab *Neohelice granulata* at concentrations of 1 mg/L by producing abnormal spermatophores and a reduction in sperm count. The authors conclude the possibility of a reduction in brood production and larvae recruitment in the natural environment. The results of spermatophore analyses of freshwater crayfish under influences of TBA and DCF from this study lead to the same conclusion and reveal a severe threat to crayfish populations.

However, not only for wild populations of freshwater crayfish sperm quality is important. Harlıoğlu et al. (2018) showed that control of male reproduction and spermatophore quality is an important matter

in crustacean aquaculture. Crustaceans represent 10 % of the global aquaculture production (FAO, 2018). In recent decades crustacean aquaculture was developing rapidly worldwide and is considered to be an important food production sector in terms of sources of animal protein, occupation and financial gain as well as foreign exchange earnings (Kozák, 2015; Wickins and O'C Lee, 2003). Different crayfish species have been assessed for artificial breeding and cultivation programs as these animals are a wholesome and desirable food (Yazicioglu et al., 2018). Hence, the impact of chemicals is not only relevant for reasons of nature conservation, but also for economy and food production.

Additionally, not only the number of sperm-cells and eggs of noble crayfish are affected by both chemicals, but also, as a sublethal parameter, changes of hepatopancreas histology were shown for every concentration for parental and juvenile noble crayfish. The hepatopancreas is the site of nutrient absorption, digestion, synthesis and secretion of digestive enzymes and reserve storage in decapods (Calvo et al., 2011; Johnston et al., 1998; Xiao et al., 2014). It is formed of numerous tubules separated by connective tissues (Abd El-Atti et al., 2019) and consists of a lumen, membranes and four types of epithelial cells. When investigating effects of potentially harmful substances, the blister-like secretory cells (B-cell), which channel off harmful substances, are of great interest. Changes in B-cell size or number would indicate a higher or lower outtake of harmful substances. Therefore, chemical load and stress originating from this load can be detected by histological changes of these cells. At the same time, these changes can lead to a damaged or non-functional organ, resulting in a flawed or decreased functionality including the defence against harmful substances. As another parameter, influences of the chemicals on the weight of hatched crayfish were shown in chapter 3 for noble crayfish exposed to high TBA concentrations starting at 1.6 mg/L. Hence, we assume that the substances, without interfering substances or circumstances, would not have an impact on hatching weight under outdoor conditions due to the much lower detected concentrations in surface waters.

In conclusion, the chemicals TBA and DCF influence sperm quantity, egg numbers, survival of embryos and the histology of the hepatopancreas, which results in combined impacts on the stability and fitness of freshwater crayfish populations under outdoor conditions and in aquaculture. Therefore, the first part of the first question asked in the aims of this study is answered:

1: Reproduction stages of freshwater crayfish are negatively influenced by the chemicals TBA and DCF.

To answer the second part of the question, whether or not reproduction stages of freshwater crayfish are influenced by the mixture of chemicals in sewage treatment plants, we have to take a look at chapter 4, where the effect of an STP outlet on noble crayfish was investigated in a field study.

The four locations used in the field study represent naturally occurring habitats with different chemical loads. They comprise a river section of approximately 20 m around the entry of the STP wastewater.

This enables the study site to serve as a model for differently polluted surface waters and at the same time provide similar environmental parameters to ensure the comparability of the data. Nevertheless, concentrations of pharmaceuticals and agricultural substances are not always limited to small areas and can also affect whole streams and large lake habitats (Kandie et al., 2020; Maasz et al., 2019; Picó et al., 2020; Wang et al., 2020). While the monitored compounds and concentrations differ at each of the four locations, influences of the compositions of residues of the human products for health and agriculture are very likely. This study shows that pollution as strong as in this small area can definitely harm crayfish reproduction and, as a consequence, crayfish population dynamics. In this case, the influences on the whole ecosystem and on present populations of crayfish in this stream are marginal due to the dilution of concentrations after a relatively short distance, as shown in the manuscript data. To transfer the findings of chapter 4 to other surface water areas, we take a closer look at the connections of measured concentrations and affected variables.

The studied parameters (survival of embryos, size of hatched animals and histology of the hepatopancreas of hatched juveniles) are all affected, at least by the chemical load in the direct influence area of the STP. The concentrations of all detected chemicals are lower than highest measured concentrations in surface waters. For example, DCF concentrations of 2,767 ± 0,125 µg/L were measured in the wastewater at location A, but were still much lower than the highest reported concentrations in surface waters of 29 µg/L (Dusi et al., 2019). However, we measured concentrations of Carbamazepin, DCF and Primidone as high or higher than the predicted non-effective concentrations (Vogel, 2011). In addition, the mixture and relatively high concentrations of the above-mentioned three pharmaceuticals or even some not-monitored substances have a relatively high impact on the study animals. The observed decrease of hatched embryos shows that the sensitive reproduction cycle is disturbed by the chemicals. Survival rate of juveniles is one, if not the most important factor for the stabilisation of populations. Therefore, a decrease of juvenile survival of more than 30.85 %, as observed in this study, can lead to a reduction of population size of the endangered species *Astacus astacus* (Edsman et al., 2015).

Not only survival, but also sublethal effects are of importance in this study under outdoor conditions, similar to the laboratory experiments. Effects of decreased size and hepatopancreas damages were discussed before, but in chapter 4 it becomes even more obvious how important the actual control of chemical input in aquatic systems can be. Even the lowest chemical doses influenced organisms that were not in the direct area of the STP output and showed these sublethal effects.

Thus, the second part of the first question is answered as well. It is proven, that DCF, TBA and also the mixture of chemicals in sewage treatment plants negatively influence the reproduction of freshwater crayfish.

Consequently, the second question phrased in the aims of this study has to be answered:

2: Are marbled crayfish suitable as a model organism in toxicological studies?

This question cannot be answered with a clear “yes” or “no”. Harzsch and Viertel (2020) as well as Linzmaier and Jeschke (2019) recently showed that marbled crayfish can serve as model organisms for immunolocalization of neurotransmitters and neuromodulators or to measure the impact of introducing truly new species into an ecosystem. Hossain et al. (2018) also showed in his review that the species is already used in toxicological studies, but that its suitability and sensitivity was not investigated before.

The studies of chapter 2 and 3 indicate that the suitability of marbled crayfish as a model organism in toxicological studies depends on the studied parameters and the used chemicals. When investigating the impact of DCF on embryonic development, marbled crayfish showed similar responses compared to noble crayfish. In contrast, noble crayfish were much more sensitive when exposed to TBA compared to marbled crayfish. For gonadal development, effects were only detectable for noble crayfish, but not for marbled crayfish.

In chapter 2, we were able to show that the effects of the exposure to the chemicals are identical in both species, even if they were connected to different concentrations of the chemicals.

Therefore, we conclude that the usage of marbled crayfish as model organism can only be recommended to transfer detected damages to other species. Not observed effects are not necessarily also missing for other species and effective concentrations might be different for other crayfish species.

For the estimation of NOEC (No Observed Effect Concentration) or PNEC (Predicted No Effect Concentration) or even $LC_{50}$-values, more sensitive organisms than marbled crayfish should be used to assure that limit values are not harmful to other species.

## 5.2 Perspective

While toxicological approaches have certainly helped advance our understanding of the impact of anthropogenic pollution on freshwater crayfish, much remains to be discovered. In a way, the findings of this thesis created the foundation to address new and exciting questions in toxicological and ecological approaches with the aim to protect the crayfish and their ecosystem.

