CONSERVATION OF THE EUROPEAN WEATHERFISH *Misgurnus fossilis*

STOCKING MEASURES, AUTECOLOGY AND POTENTIAL THREATS

by

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OVERVIEW OF PUBLICATIONS

As a cumulative dissertation, this thesis is based on the following four scientific articles that are either published or submitted for publication:

1. **Schreiber, B.,** Korte, E., Schmidt, T., Schulz, R. Reintroduction and stock enhancement of European weatherfish (*Misgurnus fossilis* L.) in Rhineland-Palatinate and Hesse, Germany. (Submitted to Fisheries Management and Ecology) [*Appendix I*]

2. **Schreiber, B.**, Monka, J., Drozd, B., Hundt, M., Weiss, M., Oswald, T., Gergs, R., Schulz, R., 2017. Thermal requirements for growth, survival and aerobic performance of weatherfish larvae *Misgurnus fossilis*. J. Fish Biol. 90, 1597–1608. doi:10.1111/jfb.13261 [*Appendix II*]

3. **Schreiber, B.**, Petrenz, M., Monka, J., Drozd, B., Hollert, H., Schulz, R., 2017. Weatherfish (*Misgurnus fossilis*) as a new species for toxicity testing? Aquat. Toxicol. 183, 46–53. doi:10.1016/j.aquatox.2016.12.006 [*Appendix III*]

4. **Schreiber, B.**, Fischer, J., Schiwy, S., Hollert, H., Schulz, R, 2017. Towards more ecological relevance in sediment toxicity testing with fish: evaluation of multiple bioassays with embryos of the benthic weatherfish (*Misgurnus fossilis*). Sci. Total Environ., Accepted. [*Appendix IV*]

CONTENTS

ABBREVIATION LIST

3Rs	Replacement, reduction and refinement of animal experiments
AL	Altrip
ASE	Accelerated solvent extraction
CI	Confidence interval
DCA	3,4-dichloroaniline
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
dph	Days post-hatch
EC_X	Effect concentration, X%
EEA	European Environment Agency
EEC	European Economic Community
ERA	Ecological risk assessment
ES	Ehrenbreitstein
EU	European Union
ex-ACE	Acetone extract
ex-PHWE	Pressurized hot water extract
FET	Acute fish embryo toxicity test
G	Mean mass-specific growth rate
GADM	Global administrative areas
GnRH	Gonadotropin-releasing hormone
HH	Hamburg
ISO	International Organization for Standardization
IUCN	International Union for Conservation of Nature
LC_X	Lethal concentration, X%
LOEC	Lowest observed effect concentration
M_D	Dry mass
$\dot{M}O_2$	Routine metabolic rate
OCR	Oxygen consumption rate
OECD	Organization for Economic Co-Operation and Development
OECD-236	Test guideline No. 236 of the OECD
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls

PCDD/Fs	Polychlorinated dibenzodioxins and dibenzofurans
PHWE	Pressurized hot water extraction
RAS	Recirculating aquaculture system
SCA	Sediment contact assay
SD	Standard deviation
SE	Standard error
ST	Streitgraben
T_C	Critical temperature
T_{Opt}	Temperature optimum
$T_{Opt}G$	Optimum temperature for growth

ABSTRACT

The European weatherfish (*Misgurnus fossilis*) is a benthic freshwater fish species belonging to the family Cobitidae, that is subjected to a considerable decline in many regions across its original distribution range. Due to its cryptic behavior and low economic value, the causes of threat to weatherfish remained partly unknown and the species is rarely at the center of conservation efforts. In order to address these concerns, the overall aim of the present thesis was to provide a comprehensive approach for weatherfish conservation, including the development of stocking measures, investigations on the species autecology and the evaluation of potential threats. The first objective was to devise and implement a regional reintroduction and stock enhancement program with hatchery-reared weatherfish in Germany. Within this program (2014-2016), a total number of 168,500 juvenile weatherfish were stocked to seven water systems. Recaptures of 45 individuals at two reintroduction sites supported the conclusion that the developed stocking strategy was appropriate. In order to broaden the knowledge about weatherfish autecology and thereby refining the rearing conditions and the selection of appropriate stocking waters, the second objective was to investigate the thermal requirements of weatherfish larvae. Here, the obtained results revealed that temperatures higher than previously suggested were tolerated by larvae, whereas low temperatures within the range of likely habitat conditions increased mortality rates. As weatherfish can be frequently found in agriculturally impacted waters (e.g. ditch systems), they are assumed to have an increased probability to be exposed to chemical stress. Since the resulting risk has not yet been investigated with a focus on weatherfish, the third objective was to provide a methodical foundation for toxicity testing that additionally complies with the requirements of alternative test methods. For this purpose, the acute fish embryo toxicity test was successfully transferred to weatherfish and first results exhibited that sensitivity of weatherfish towards a tested reference substance (3,4-dichloroaniline) was highest compared to other species. On the basis of these findings, the fourth objective was to apply weatherfish embryos for multiple sediment bioassays in order to investigate teratogenic effects derived from sediment-associated contaminants. In this context, weatherfish revealed particular sensitivity to water extractable substances, indicating that sediment contamination might pose a considerable risk. Moreover, as an endangered benthic fish species with high ecological relevance for European waters that are specifically exposed to hazardous contaminants, the weatherfish might be a prospective species for an ecological risk assessment of sediment toxicity. Overall, the present thesis contributed to the conservation of weatherfish by considering a variety of aspects that interact and reinforce one another in order to achieve improvements for the species situation.

CHAPTER

INTRODUCTION, OBJECTIVES AND THESIS OUTLINE

1.1 INTRODUCTION

Fishes are the most abundant and diverse of the vertebrate groups, including more than 30,000 described species that inhabit an enormous variety of aquatic habitats (Lévêque et al., 2008; FishBase, 2017a). The fundamental (e.g. nutrient cycling) and demand-derived ecosystem services (e.g. economic and recreational values) provided by fish populations are undoubtedly of significant value for human societies (Holmlund & Hammer, 1999; Ormerod, 2003). However, the worlds ichthyofauna is increasingly threatened by consequences of the expansion of human populations, including (i) competition for water, (ii) habitat alteration, (iii) pollution, (iv) introduction of exotic species and (v) commercial exploitation (Moyle & Leidy, 1992; Clausen & York, 2008). On the European red list of freshwater fishes (2011), 37% of 531 occurring native species are listed as threatened at a continental scale and 17% show declining populations (Freyhof & Brooks, 2011). In contrast to fish species of high public interest like Atlantic salmon (*Salmo salar*) or European sturgeon (*Acipenser sturio*) that are used as conservation flagships for European major rivers (Ludwig, 2006; Monnerjahn, 2011), smaller endangered species with low economic value and cryptic behavior might be overlooked and neglected by conservation efforts (Kalinkat et al., 2016). A species that can be found among this latter category, is the European weatherfish (*Misgurnus fossilis*).

The weatherfish is a member of the family Cobitidae with a wide distribution range throughout large parts of Europe - originally ranging from northwest France to the Volga basin (Lelek, 1987; Kottelat & Freyhof, 2007). Primary habitats are oxbows, periodically flooded meadows or backwaters, that all contain dense aquatic vegetation as well as muddy sediments (Pekárik et al., 2008; Hartvich et al., 2010). In modern landscapes that are highly influenced by agriculture, weatherfish populations can be found in temporarily filled ponds and ditches with conditions comparable to those of their primary habitats (Meyer & Hinrichs, 2000; Sigsgaard et al., 2015). The weatherfish is a demersal species that burrows into the sediment by day, desiccation or to avoid predation. Furthermore, it is physiologically and morphologically adapted to hypoxic conditions, including dependence on cutaneous respiration, oxygen-uptake via the gut and development of external filamentous gills a few days after hatching (Kottelat & Freyhof, 2007). These adaptations attracted the attention of scientists in the early years of ichthyological research (Baumert, 1855; Grieb, 1936), however, it could not protect the species

from a distinctive decline exhibited in many regions across its original distribution range (Hartvich et al., 2010). Consequently, the species is listed as vulnerable or nearly extinct in many European countries, including Austria, Czech Republic, Denmark, Germany, Hungary, Slovakia, Slovenia and Switzerland (Zulka & Wallner, 2006; Haupt et al., 2009; Sigsgaard et al., 2015). Furthermore, the European Union (EU) listed the weatherfish under Annex II of the Council Directive 92/43/EEC, representing species of community interest that need special areas of conservation (EU, 1992). The main reasons for the species decline are assumed to be habitat loss, degradation of water quality by pollution and ditch maintenance practices (e.g. dredging) (Lelek, 1987; Kouril et al., 1996; Meyer & Hinrichs, 2000; Hartvich et al., 2010). However, only few of the mentioned reasons have so far been investigated with specific relation to the conservation of weatherfish.