Especially the results described in chapter 4 of this study showed the importance of future studies examining ecotoxicological effects on freshwater systems. The amount of chemicals found in the study site and the poor amount of available data on their toxicological risk on freshwater crayfish underline the importance of further studies in order to understand and evaluate the effects of the chemical load

on freshwater crayfish. In this context, the estimation of mixture effects of substances on aquatic organisms will be of special interest. Many studies and reviews are already addressing the problem and point out the need of future studies in this field. For instance, Overturf et al. (2015) show the increasing relevance of studies concerning the mixture of chemicals originating from sewage treatment plants, similar to this study. Vasquez et al. (2014) demonstrated the importance of chronic exposure of pharmaceuticals in surface waters and the assessment of cocktail effects. Many more studies show how difficult but important the understanding of chemical mixtures and the effects are for ecological health (Ahrens et al., 2016; Altenburger et al., 2018; Orton et al., 2014).

Nilsen et al. (2019) analyse data of contaminants of emerging concern (CECs) from several toxicological studies. The authors formulate the following challenges for future approaches for a better understanding of toxicological effects:

1) more detailed information on the complexity of mixtures of CECs in the aquatic environment,
2) a greater understanding of the sublethal effects of CECs on a wide range of aquatic organisms,
3) an ascertaining of the biological consequences of variable duration CEC exposures within and across generations in aquatic species,
4) a linkage of multiple stressors with CEC exposure in aquatic systems and
5) a documenting of the trophic consequences of CEC exposure across aquatic food webs.

As shown in chapter 4, only the first two challenges may lead to a nearly unlimited number of studies due to the big number of chemicals and organisms that need to be included to gather all the information needed. This challenge was also addressed in all chapters of this thesis. Nevertheless, the possibilities of sublethal effects are vast and many have probably not even been discovered yet. To expand our knowledge, further parameters, for example regarding effects on stress proteins (Dix, 1997), receptor pathways (Baldwin et al., 2003) and behaviour (Little and Finger, 1990), should be included.

In addition, challenges 3-5 posed by Nilsen et al. (2019) can and should also be processed for as many organisms and chemicals as possible. Only by assessing every possible combination of pollutant and effect we can fully understand the consequences resulting from pollutants in aquatic environments. In this present study, we were able to show effects of two single chemicals and the specific mixture of the STP on freshwater crayfish. We can assume that the possibly resulting decreasing population sizes of the “ecosystem engineers” and “key species” noble crayfish (Weinländer and Füreder, 2016) have a major impact on their habitat. However, to fully confirm and understand the meaning of pollutants for these animals many more studies have to be carried out in the future.

## 5.3 References Overall discussion and perspective

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# 6 Appendix

## 6.1 List of Figures

## 6.2 List of Tables

## 6.3 List of Abriviations

ATPS ..... *aqueous two-phase system*
B-cell ..... *Blister-like Secretory Cells*
CEC ..... *Contaminants of Emerging Concern*
DCF ..... *Diclofenac*
$EC_{50}$ ..... *Median Effective Concentration*
E-cell ..... *embryonic cells*
F-cell ..... *fibrillar cells*
HPLC ..... *High Perprmance Liquid Chromatography*
LOEC ..... Lowest Observed Effective Concentration
log KOW ..... *Octanol–Water Partition Coefficient*
MEC ..... *Measured Environmental Concentration*
NOEC ..... *No Observed Effect Concentration*
NSAID ..... *Non-Steroidal Anti Inflammatory Drug*
pH ..... *decimal logarithm of the reciprocal of the hydrogen ion activity, aH+, in a solution*
PNEC ..... *Predicted No Effect Concentration*
R-cell ..... *resorptive lipid cells*
STP ..... *Sewage Treatment Plant*
TBA ..... *Terbuthylazine*
UBA ..... *German Environment Agency*
UV ..... *Ultra Violet*
WFD ..... *Water Framework Directive*

## 6.4 Supplemental Material Chapter 2

*Table 6.1: weights of noble crayfish in g*

| Animal/Conc. | 0 | 0.025 T | 0.4 T | 1.6 T | 6.4 T | 0.04 D | 0.16 D | 2.56 D | 10.24 D |
|---|---|---|---|---|---|---|---|---|---|
| W1 | 38.37 | - | 43.8 | 47.47 | - | 33.08 | 37.81 | - | - |
| W2 | 31.37 | 40.19 | 38.94 | 34.83 | - | 26.13 | 33.32 | 35.78 | 39.1 |
| W3 | 40.96 | 28.07 | 49.61 | 45.8 | 38.45 | 29.75 | 37.1 | - | 42.87 |
| W4 | 47.66 | 45.43 | 42.93 | 36.09 | 44.98 | 34.64 | 28.59 | 23.51 | 31.02 |
| M1 | 48.01 | 27.79 | 36.03 | 23.47 | 38.44 | 37.3 | 32.8 | 27.19 | 48.39 |
| M2 | 34.26 | 34.69 | 38.52 | 29.61 | 29.3 | 35.84 | 37.54 | 27.39 | 36.25 |

*Table 6.2: sizes of noble crayfish in cm*

| Animal/Conc. | 0 | 0.025 T | 0.4 T | 1.6 T | 6.4 T | 0.04 D | 0.16 D | 2.56 D | 10.24 D |
|---|---|---|---|---|---|---|---|---|---|
| W1 | 5.22 | 5.14 | 5.7 | 5.5 | - | 5.2 | 5.31 | - | 5.29 |
| W2 | 4.91 | 5.56 | 5.38 | 5.35 | - | 4.89 | 5.12 | 4.91 | 5.18 |
| W3 | 5.51 | 5.03 | 6.05 | 5.7 | 5.49 | 4.82 | 5.22 | - | 5.52 |
| W4 | 5.8 | 5.8 | 5.49 | 5.49 | 5.7 | 5.21 | 4.85 | 4.51 | 4.89 |
| M1 | 6.1 | 4.9 | 5.25 | 4.36 | 5.21 | 5.21 | 5.2 | 4.72 | 5.23 |
| M2 | 5.14 | 5.18 | 5.19 | 4.96 | 4.59 | 5.39 | 5.2 | 5.08 | 4.9 |

*Table 6.3: Laid eggs of female noble crayfish*

| Animal/Conc. | 0 | 0.025 T | 0.4 T | 1.6 T | 6.4 T | 0.04 D | 0.16 D | 2.56 D | 10.24 D |
|---|---|---|---|---|---|---|---|---|---|
| W1 | 287 | 278 | 136 | 110 | 97 | 4 | 89 | 0 | 0 |
| W2 | 187 | 230 | 198 | 94 | 89 | 159 | 68 | 0 | 0 |
| W3 | 245 | 189 | 187 | 134 | 124 | 210 | 124 | 45 | 0 |
| W4 | 245 | 179 | 198 | 102 | 0 | 232 | 145 | 65 | 0 |

*Table 6.4: Hatched juveniles of noble crayfish*

| Animal/Conc. | 0 | 0.025 T | 0.4 T | 1.6 T | 6.4 T | 0.04 D | 0.16 D | 2.56 D | 10.24 D |
|---|---|---|---|---|---|---|---|---|---|
| W1 | 145 | 152 | 65 | 50 | 23 | 0 | 30 | 0 | 0 |
| W2 | 110 | 123 | 100 | 0 | x | 86 | 0 | 0 | 0 |
| W3 | 165 | 145 | 89 | 0 | x | 93 | 56 | x | x |
| W4 | 0 | x | x | x | x | x | x | x | x |