Restoration of habitats or removal of invasive species are key strategies to support the viability of existing freshwater fish populations (Cochran-Biedermann et al., 2015). However, the increasing number of eradications of fish species from parts of their historical range led to enhanced use of reintroduction measures for conservation purposes (Armstrong & Seddon, 2007). In the case of weatherfish, Lelek (1987) also recommended the reintroduction of hatchery-reared individuals to waters with suitable conditions where the decisive reasons for its decline can be excluded. In order to implement such measures, artificial propagation of weatherfish and subsequent rearing of fry under controlled conditions in aquaculture systems can provide a suitable foundation for the production of stocking material (Geldhauser et al., 1992; Kouril et al., 1996; Demény et al., 2009). In addition, effective stocking measures presuppose an identification of locally sourced broodstock, a critical assessment of stocking habitats, and an implementation of perennial stocking (Cochran-Biedermann et al., 2015). In order to meet many of these requirements, the species autecology has to be thoroughly considered. This is particularly important for early life stages, since they often represent the most sensitive period in the life cycle of fishes and are therefore considered a bottleneck for maintenance of stable populations (Houde, 2002). In the context of fish autecology, water temperature is an important abiotic factor because it influences functions at various levels in ectothermic organisms, including foraging ability, behaviour, growth and survival (Beitinger et al. 2000; Angiletta et al., 2002; Killen et al., 2013). Furthermore, thermal tolerance of fishes is typically narrowest in eggs and early larval stages (Brett, 1956; Rombough, 1997; Pörtner & Farrell, 2008). Due to the low public interest in weatherfish, only little information is available about thermal requirements of this species (Drozd et al., 2009; FishBase, 2017b).

Apart from stocking measures conducted for a strengthening of populations, it is also important to consider and evaluate potential causes of threat (Cochran-Biedermann et al., 2015), especially because reasons for weatherfish declines partly remained unexplained. A threat that was frequently assumed as decisive but has not been previously investigated with a focus on weatherfish arises from chemical stress induced by water contamination (Hartvich et al., 2010; Drozd et al., 2009). This aspect, however, deserves special consideration because weatherfish might have an increased probability for an exposure to hazardous contaminants for two main reasons: firstly, agriculturally impacted ditch systems that are populated by weatherfish as secondary habitats (Meyer & Hinrichs, 2000) are known to be particularly exposed to contaminants such as pesticides as they are constructed to drain water from agriculture land (Herzon & Helenius, 2008; Dollinger et al., 2015). Secondly, sediments that serve as retreat and feeding ground for weatherfish have the ability to accumulate chemicals up to years (Power & Chapman, 1992), making them a 'major hazard for the environment' (Hallare et al., 2011). Hence, the habitat requirements and benthic lifestyle of weatherfish increases their proximity to potential hazard and possibly exposes them to sediment-associated contaminants via multiple pathways (e.g. aqueous and dietary exposure) (Costa et al., 2011; Egeler et al., 2001; Goksøyr et al. 1996; Hellou et al., 1994).

For an ecological risk assessment (ERA) of hazardous contaminants, a wide variety of ecotoxicological bioassays can generally be applied for different fish species (Powers, 1989; van der Oost et al., 2003). However, a majority of studies is carried out with standard test organisms like zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*) or Japanese medaka (*Oryzias latipes*), irrespective of site- or species-specific characteristics to be investigated. More specifically, the restricted ecological relevance of these species can be scrutinized as fundamental inconsistencies exist concerning their origin (exotic vs. temperate) and lifestyle (pelagic vs. benthic) (Chapman, 2002; Chapman et al., 2002; Hallare et al., 2011; Wedekind et al., 2007). Furthermore, regardless of which fish species is selected for toxicity tests, the question of ethical concerns about bioassays with vertebrates arises (Scholz et al., 2013). A promising assay that complies with the requirements of alternative test methods according to the 3Rs principle (replacement, reduction and refinement of animal experiments) (Russel & Burch, 1959) is the acute fish embryo toxicity test (FET) because EU animal welfare legislation protects fish as experimental organism from the onset of exogenous feeding, which is not applicable to embryonic life stages (EU, 2010; Strähle et al., 2012). The successful transfer of a FET protocol to weatherfish embryos could bridge the gap of ecological relevance regarding standard test organisms and overcome animal welfare concerns related to fish as

vertebrates. Furthermore, it could provide the methodical foundation to investigate adverse effects on weatherfish that arise from chemicals dissolved in water.