*Table 6.5: Quantification Spermatozoa TBA*

| Konz | 0.00 | 0.00 | TBA 0.025 | TBA 0.4 | TBA 0.4 | TBA 1.6 | TBA 6.4 |
|---|---|---|---|---|---|---|---|
| Tier | K1 | K2 | T1 | T3 | T4 | T5 | T7 |
| Spermatozoa 1 | 222 | 129 | 30 | 245 | 101 | 127 | 150 |
| | 217 | 145 | 33 | 288 | 132 | 119 | 107 |
| | 172 | 158 | 30 | 259 | 80 | 123 | 135 |
| | 224 | 161 | 21 | 229 | 86 | 125 | 85 |
| Spermatozoa 2 | 254 | 130 | 43 | 219 | 85 | 136 | 133 |
| | 242 | 143 | 45 | 260 | 84 | 102 | 111 |
| | 248 | 158 | 34 | 181 | 98 | 129 | 96 |
| | 240 | 158 | 30 | 277 | 76 | 150 | 122 |
| Spermatozoa 3 | 227 | 188 | 32 | 163 | 77 | 86 | 51 |
| | 190 | 159 | 51 | 144 | 83 | 74 | 82 |
| | 235 | 198 | 52 | 157 | 91 | 83 | 54 |
| | 240 | 230 | 48 | 152 | 78 | 99 | 34 |
| Spermatozoa 4 | 241 | 221 | 46 | 208 | 58 | 173 | 11 |
| | 272 | 171 | 41 | 231 | 62 | 99 | 20 |
| | 217 | 173 | 35 | 247 | 70 | 122 | 23 |
| | 271 | 167 | 41 | 181 | 68 | 128 | 23 |
| Spermatozoa 5 | 188 | 271 | 65 | 256 | 107 | 156 | 135 |
| | 248 | 218 | 85 | 177 | 72 | 144 | 95 |
| | 223 | 260 | 59 | 240 | 96 | 103 | 115 |
| | 199 | 236 | 69 | 208 | 96 | 124 | 145 |
| Spermatozoa 6 | 197 | 178 | 57 | 202 | 77 | 130 | 158 |
| | 200 | 257 | 86 | 194 | 129 | 102 | 133 |
| | 186 | 258 | 66 | 244 | 77 | 98 | 141 |
| | 206 | 166 | 56 | 241 | 96 | 114 | 167 |
| Spermatozoa 7 | 269 | 186 | 38 | 268 | 84 | 111 | 156 |
| | 190 | 150 | 52 | 196 | 102 | 81 | 172 |
| | 218 | 194 | 36 | 267 | 92 | 96 | 133 |
| | 210 | 160 | 47 | 223 | 93 | 81 | 119 |
| Spermatozoa 8 | 229 | 150 | 33 | 254 | 72 | 64 | 159 |
| | 251 | 154 | 54 | 236 | 95 | 69 | 135 |
| | 225 | 155 | 41 | 218 | 81 | 55 | 91 |
| | 203 | 109 | 48 | 276 | 91 | 88 | 122 |

*Table 6.6: Quantification Spermatozoa DCF*

| Konz | D 0.04 | D0.04 | D 0.16 | D 0.16 | D 2.56 | D 10.24 |
|---|---|---|---|---|---|---|
| Tier | D9 | D10 | D11 | D12 | D13 | D14 |
| Spermatozoa 1 | 39 | 37 | 19 | 96 | 76 | 11 |
| | 50 | 30 | 27 | 95 | 60 | 19 |
| | 45 | 45 | 15 | 104 | 61 | 18 |
| | 53 | 43 | 14 | 133 | 51 | 13 |
| Spermatozoa 2 | 61 | 26 | 19 | 86 | 56 | 17 |
| | 62 | 50 | 33 | 124 | 40 | 18 |
| | 51 | 30 | 29 | 89 | 45 | 22 |
| | 55 | 31 | 20 | 134 | 46 | 16 |
| Spermatozoa 3 | 93 | 16 | 22 | 48 | 26 | 7 |
| | 80 | 41 | 23 | 95 | 39 | 14 |
| | 119 | 29 | 29 | 89 | 40 | 10 |
| | 98 | 35 | 20 | 71 | 29 | 21 |
| Spermatozoa 4 | 158 | 78 | 9 | 106 | 47 | 16 |
| | 151 | 64 | 14 | 136 | 49 | 14 |
| | 147 | 71 | 24 | 124 | 51 | 24 |
| | 161 | 70 | 14 | 130 | 64 | 11 |
| Spermatozoa 5 | 87 | 45 | 10 | 61 | 31 | 10 |
| | 101 | 53 | 23 | 92 | 27 | 12 |
| | 123 | 34 | 16 | 70 | 36 | 12 |
| | 126 | 19 | 16 | 64 | 33 | 13 |
| Spermatozoa 6 | 232 | 25 | 13 | 79 | 33 | 8 |
| | 233 | 32 | 17 | 77 | 34 | 16 |
| | 177 | 30 | 20 | 69 | 23 | 10 |
| | 201 | 26 | 15 | 80 | 27 | 14 |
| Spermatozoa 7 | 42 | 38 | 27 | 92 | 12 | 11 |
| | 36 | 47 | 16 | 73 | 13 | 19 |
| | 61 | 44 | 13 | 104 | 15 | 5 |
| | 59 | 36 | 19 | 78 | 19 | 13 |
| Spermatozoa 8 | 69 | 24 | 13 | 69 | 59 | 6 |
| | 73 | 17 | 16 | 85 | 79 | 13 |
| | 50 | 30 | 13 | 84 | 74 | 7 |
| | 60 | 31 | 18 | 68 | 51 | 15 |

*Table 6.7: Percentage of living Sperm Cells*

| Sample/Conc. | 0 | 0.025 T | 0.4 T | 1.6 T | 6.4 T | 0.04 D | 0.16 D | 2.56 D | 10.24 D |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 54.55 | 100.00 | 46.67 | 50.00 | 50.00 | 0.00 | 16.67 | 50.00 | 14.29 |
| 2 | 41.67 | 33.33 | 56.52 | 50.00 | 46.67 | 0.00 | 200.00 | 71.43 | 0.00 |
| 3 | 58.33 | 100.00 | 54.17 | 50.00 | 23.53 | 50.00 | 25.00 | 60.00 | 10.00 |
| 4 | 60.00 | 60.00 | 47.62 | 20.00 | 28.57 | 50.00 | 27.27 | 62.50 | 25.00 |
| 5 | 50.00 | 50.00 | 57.89 | 16.67 | 9.09 | 28.57 | 7.14 | 80.00 | 30.00 |
| 6 | 40.00 | 71.43 | 44.44 | 0.00 | 50.00 | 58.62 | 7.69 | 71.43 | 28.57 |
| 7 | 60.00 | 100.00 | 50.00 | 25.00 | 30.77 | 28.57 | 0.00 | 52.00 | 0.00 |
| 8 | 70.00 | 100.00 | 46.67 | 25.00 | 11.11 | 44.44 | 33.33 | 33.33 | 0.00 |
| 9 | 28.57 | | 50.00 | | | 0.00 | 81.82 | | |
| 10 | 40.00 | | 75.00 | | | 62.50 | 66.67 | | |
| 11 | 50.00 | | 66.67 | | | 57.14 | 50.00 | | |
| 12 | 100.00 | | 44.44 | | | 100.00 | 33.33 | | |
| 13 | 75.00 | | 100.00 | | | 75.00 | 60.00 | | |
| 14 | 50.00 | | 66.67 | | | 75.00 | 33.33 | | |
| 15 | 85.71 | | 50.00 | | | 57.14 | 60.00 | | |
| 16 | 66.67 | | 66.67 | | | 80.00 | 60.00 | | |
| Average | 58.16 | 76.85 | 57.71 | 29.58 | 31.22 | 47.94 | 47.64 | 60.09 | 13.48 |

*Table 6.8: B-Cell sizes*

| | 0 | TBA 0.025 | TBA 0.4 | TBA 1.6 | TBA 6.4 | D 0.04 | D 0.16 | D 2.56 | D 10.24 |
|---|---|---|---|---|---|---|---|---|---|
| Average | 26.86 | 39.60 | 53.98 | 68.15 | 75.58 | 41.53 | 44.10 | 50.20 | 73.90 |
| SD | 9.90 | 11.51 | 14.57 | 16.44 | 23.53 | 11.32 | 11.98 | 10.25 | 18.21 |

## 6.5 Supplemental Material Chapter 3

### 6.5.1 Noble crayfish are more sensitive to Terbuthylazine than parthenogenetic marbled crayfish

*Table 6.9: Number of B-Cells/Hepathopancreas Cell regarding to concentration for noble crayfish juveniles exposed to TBA*

| Sample/Conc. | Control | 0.025 | 0.1 | 0.4 | 1.6 | 6.4 | 12.8 |
|---|---|---|---|---|---|---|---|
| 1 | 25 | 80 | 75 | 67 | 80 | 66 | 47 |
| 2 | 24 | 62 | 72 | 76 | 79 | 66 | 29 |
| 3 | 26 | 73 | 62 | 62 | 70 | 67 | 34 |
| 4 | 28 | 77 | 69 | 60 | 78 | 64 | 45 |
| 5 | 29 | 70 | 70 | 66 | 74 | 80 | 50 |
| 6 | 24 | 73 | 60 | 77 | 68 | 75 | 60 |
| 7 | 23 | 76 | 77 | 68 | 76 | 80 | 46 |
| 8 | 25 | 74 | 61 | 74 | 76 | 77 | 44 |
| 9 | 21 | 64 | 79 | 79 | 68 | 74 | 51 |
| 10 | 25 | 72 | 71 | 65 | 77 | 66 | 58 |
| 11 | 26 | 64 | 78 | 67 | 68 | 61 | 40 |
| 12 | 28 | 77 | 77 | 63 | 72 | 68 | 44 |
| 13 | 27 | 61 | 75 | 64 | 74 | 62 | 39 |
| 14 | 22 | 79 | 78 | 75 | 72 | 71 | 30 |
| 15 | 23 | 62 | 65 | 77 | 63 | 63 | 27 |
| 16 | 25 | 77 | 65 | 69 | 76 | 72 | 41 |
| 17 | 26 | 61 | 73 | 67 | 72 | 67 | 58 |
| 18 | 22 | 63 | 72 | 74 | 64 | 61 | 51 |
| 19 | 21 | 65 | 70 | 80 | 65 | 66 | 29 |
| 20 | 24 | 74 | 68 | 69 | 78 | 80 | 43 |
| 21 | 25 | 77 | 74 | 67 | 77 | 75 | 40 |
| 22 | 27 | 60 | 69 | 73 | 76 | 68 | 26 |
| 23 | 22 | 65 | 60 | 78 | 75 | 61 | 32 |
| 24 | 23 | 73 | 61 | 71 | 63 | 75 | 42 |
| 25 | 25 | 63 | 64 | 76 | 76 | 61 | 28 |
| 26 | 29 | 79 | 75 | 67 | 70 | 71 | 43 |
| 27 | 28 | 64 | 71 | 70 | 60 | 61 | 43 |
| 28 | 22 | 71 | 68 | 65 | 60 | 64 | 51 |
| 29 | 22 | 71 | 73 | 69 | 79 | 72 | 56 |
| 30 | 21 | 69 | 64 | 63 | 61 | 75 | 29 |
| Average | 24.60 | 69.87 | 69.87 | 69.93 | 71.57 | 68.97 | 41.87 |

### 6.5.2 Effects of Diclofenac on the embryonic development of freshwater crayfish

*Table 6.10: Number of B-Cells/Hepathopancreas Cell regarding to concentration for noble crayfish juveniles exposed to Diclofenac*

| Sample/Conc. | Control | 0.01 | 0.04 | 0.16 | 0.64 | 2.56 | 10.24 | 40.96 |
|---|---|---|---|---|---|---|---|---|
| 1 | 25 | 24 | 26 | 30 | 25 | 34 | 32 | 47 |
| 2 | 24 | 24 | 31 | 25 | 28 | 37 | 47 | 29 |
| 3 | 26 | 27 | 25 | 27 | 29 | 33 | 32 | 34 |
| 4 | 28 | 28 | 31 | 27 | 28 | 38 | 37 | 45 |
| 5 | 29 | 29 | 28 | 26 | 29 | 32 | 38 | 50 |
| 6 | 24 | 27 | 30 | 28 | 31 | 34 | 36 | 60 |
| 7 | 23 | 25 | 31 | 29 | 31 | 36 | 41 | 46 |
| 8 | 25 | 25 | 30 | 29 | 26 | 35 | 44 | 44 |
| 9 | 21 | 26 | 30 | 29 | 28 | 37 | 34 | 51 |
| 10 | 25 | 24 | 27 | 25 | 27 | 30 | 48 | 58 |
| 11 | 26 | 26 | 29 | 28 | 27 | 29 | 38 | 40 |
| 12 | 28 | 26 | 26 | 31 | 26 | 29 | 45 | 44 |
| 13 | 27 | 30 | 28 | 26 | 24 | 39 | 42 | 39 |
| 14 | 22 | 26 | 24 | 30 | 28 | 36 | 47 | 30 |
| 15 | 23 | 27 | 29 | 31 | 26 | 35 | 38 | 27 |
| 16 | 25 | 27 | 26 | 24 | 30 | 32 | 38 | 41 |
| 17 | 26 | 25 | 30 | 31 | 27 | 29 | 34 | 58 |
| 18 | 22 | 28 | 31 | 27 | 27 | 26 | 45 | 51 |
| 19 | 21 | 24 | 26 | 30 | 29 | 37 | 37 | 29 |
| 20 | 24 | 29 | 29 | 26 | 26 | 29 | 37 | 43 |
| 21 | 25 | 28 | 25 | 29 | 31 | 29 | 43 | 40 |
| 22 | 27 | 29 | 25 | 31 | 26 | 38 | 33 | 26 |
| 23 | 22 | 26 | 29 | 31 | 27 | 31 | 34 | 32 |
| 24 | 23 | 27 | 30 | 30 | 31 | 28 | 48 | 42 |
| 25 | 25 | 30 | 31 | 31 | 24 | 33 | 34 | 28 |
| 26 | 29 | 24 | 27 | 27 | 31 | 30 | 34 | 43 |
| 27 | 28 | 27 | 25 | 28 | 28 | 29 | 39 | 43 |
| 28 | 22 | 30 | 30 | 25 | 31 | 33 | 38 | 51 |
| 29 | 22 | 29 | 27 | 25 | 31 | 27 | 43 | 56 |
| 30 | 21 | 26 | 31 | 27 | 27 | 29 | 42 | 29 |
| Average | 24.60 | 26.77 | 28.23 | 28.10 | 27.97 | 32.47 | 39.27 | 41.87 |

*Table 6.11: Weights of hatched noble crayfish exposed to different Concentrations of TBA*

| Animal/Conc. | 40.96 | 10.24 | 2.56 | 0.64 | 0.16 | 0.04 | 0.01 | LSM | 0 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.03 | 0.02 | 0.03 | 0.03 | 0.02 | 0.03 | 0.02 | 0.02 | 0.02 |
| 2 | 0.02 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.02 | 0.03 | 0.02 |
| 3 | 0.02 | 0.02 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| 4 | 0.02 | 0.02 | 0.02 | 0.03 | 0.03 | 0.02 | 0.03 | 0.03 | 0.02 |
| 5 | 0.03 | 0.02 | 0.02 | 0.02 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 |
| 6 | 0.02 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.02 |
| 7 | 0.02 |  | 0.02 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.03 |
| 8 | 0.02 |  |  |  |  | 0.03 | 0.03 | 0.02 | 0.03 |
| 9 |  |  |  |  |  | 0.02 | 0.03 | 0.02 | 0.03 |
| 10 |  |  |  |  |  | 0.03 | 0.03 | 0.03 | 0.02 |
| 11 |  |  |  |  |  | 0.03 | 0.03 | 0.03 |  |
| 12 |  |  |  |  |  | 0.03 | 0.03 |  |  |
| 13 |  |  |  |  |  | 0.03 |  |  |  |
| Average | 0.02 | 0.03 | 0.03 | 0.03 | 0.02 | 0.03 | 0.03 | 0.03 | 0.02 |