Due to the particular hazard potential of sediment-associated contaminants to which the benthic weatherfish might be exposed, a further critical issue comes into effect for an ERA: the selection of an appropriate exposure scenario for sediment toxicity testing with fish (Burton, 2013; Chapman et al., 2002; Hallare et al., 2011). Moreover, options are limited as a decision in favor of fish embryos because of animal welfare concerns has high influence on the choice of potential test systems. The above-mentioned FET could be applied for sediment toxicity testing as long as aqueous solutions of particle bound contaminants are prepared. This can be achieved by a variety of extraction methods and subsequent exposure to the gained extract (Hallare et al., 2011). In this context, organic solvent extraction (e.g. with acetone) can be used to investigate the overall hazard potential of a sediment (i.e. worst-case scenario) as also hardly bioavailable fractions are extracted (Hallare, et al., 2005; Kosmehl et al., 2007). An intermediate extraction method where the naturally occurring solvent water is used under supernatural extraction conditions (i.e. high temperature and pressure) might be represented by pressurized hot water extraction (PHWE) (Hawthorne et al., 2000). However, PHWE was not yet applied for sediment toxicity bioassays with fish. As a further development of the FET, Hollert et al. (2003) presented the sediment contact assay (SCA) where fish embryos are incubated in direct contact to whole freeze-dried sediment samples. This exposure scenario is assumed to be most realistic for sediment toxicity testing with fish embryos (Hallare et al., 2011) but methodical problems associated with high oxygen consumption rates of native sediments were reported in applications with zebrafish (Rocha et al., 2011; Schiwy et al., 2015; Strecker, 2013). A comparative evaluation of various sediment bioassays with embryos of the weatherfish might help to identify a suitable test system for the investigation of potential threats posed by sediment-associated contaminants. Furthermore, the ecological relevance of weatherfish for sediment contamination might qualify the species for an ERA of sediment toxicity.

1.2 OBJECTIVES AND THESIS OUTLINE

Overall, the present thesis followed a holistic approach for the conservation of weatherfish by combining reintroduction and stock enhancement measures with underlying autecological research, as well as with concepts for the evaluation of chemical stress posed by both contaminants dissolved in water and contaminants associated to sediments. For this purpose,

two key areas were defined: (i) stocking measures and autecology and (ii) chemical stress as a potential threat. In each of the key areas, two research objectives were addressed (Fig. 1).

The framework for the first key area (Chapter 3) was provided by the conceptualization of a regional reintroduction and stock enhancement program for the German federal states of Rhineland-Palatinate and Hesse. One of the associated research objectives was related to fisheries management issues, including identification and sampling of brood stocks, establishment of a rearing program, implementation of stocking measures and monitoring for stocking success. On the basis of current scientific literature, a management plan was developed and implemented that addressed these concerns. Furthermore, achieved results and remaining challenges of the program were evaluated regarding potential improvements. In order to advance the rearing conditions and to refine the identification of suitable stocking habitats, a second research objective within this key area was to study the autecology of early life stages. For this purpose, effects of different temperatures on survival, growth and oxygen consumption rate (OCR) of larval weatherfish were investigated. The experimental approaches included temperature ranges at the limits of the larvae physiological capabilities as well as at their optimum and covered acute and chronic exposures to different temperatures.

The second key area (Chapter 4) of the thesis generally focused on possibilities to assess potential threats posed by chemical stress to weatherfish based on experimental assays that meet the requirements demanded for alternative test methods. Moreover, the associated research objectives considered possible improvements for an ERA in Europe, regarding the ecological relevance of fish species used for toxicity testing. For this purpose, one objective was to investigate the suitability of weatherfish for FET, including the practical feasibility, their sensitivity towards contamination and their tolerance against dissolved oxygen (DO) as an environmental condition of concern. The thereby developed methical foundations were used to apply weatherfish embryos for an ERA of sediment toxicity. The objective here was to comparatively evaluate different bioassays applicable together with weatherfish embryos, regarding the simulation of realistic exposure scenarios. In this context, PHWE was studied as an alternative method to extract sediment-associated contaminants for an application in fish bioassays. Additionally, a comparison to zebrafish embryos served to study species-specific differences concerning their sensitivity to sediment-associated contaminants.

Since all experiments as well as stocking measures described in Chapters 3 and 4 were carried out with artificially propagated weatherfish of different life stages (i.e. embryos, larvae or juveniles), a methical background about the underlying procedure will initially be provided in Chapter 2.
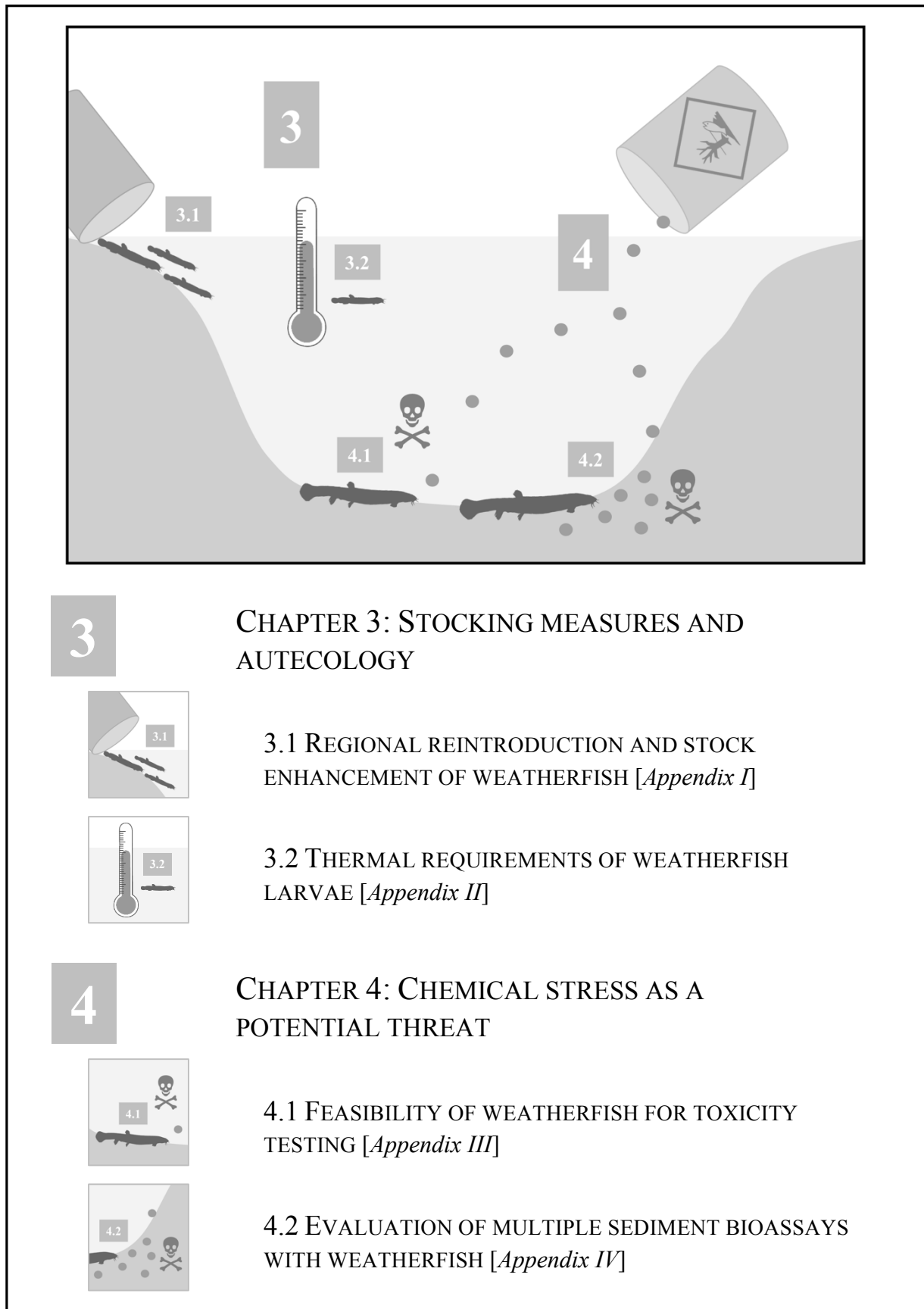
Fig. 1. Schematic overview of the thesis outline, including the two key areas (Chapter 3 & 4) and the four research objectives (3.1, 3.2, 4.1, 4.2). The underlying publications can be found in the appendices (*Appendix I – IV*).

METHODICAL BACKGROUND

For an artificial propagation of weatherfish, adult spawners were caught with baited fish traps from stable wild populations at the beginning of the spawning season (between March and May, depending on the climatic conditions) and transported to the research facilities of the "Ökosystemforschung Anlage Eußerthal", University of Koblenz-Landau, Germany. Localization and catching of weatherfish spawners were carried out in cooperation with Egbert Korte from "INGA – Institut für Gewässer- und Auenökologie GbR", Riedstadt, Germany.