*Table 6.12: Weights of hatched marbled crayfish exposed to different Concentrations of TBA*

| Animal/Conc. | 2.56 | 0.64 | 0.16 | 0.04 | 0.01 | 0 |
|---|---|---|---|---|---|---|
| 1 | 0.0038 | 0.0046 | 0.0044 | 0.003 | 0.0037 | 0.0043 |
| 2 | 0.004 |  | 0.0038 | 0.0038 |  | 0.004 |
| 3 | 0.0039 |  | 0.0033 |  |  | 0.0047 |
| 4 | 0.0052 |  |  |  |  | 0.0039 |
| 5 |  |  |  |  |  | 0.0038 |
| 6 |  |  |  |  |  | 0.005 |
| 7 |  |  |  |  |  | 0.0038 |
| Average | 0.0042 | 0.0046 | 0.0038 | 0.0034 | 0.0037 | 0.0042 |

## 6.6 Supplemental Material Chapter 4

### 6.6.1 Agrolab study site Data

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Am Botanischen Garten 1-9
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Datum 07.04.2020
Kundennr. 20066508

## PRÜFBERICHT 2020177 - 789215

| | |
|---|---|
| Auftrag | 2020177 Oberflächenwasseruntersuchung, Projekt Edelkrebs |
| Analysennr. | 789215 Oberflächenwasser |
| Probeneingang | 31.03.2020 |
| Probenahme | 31.03.2020 12:50 |
| Kunden-Probenbezeichnung | KA-A |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,83 | 2 | | | DIN EN ISO 10523 : 2012-04 |
| Wassertemperatur (vor Ort) | °C | 10,8 | 0 | | | DIN 38404-4 : 1976-12 |
| Leitfähigkeit (vor Ort) bei 25°C | µS/cm | 1080 | 10 | | | DIN EN 27888 : 1993-11 |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 59 | | | | Berechnung |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[m1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | <0,030 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | 0,14 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | 2,7 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | 2,6 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <30 (+) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <30 (+) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,30 (+)[m1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | 0,166 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | 1,87 | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | 0,23 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | 60 | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | 0,23 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | 0,78 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,50[m1] | 0,5 | | | DIN 38407-47 : 2017-07(BS) |
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | 1,1 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |

Seite 1 von 2

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Dr. Paul Winnefeld
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Datum 07.04.2020
Kundennr. 20086508

**PRÜFBERICHT 2020177 - 789215**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 4-Acetamidoantipyrin | µg/l | 1,7 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Aminoantipyrin | µg/l | 2,0 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Dimethylaminoantipyrin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Formylaminoantipyrin | µg/l | 10 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,49 | 0,03 | | | DIN ISO 16308 : 2017-09(B8) u) |
| Glyphosat | µg/l | 0,061 | 0,03 | | | DIN ISO 16308 : 2017-09(B8) u) |
| Terbuthylazin | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-36 : 2014-09(B8) u) |

*nn) Die Bestimmungs-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

*u) Vergabe an ein akkreditiertes Agrolab-Gruppen-Labor*

**Agrolab-Gruppen-Labore**
**Untersuchung durch**
(B8) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee
Methoden
DIN 38407-47 : 2017-07

(B8) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 31.03.2020*
*Ende der Prüfungen: 07.04.2020*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich gemäß Auftragsbestätigung getroffenen Vereinbarung in vereinfachter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

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Datum 07.04.2020
Kundennr. 20066508

# PRÜFBERICHT 2020177 - 789216

| | |
|---|---|
| Auftrag | **2020177 Oberflächenwasseruntersuchung, Projekt Edelkrebs** |
| Analysennr. | **789216 Oberflächenwasser** |
| Probeneingang | **31.03.2020** |
| Probenahme | **31.03.2020** |
| Kunden-Probenbezeichnung | **KA-C** |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,93 | 2 | | | DIN EN ISO 10523 : 2012-04 |
| Wassertemperatur (vor Ort) | °C | 6,5 | 0 | | | DIN 38404-4 : 1976-12 |
| Leitfähigkeit (vor Ort) bei 25 °C | µS/cm | 524 | 10 | | | DIN EN 27888 : 1993-11 |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 65 | | | | Berechnung |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[#1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | <0,010 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,10 (NWG)[#1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | 0,09 | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,10 | 0,1 | | | DIN 38407-47 : 2017-07(BS) |
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |

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**PRÜFBERICHT 2020177 - 789216**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 4-Acetamidoantipyrin | µg/l | 0,07 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Aminoantipyrin | µg/l | 0,04 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Dimethylaminoantipyrin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Formylaminoantipyrin | µg/l | 0,04 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,03 | 0,03 | | | DIN ISO 16308 : 2017-09(B8) u) |
| Glyphosat | µg/l | <0,010 (NWG) | 0,03 | | | DIN ISO 16308 : 2017-09(B8) u) |
| Terbuthylazin | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-36 : 2014-09(B8) u) |

*nw) Die Bestimmungs-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

*u) Vergabe an ein akkreditiertes Agrolab-Gruppen-Labor.*

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Methoden
DIN 38407-47 : 2017-07

(B8) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 31.03.2020*
*Ende der Prüfungen: 07.04.2020*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich gemäß Auftragsbestätigung getroffenen Vereinbarung in vereinfachter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

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24118 Kiel

Datum 07.04.2020
Kundennr. 20066508

# PRÜFBERICHT 2020177 - 789217

| | |
|---|---|
| Auftrag | **2020177 Oberflächenwasseruntersuchung, Projekt Edelkrebs** |
| Analysennr. | **789217 Oberflächenwasser** |
| Probeneingang | **31.03.2020** |
| Probenahme | **31.03.2020** |
| Kunden-Probenbezeichnung | **KA-D** |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,96 | 2 | | | DIN EN ISO 10523 : 2012-04 |
| Wassertemperatur (vor Ort) | °C | 6,6 | 0 | | | DIN 38404-4 : 1976-12 |
| Leitfähigkeit (vor Ort) bei 25 °C | µS/cm | 524 | 10 | | | DIN EN 27888 : 1993-11 |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 88 | | | | Berechnung |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[m1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | <0,010 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,10 (NWG)[m1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | 0,09 | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,10 | 0,1 | | | DIN 38407-47 : 2017-07(BS) |
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |

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**PRÜFBERICHT 2020177 - 789217**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 4-Acetamidoantipyrin | µg/l | 0,07 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Aminoantipyrin | µg/l | <0,02 (NWG) m) | 0,06 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Dimethylaminoantipyrin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Formylaminoantipyrin | µg/l | 0,05 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,03 | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Glyphosat | µg/l | <0,030 (+) | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Terbuthylazin | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-36 : 2014-09(BB) u) |

*m) Die Nachweis-, bzw. Bestimmungsgrenze musste erhöht werden, da Matrixeffekte bzw. Substanzüberlagerungen eine Quantifizierung erschweren.*
*mv) Die Bestimmung-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

*u) Vergabe an ein akkreditiertes Agrolab-Gruppen-Labor*

**Agrolab-Gruppen-Labore**
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(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee
Methoden
DIN 38407-47 : 2017-07

(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 31.03.2020*
*Ende der Prüfungen: 07.04.2020*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich getroffenen Vereinbarung in vereinfachter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

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24118 Kiel

Datum 11.05.2020
Kundennr. 20066508

# PRÜFBERICHT 2026685 - 804157

| | |
|---|---|
| Auftrag | **2026685 Oberflächenwasseruntersuchung, Projekt Edelkrebs** |
| Analysennr. | **804157 Oberflächenwasser** |
| Probeneingang | **27.04.2020** |
| Probenahme | **27.04.2020 13:50** |
| Probenehmer | **Jan Lorenzen** |
| Kunden-Probenbezeichnung | **KA-A** |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,83 | 2 | | | DIN EN ISO 10523 : 2012-04 |
| Wassertemperatur (vor Ort) | °C | 14,7 | 0 | | | DIN 38404-4 : 1976-12 |
| Leitfähigkeit (vor Ort) bei 25°C | µS/cm | 1110 | 10 | | | DIN EN 27888 : 1993-11 |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 59 | | | | Berechnung |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[#1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | 0,041 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | 0,17 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | 2,5 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | 2,9 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,30 (+)[#1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | 0,167 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | 1,83 | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | 0,23 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | 43 | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | 0,27 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | 0,90 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,20 [#1] | 0,2 | | | DIN 38407-47 : 2017-07(BS) |

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**PRÜFBERICHT 2026685 - 804157**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | 1,3 | 0,03 | | | DIN 38407-47 : 2017-07(BB) |
| 4-Acetamidoantipyrin | µg/l | 1,8 | 0,03 | | | DIN 38407-47 : 2017-07(BB) |
| 4-Aminoantipyrin | µg/l | 0,89 | 0,03 | | | DIN 38407-47 : 2017-07(BB) |
| 4-Dimethylaminoantipyrin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BB) |
| 4-Formylaminoantipyrin | µg/l | 12 | 0,03 | | | DIN 38407-47 : 2017-07(BB) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,74 | 0,03 | | | DIN ISO 16308 : 2017-09(BB) |
| Glyphosat | µg/l | 0,16 | 0,03 | | | DIN ISO 16308 : 2017-09(BB) |
| Terbuthylazin | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-36 : 2014-09(BB) |

*m) Die Nachweis-, bzw. Bestimmungsgrenze musste erhöht werden, da Matrixeffekte bzw. Substanzüberlagerungen eine Quantifizierung erschweren.*
*mv) Die Bestimmungs-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

*u) Vergabe an ein akkreditiertes Agrolab-Gruppen-Labor*

**Agrolab-Gruppen-Labors**
**Untersuchung durch**
(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee
Methoden
DIN 38407-47 : 2017-07

(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 27.04.2020*
*Ende der Prüfungen: 11.05.2020*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich gemäß Auftragsbestätigung getroffenen Vereinbarung in vereinfachter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

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Datum 11.05.2020
Kundennr. 20066508

# PRÜFBERICHT 2026685 - 804158

| | |
|---|---|
| Auftrag | **2026685 Oberflächenwasseruntersuchung, Projekt Edelkrebs** |
| Analysennr. | **804158 Oberflächenwasser** |
| Probeneingang | **27.04.2020** |
| Probenahme | **27.04.2020 14:20** |
| Probenehmer | **Jan Lorenzen** |
| Kunden-Probenbezeichnung | **KA-D** |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,97 | 2 | | | DIN EN ISO 10523 : 2012-04 |
| Wassertemperatur (vor Ort) | °C | 11,4 | 0 | | | DIN 38404-4 : 1976-12 |
| Leitfähigkeit (vor Ort) bei 25°C | µS/cm | 577 | 10 | | | DIN EN 27888 : 1993-11 |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 65 | | | | Berechnung |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[#1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | <0,010 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,10 (NWG)[#1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | <0,05 (+) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,10 | 0,1 | | | DIN 38407-47 : 2017-07(BS) |

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**PRÜFBERICHT 2026685 - 804158**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Acetamidoantipyrin | µg/l | 0,05 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Aminoantipyrin | µg/l | 0,04 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Dimethylaminoantipyrin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Formylaminoantipyrin | µg/l | 0,09 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,03 | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Glyphosat | µg/l | <0,010 (NWG) | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Terbuthylazin | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-36 : 2014-09(BB) u) |

*nn) Die Bestimmung-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<....(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<....(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

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Methoden
DIN 38407-47 : 2017-07

(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 27.04.2020*
*Ende der Prüfungen: 11.05.2020*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich gemäß Auftragsbestätigung getroffenen Vereinbarung in vereinfachter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

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Am Botanischen Garten 1-9
24118 Kiel

Datum 11.05.2020
Kundennr. 20066508

# PRÜFBERICHT 2026685 - 804159

| | |
|---|---|
| Auftrag | **2026685 Oberflächenwasseruntersuchung, Projekt Edelkrebs** |
| Analysennr. | **804159 Oberflächenwasser** |
| Probeneingang | **27.04.2020** |
| Probenahme | **27.04.2020 14:15** |
| Probenehmer | **Jan Lorenzen** |
| Kunden-Probenbezeichnung | **KA-B** |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,93 | 2 | | | DIN EN ISO 10523 : 2012-04 |
| Wassertemperatur (vor Ort) | °C | 11,2 | 0 | | | DIN 38404-4 : 1976-12 |
| Leitfähigkeit (vor Ort) bei 25°C | µS/cm | 578 | 10 | | | DIN EN 27888 : 1993-11 |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 88 | | | | Berechnung |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[#1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | <0,010 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,10 (NWG)[#1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | <0,05 (+) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,10 | 0,1 | | | DIN 38407-47 : 2017-07(BS) |

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**PRÜFBERICHT 2026685 - 804159**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Acetamidoantipyrin | µg/l | 0,04 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Aminoantipyrin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Dimethylaminoantipyrin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Formylaminoantipyrin | µg/l | 0,12 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,04 | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Glyphosat | µg/l | <0,010 (NWG) | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Terbuthylazin | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-36 : 2014-09(BB) u) |

*nn) Die Bestimmung-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

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Methoden
DIN 38407-47 : 2017-07

(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 27.04.2020*
*Ende der Prüfungen: 11.05.2020*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich gemäß Auftragsbestätigung getroffenen Vereinbarung in vereinbarter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

**AGROLAB Agrar&Umwelt Frau Melina Göllner, Tel. 0431/22138-546**
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24118 Kiel

Datum 25.08.2020
Kundennr. 20066508

# PRÜFBERICHT 2030849 - 836570

| | |
|---|---|
| Auftrag | 2030849 Oberflächenwasseruntersuchung, Projekt Edelkrebs |
| Analysennr. | 836570 Oberflächenwasser |
| Probeneingang | 12.06.2020 |
| Probenahme | 12.06.2020 10:00 |
| Kunden-Probenbezeichnung | KA-A |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,95 | 2 | | | Kundeninformation |
| Wassertemperatur (vor Ort) | °C | 17,4 | 0 | | | Kundeninformation |
| Leitfähigkeit (vor Ort) bei 25 °C | µS/cm | 1090 | 10 | | | Kundeninformation |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 59 | | | | Kundeninformation |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[m1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | <0,010 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | 0,04 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | 3,1 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | 2,8 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,10 (NWG)[m1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <30 (+) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,05 (+) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | 0,177 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | 0,63 | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | 0,08 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,10 | 0,1 | | | DIN 38407-47 : 2017-07(BS) |
| 10H-phenyl-10,11-dihydrocarbamazepin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |

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**PRÜFBERICHT 2030849 - 836570**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 4-Acetamidoantipyrin | µg/l | 1,0 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Aminoantipyrin | µg/l | 1,3 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Dimethylaminoantipyrin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| 4-Formylaminoantipyrin | µg/l | 10 | 0,03 | | | DIN 38407-47 : 2017-07(B8) u) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 1,5 | 0,03 | | | DIN ISO 16308 : 2017-09(B8) u) |
| Glyphosat | µg/l | 5,8 | 0,03 | | | DIN ISO 16308 : 2017-09(B8) u) |
| Terbuthylazin | µg/l | <0,030 (+) | 0,03 | | | DIN 38407-36 : 2014-09(B8) u) |

*nn) Die Bestimmungs-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(MW/G)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

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**Untersuchung durch**
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Methoden
DIN 38407-47 : 2017-07

(B8) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 12.06.2020*
*Ende der Prüfungen: 25.06.2020*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich gemäß Auftragsbestätigung getroffenen Vereinbarung in vereinfachter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