After arriving in Eußerthal, weatherfish were kept separated by populations for a 30-day acclimation period at $\leq 11°$ C. In order to induce stripping, male and female spawners were isolated from each other and water temperature was gradually increased over a period of 4 days (11 to 18° C). Subsequently, a gonadotropin-releasing hormone (GnRH) analogue and metoclopramide-containing preparation (Ovopel, Unic-trade, Hungary) was intramuscularly injected into fishes of both sexes. The total dose of 1 pellet/kg body mass was administered in two consecutive doses with a time interval of 12 h. Approximately 24 h after the second injection, spawners were stripped and eggs of one female were fertilized with sperm of two males using the dry method (Drozd et al., 2009). At this point in time, embryos that were selected for experiments (Chapter 4) were transferred to artificial water prepared according to ISO 7346-3 (ISO, 1996) and subsequently treated pursuant to the respective experimental approach. Incubation of embryos that were not used for experimental investigations were incubated in hatching troughs connected to a recirculating aquaculture system (RAS) at a water temperature of 18° C. Hatching from these eggs occurred 62 – 80 h after fertilization and larvae were fed with decapsulated *Artemia salina* nauplii from the beginning of 3 days post-hatch (dph). For experiments carried out with weatherfish larvae, fish were transferred at this stage to experimental aquaria. All other individuals that were intended for stocking measures, were cultured for four to eight weeks in the RAS at 20-21° C, before they were released to designated water bodies.

STOCKING MEASURES AND AUTECOLOGY

3.1 REGIONAL REINTRODUCTION AND STOCK ENHANCEMENT OF WEATHERFISH

In 2014, a reintroduction and stock enhancement program for hatchery-reared weatherfish fry was launched in the German federal states of Rhineland-Palatinate and Hesse, representing a regional joint conservation program. At the beginning of the program, a monitoring served to identify local broodstock populations and to assess habitats regarding their ecological suitability for weatherfish reintroduction (e.g. presence of spawning habitats, year-round water bearing, absence of nocturnal predators). In a second step, spawners were caught and treated for artificial propagation according to Chapter 2. Weatherfish fry were cultured until they reached a size appropriate for stocking (2 to 4 cm) and released into previously selected waters. In a last step, waters used for reintroduction were bi-annually monitored for stocking success. The whole procedure was repeated in three consecutive years (2014-2016).
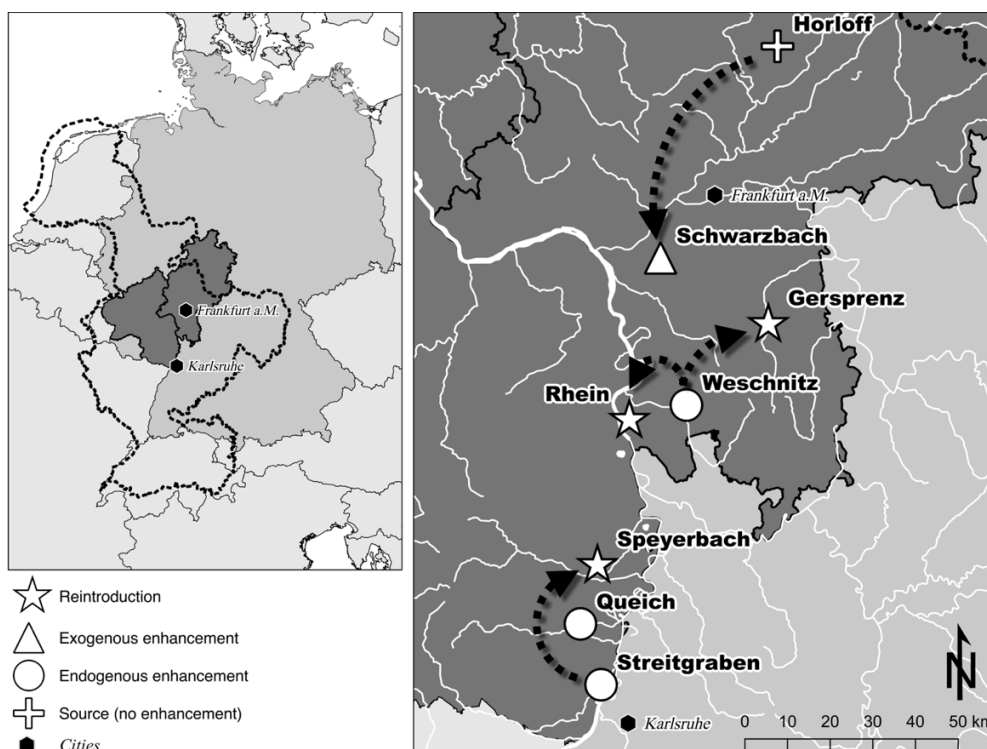