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Limnologie, Kai Lehmann
Am Botanischen Garten 1-9
24118 Kiel

Datum 25.06.2020
Kundennr. 20066508

# PRÜFBERICHT 2030849 - 836571

| | |
|---|---|
| Auftrag | **2030849 Oberflächenwasseruntersuchung, Projekt Edelkrebs** |
| Analysennr. | **836571 Oberflächenwasser** |
| Probeneingang | **12.06.2020** |
| Probenahme | **12.06.2020** |
| Kunden-Probenbezeichnung | **KA-D** |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 65 | | | | Kundeninformation |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[m1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(B5) |
| Acetylsulfamethoxazol | µg/l | <0,010 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Bezafibrat | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Carbamazepin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(B5) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Diclofenac | µg/l | 0,04 | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(B5) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(B5) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(B5) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(B5) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Ibuprofen | µg/l | <0,10 (NWG)[m1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(B5) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(B5) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(B5) |
| Lidocain | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Metformin | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(B5) |
| Naproxen | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Paracetamol | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(B5) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(B5) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Phenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Primidon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| Tris-2-Chlorethylphosphat | µg/l | <0,10 | 0,1 | | | DIN 38407-47 : 2017-07(B5) |
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | 0,03 | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| 4-Acetamidoantipyrin | µg/l | 0,09 | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| 4-Aminoantipyrin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| 4-Dimethylaminoantipyrin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(B5) |
| 4-Formylaminoantipyrin | µg/l | 0,35 | 0,03 | | | DIN 38407-47 : 2017-07(B5) |

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**PRÜFBERICHT 2030849 - 836571**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,07 | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u |
| Glyphosat | µg/l | <0,030 (+) | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u |
| Terbuthylazin | µg/l | <0,030 (+) | 0,03 | | | DIN 38407-36 : 2014-09(BB) u |

*mn) Die Bestimmung-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

*u) Vergabe an ein akkreditiertes Agrolab-Gruppen-Labor*

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**Untersuchung durch**
(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee
Methoden
DIN 38407-47 : 2017-07

(BB) AGROLAB Standort Eching / Ammersee, Moosstrasse 6 a, 82279 Eching / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 12.06.2020*
*Ende der Prüfungen: 25.06.2020 (Verlängerung wg. Nacherfassung und/oder Plausibilitätsprüfung)*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich getroffenen Vereinbarung in vereinfachter Weise (z.B. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 beachtet.*

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24118 Kiel

Datum 25.08.2020
Kundennr. 20066508

# PRÜFBERICHT 2030849 - 836572

| | |
|---|---|
| Auftrag | 2030849 Oberflächenwasseruntersuchung, Projekt Edelkrebs |
| Analysennr. | 836572 Oberflächenwasser |
| Probeneingang | 12.06.2020 |
| Probenahme | 12.06.2020 10:10 |
| Kunden-Probenbezeichnung | KA-B |

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| **Physikalisch-chemische Parameter** | | | | | | |
| pH-Wert (vor Ort) | | 7,81 | 2 | | | Kundeninformation |
| Wassertemperatur (vor Ort) | °C | 14,0 | 0 | | | Kundeninformation |
| Leitfähigkeit (vor Ort) bei 25°C | µS/cm | 617 | 10 | | | Kundeninformation |
| **Gasförmige Komponenten** | | | | | | |
| Sauerstoffsättigungsindex (vor Ort) | % | 88 | | | | Kundeninformation |
| **Arzneimittelrückstände - Analgetika, Lipidsenker, u.a.** | | | | | | |
| Acetylsalicylsäure (ASS) | ng/l | <100 (NWG)[#1] | 300 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Acetylsulfamethoxazol | µg/l | <0,010 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Bezafibrat | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Carbamazepin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Clofibrinsäure | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Crotamiton | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diazepam | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Diclofenac | µg/l | 0,04 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Etofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrat | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenofibrinsäure | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Fenoprofen | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Gemfibrozil | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Heptabarbital | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Ibuprofen | µg/l | <0,10 (NWG)[#1] | 0,3 | | | DIN 38407-47 : 2017-07 (mod.)(BS) |
| Indometacin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Ketoprofen | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Lidocain | µg/l | <0,015 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Metformin | µg/l | <0,03 (NWG) | 0,05 | | | DIN 38407-47 : 2017-07(BS) |
| Naproxen | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Norethindron | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Paracetamol | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Pentoxifyllin | ng/l | <10 (NWG) | 30 | | | DIN 38407-47 : 2017-07(BS) |
| Phenacetin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Phenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Primidon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Propyphenazon | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BS) |
| Tris-2-Chlorethylphosphat | µg/l | <0,10 | 0,1 | | | DIN 38407-47 : 2017-07(BS) |
| 10-Hydroxy-10,11-dihydrocarbamazepin | µg/l | 0,03 | 0,03 | | | DIN 38407-47 : 2017-07(BS) |

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**PRÜFBERICHT 2030849 - 836572**

| | Einheit | Ergebnis | Best.-Gr. | Grenzwert | Bewertung | Methode |
|---|---|---|---|---|---|---|
| 4-Acetamidoantipyrin | µg/l | 0,08 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Aminoantipyrin | µg/l | <0,03 (+) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Dimethylaminoantipyrin | µg/l | <0,01 (NWG) | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| 4-Formylaminoantipyrin | µg/l | 0,34 | 0,03 | | | DIN 38407-47 : 2017-07(BB) u) |
| **Pflanzenschutzmittel und Biozidprodukte (PSM)** | | | | | | |
| AMPA | µg/l | 0,08 | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Glyphosat | µg/l | <0,030 (+) | 0,03 | | | DIN ISO 16308 : 2017-09(BB) u) |
| Terbuthylazin | µg/l | <0,030 (+) | 0,03 | | | DIN 38407-36 : 2014-09(BB) u) |

*nw) Die Bestimmungs-, bzw. Nachweisgrenze musste erhöht werden, da zur Analyse das zu vermessende Material aufgrund seiner Probenbeschaffenheit verdünnt werden musste.*
*Erläuterung: Das Zeichen "<" oder n.b. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Bestimmungsgrenze nicht quantifizierbar.*
*Das Zeichen "<...(NWG)" oder n.n. in der Spalte Ergebnis bedeutet, der betreffende Stoff ist bei nebenstehender Nachweisgrenze nicht nachzuweisen.*
*Das Zeichen "<...(+)" in der Spalte Ergebnis bedeutet, der betreffende Stoff wurde im Bereich zwischen Nachweisgrenze und Bestimmungsgrenze qualitativ nachgewiesen.*
*Die parameterspezifischen Messunsicherheiten sowie Informationen zum Berechnungsverfahren sind auf Anfrage verfügbar, sofern die berichteten Ergebnisse oberhalb der parameterspezifischen Bestimmungsgrenze liegen.*

*u) Vergabe an ein akkreditiertes Agrolab-Gruppen-Labor*

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Methoden
DIN 38407-47 : 2017-07

(BB) AGROLAB Standort Etting / Ammersee, Moosstrasse 6 a, 82279 Etting / Ammersee, für die zitierte Methode akkreditiert nach ISO/IEC 17025:2005, Akkreditierungsurkunde: D-PL-14289_01_00
Methoden
DIN ISO 16308 : 2017-09; DIN 38407-36 : 2014-09; DIN 38407-47 : 2017-07; DIN 38407-47 : 2017-07 (mod.)