Fig. 2. Overview map on the left shows the location of Rhineland-Palatinate and Hesse (dark grey) in Germany (grey) and Europe (light grey; GADM, 2012). Dotted black line indicates the international Rhine river basin district (EEA, 2011). Detailed map on the right shows the sites used for different stocking measures (reintroduction, exogenous enhancement, endogenous enhancement) and the broodstock source used where no enhancement took place. Black arrows indicate the broodstock origin used for reintroductions. White lines show main rivers (GADM, 2012). Figure taken from *Appendix I.*

Within the study period, four water systems were identified that provided weatherfish populations for broodstock sampling, namely Streitgraben, Weschnitz, Queich and Horloff (Fig. 2). Hence, despite the critical situation of weatherfish within the study area, local broodstock could be used for the artificial propagation of the program, which is considered important in the context of species translocations from a genetic point of view (Weeks et al., 2011). Furthermore, numerous waters (in particular ditch systems) were assessed as suitable for weatherfish, but missing spawning habitats excluded them from a selection for reintroduction measures (Cochran-Biedermann, 2014). Finally, only Speyerbach, Gersprenz and Rhein were selected as water systems for reintroduction (Fig. 2).

Table 1. Number of stocked weatherfish subdivided into stock enhancement and reintroduction measures, including year of stocking, stocked catchment and catchment of broodstock origin. For a geographical orientation, the reader is referred to Fig. 2. Table taken from *Appendix I*.

Year	Stock enhancement		Reintroduction		
	Stocked catchment	No. of stocked individuals	Stocked catchment	No. of stocked individuals	Catchment of broodstock origin
2014	Streitgraben[†]	24,000	Speyerbach	11,000	Streitgraben
2014	Weschnitz[†]	12,000	Gersprenz	13,000	Weschnitz
Total 2014	-	36,000	-	24,000	-
2015	Streitgraben[†]	13,000	Speyerbach	7,500	Streitgraben
2015	Queich[†]	10,000	Gersprenz	8,000	Weschnitz
2015	-	-	Rhein	18,000	Weschnitz
Total 2015	-	23,000	-	33,500	-
2016	Streitgraben[†]	6,000	Speyerbach	14,000	Streitgraben
2016	Schwarzbach[‡]	20,000	Gersprenz	5,000	Horloff
2016	-	-	Rhein	7,000	Horloff
Total 2016	-	26,000	-	26,000	-
Total	-	85,000	-	83,500	-

[†] Stocking site is equivalent to the site of broodstock origin (i.e. endogenous enhancement)
[‡] Broodstock originates from the Horloff catchment (i.e. exogenous enhancement)

The total number of approximately 168,500 juvenile weatherfish was gained from 38 female spawners (approximately 275,000 eggs) and stocked during the study period (2014-2016) (Table 1). These numbers approve the good feasibility of artificial propagation of weatherfish as reported in numerous studies (Demény et al., 2009; Kouril et al., 1996; Schauer et al., 2013) and ensured an effective perennial stocking in the program area (Cochran-Biedermann, 2014).

Table 2. Number of recaptured individuals from weatherfish monitoring conducted in waters used for reintroduction, including year of monitoring, monitoring catchment, catching effort, and size class of recaptured individuals.

Year	Catchment	Catching effort (No. of traps)	No. of recaptured individuals	Size class (cm)
2015	Gersprenz	40	7	7-8
2016	Rhein	40	34	10-14
			3	16-18
2016	Gersprenz	50	1	10
Total	-	130	45	-

During the monitoring for stocking success, 45 individuals were recaptured in two waters that were used for reintroduction measures (i.e. Gersprenz and Rhein; Table 2). As the capturing of weatherfish was frequently reported as inefficient because of their behavior to burry into the sediment (Meyer & Hinrichs, 2000; Sigsgaard et al., 2015), this amount can be evaluated as relatively high. Therefore, the results indicate perennial reintroduction success in Gersprenz and Rhein. However, reproduction - the criteria used by the International Union for Conservation of Nature (IUCN, 1998) for a successful reintroduction of self-sustainable populations - could not yet be confirmed in these systems. Furthermore, an evaluation of stock enhancement is still pending, as the released individuals were not marked and hence, could not be distinguished from previously existing fish. Chemical marking of fry used for stock enhancement (Skalski et al., 2009) or a parental assignment based on microsatellite markers (Zhao et al., 2015) could provide methods to solve the latter challenges.