*Beginn der Prüfungen: 12.06.2020*
*Ende der Prüfungen: 25.06.2020 (Verlängerung ag. Nacherfassung und/oder Plausibilitätsprüfung)*

*Die Ergebnisse beziehen sich ausschließlich auf die geprüften Gegenstände. In Fällen, wo das Prüflabor nicht für die Probenahme verantwortlich war, gelten die berichteten Ergebnisse für die Proben wie erhalten. Die auszugsweise Vervielfältigung des Berichts ohne unsere schriftliche Genehmigung ist nicht zulässig. Die Ergebnisse in diesem Prüfbericht werden gemäß der mit Ihnen schriftlich getroffenen Vereinbarung in vereinfachter Weise i.S. der DIN EN ISO/IEC 17025:2018, Abs. 7.8.1.3 berichtet.*

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Ust./VAT-ID-Nr.
DE 188 666 523

Geschäftsführer
Dr. Paul Wimmer
Dr. Jens Radtke
Dr. Carlo C. Pelot

*Table 6.13: Number of B-Cells/Hepathopancreas Cell of noble crayfish juveniles exposed to different areas near a STP influence*

| Sample/Group | A | B | C | D |
|---|---|---|---|---|
| 1 | 26 | 40 | 40 | 48 |
| 2 | 17 | 43 | 47 | 38 |
| 3 | 18 | 54 | 53 | 49 |
| 4 | 24 | 54 | 45 | 42 |
| 5 | 17 | 51 | 43 | 41 |
| 6 | 25 | 52 | 37 | 46 |
| 7 | 18 | 44 | 40 | 35 |
| 8 | 17 | 47 | 41 | 41 |
| 9 | 23 | 49 | 42 | 38 |
| 10 | 20 | 51 | 44 | 52 |
| 11 | 19 | 40 | 54 | 45 |
| 12 | 17 | 48 | 48 | 43 |
| 13 | 24 | 44 | 47 | 50 |
| 14 | 17 | 36 | 37 | 53 |
| 15 | 17 | 44 | 49 | 53 |
| 16 | 19 | 39 | 38 | 51 |
| 17 | 20 | 50 | 51 | 51 |
| 18 | 24 | 54 | 38 | 44 |
| 19 | 18 | 50 | 48 | 45 |
| 20 | 20 | 35 | 50 | 38 |
| 21 | 19 | 37 | 55 | 34 |
| 22 | 19 | 50 | 36 | 47 |
| 23 | 23 | 37 | 46 | 50 |
| 24 | 21 | 49 | 39 | 51 |
| 25 | 26 | 52 | 40 | 36 |
| 26 | 19 | 35 | 50 | 44 |
| 27 | 19 | 46 | 54 | 39 |
| 28 | 25 | 40 | 48 | 50 |
| 29 | 24 | 44 | 36 | 52 |
| 30 | 18 | 38 | 44 | 36 |
| 31 | 16 | 50 | 43 | 46 |
| 32 | 20 | 52 | 51 | 44 |
| 33 | 22 | 38 | 48 | 46 |
| 34 | 25 | 35 | 50 | 42 |
| 35 | 26 | 37 | 45 | 48 |
| 36 | 24 | 37 | 48 | 45 |
| 37 | 21 | 35 | 40 | 49 |
| 38 | 24 | 44 | 50 | 49 |
| 39 | 24 | 44 | 43 | 47 |
| 40 | 21 | 41 | 44 | 47 |

*Table 6.14: continuation of: Number of B-Cells/Hepathopancreas Cell of noble crayfish juveniles exposed to different areas near a STP influence*

| Sample/Group | A | B | C | D |
|---|---|---|---|---|
| 41 | 23 | 41 | 37 | 50 |
| 42 | 25 | 48 | 40 | 51 |
| 43 | 22 | 47 | 49 | 45 |
| 44 | 21 | 39 | 53 | 38 |
| 45 | 19 | 39 | 49 | 37 |
| 46 | 22 | 51 | 49 | 34 |
| 47 | 16 | 54 | 49 | 45 |
| 48 | 24 | 35 | 55 | 47 |
| 49 | 26 | 42 | 39 | 40 |
| 50 | 21 | 35 | 43 | 44 |
| 51 | 18 | 35 | 48 | 37 |
| 52 | 24 | 39 | 41 | 53 |
| 53 | 19 | 48 | 53 | 47 |
| 54 | 22 | 52 | 36 | 37 |
| 55 | 23 | 46 | 54 | 34 |
| 56 | 25 | 50 | 46 | 50 |
| 57 | 16 | 42 | 38 | 46 |
| 58 | 25 | 43 | 45 | 48 |
| 59 | 26 | 54 | 38 | 35 |
| 60 | 26 | 49 | 47 | 52 |
| Average | 20.90 | 44.15 | 45.05 | 45.13 |
| SD | 3.08 | 6.23 | 5.32 | 5.27 |

## 6.7 Acknowledgments

By the end of my dissertation, a long list of people has emerged to whom I am grateful for their support in completing this work and overcoming all the other problems that have arisen in recent years. So, I would like to take this opportunity to thank some of them.

First and foremost, of course, **Prof. Dr. Heinz Brendelberger**. Without your support right from the beginning, from the elaboration of the topic and the application to the DBU, to the corrections of the manuscripts, to the help with the dissertation itself. Who would have expected 7 years ago, when I first entered the working group, that in the end a whole dissertation would come out? Thank you very much for your trust, without which I would never have come so far.

And so, I stood there at the beginning. An idea for a doctoral thesis that could even contribute to nature conservation, a working group in which I could implement this idea, but no idea how to finance me and the investigation. Fortunately, for such cases there is the "**Deutsche Bundesstiftung Umwelt**". But here I am not only grateful for the financing. Through the seminars and the supervision by **Dr. Wachendörfer** I was able to make contacts that opened up new worlds of my subject. I hope that many more committed scientists can benefit from the good work of the DBU, as I did.

I would also like to thank **Prof. Dr. Carsten Schulz** for the sacrificed time of the second correction.

All co-authors: **Kai Lehmann**, **Arne Georg**, **Lena Lietz** and **Thekla Schultheiß** and **Prof Dr. Brendelberger**, for the productive discussions, the insightful inputs and constantly pleasant collaborations and **Jessica Lichte,** who was a huge help at the laboratory and with the histological studies.

The members of the Limnology group. During the long time in the department I have not only met colleagues but also close friends. In particular I would like to mention **Jessika Lichte**, **Sophie Bodenstein**, **Kai Lehman** and **Arne Georg** as constant companions. But also, **Nadine Lefering**, **Johanna Fuhrmann**, **Jannis Hofmann** and **Adrian Schörle** should not remain unmentioned. Through this great group, the work, but especially my social life was significantly enhanced. I also have to apologize for all your pencils that I have lost. *I'm sure they'll turn up again some time*.

For the cooperation's with **Helmut Jeske**, who provided us with experimental animals and also taught me one or two things from practical experience and the **LLUR** whose data significantly advanced the planning of the experiments.

My family, but especially my **mother Maria** and**, sisters Vera and Silke** and all my friends, who always reminded me that life is not only about crayfish, but still endured it when I could not stop talking about it. It is thanks to you that I have become like this. I think that went quite well

And now to the most important person of all: **Judith**, I was allowed to experience so much support from you. You have helped me up again after every setback and given me the best motivation in the world. I can hardly wait for the future with you and our little family. Thank you, Judith.

# 7 Eidesstattliche Erklärung

Hiermit bestätige ich, Jan Laurenz, dass die folgende Dissertation

Sublethal and lethal effects of pharmaceuticals and agricultural chemicals on the reproduction of freshwater crayfish

von mir, unter Beratung meines Betreuers, selbstständig verfasst wurde, nach Inhalt und Form meine eigene Arbeit ist und keine weiteren Quellen und Hilfsmittel als die angegebenen verwendet wurden.

Die vorliegende Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden und wurde nicht im Rahmen eines Prüfungsverfahrens an anderer Stelle vorgelegt. Zur Veröffentlichung eingereichte Manuskripte wurden kenntlich gemacht. Mir wurde kein akademischer Grad entzogen

Kiel, 25.09.2020 _____

Jan Laurenz