BOX 3.1: CONCLUSIONS – STOCKING MEASURES

The presented program effectively reintroduced weatherfish to habitats where the conditions for a development of stable populations are given and supported existing populations by stock enhancement. The documented recapturing success showed that measures applied in the program – from breeding to stocking – already resulted in quantifiable improvements for the species within the study area. Beyond that, ongoing stocking activities as well as implementations of proposed refinements might contribute to weatherfish conservation on larger temporal and spatial scales.
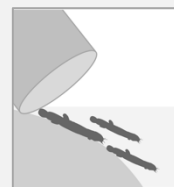
3.2 THERMAL REQUIREMENTS OF WEATHERFISH LARVAE

In order to support the management of stocking measures, autecological aspects were investigated that might help to improve rearing conditions and to identify suitable waters for stocking. For this purpose, thermal requirements of weatherfish larvae were assessed in a combination of two experimental approaches. On the one hand, three dph larvae were acclimated for 25 days to environmentally realistic temperatures (11, 15, 19, 23, 27° C) that were mainly chosen on the basis of the optimal range (15 to 24° C) reported for weatherfish embryos (Drozd et al., 2009). This approach represented a chronic exposure duration adapted to seasonal temperature fluctuations (Selong et al., 2001; Zale; 1984) and the monitored endpoints were growth (i.e. dry mass (M_D) and total length; every 5 days) and survival (twice daily). On the other hand, routine metabolic rate ($\dot{M}O_2$) of unfed weatherfish larvae (3 dph) were measured as OCR to detect acute physiological responses to thermal stress (Clark et al., 2013; Li et al., 2014; Pörtner & Peck, 2010). For this purpose, exposure time was set to 30 minutes to 1 hour and the temperature range was expanded to 7 test temperatures (11, 15, 19, 23, 27, 31, 35° C).
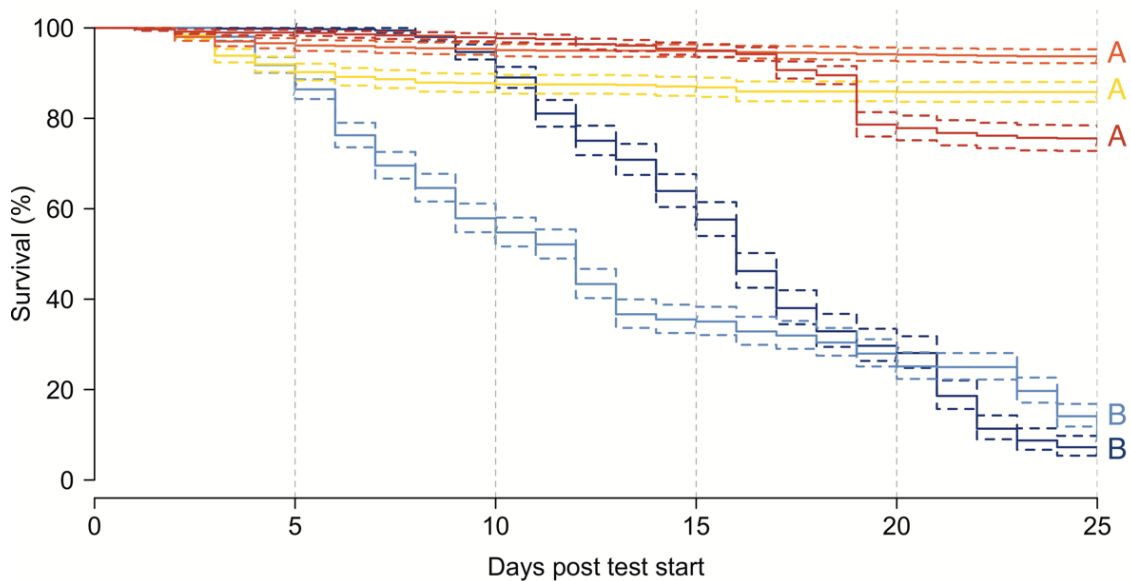

Fig. 3. Survival rates (± 95% confidence intervals) of weatherfish larvae during 25 days lasting thermal acclimation to 11 (●), 15 (●), 19 (●), 23 (●) and 27° C (●). Dotted grey vertical lines indicate dates when subsamples for growth analyses were taken. Different capital letters indicate significant differences between treatments (α = 0.05). All treatments were replicated four times (except 11° C with n = 3). Figure taken from *Appendix II*.

Survival rates of weatherfish larvae during 25 days of acclimation varied significantly among lower (11 and 15° C) and upper temperature treatments (19, 23 and 27° C) and showed a maximum rate at 23° C (Fig. 3). When comparing these results to the temperature tolerance reported for survival of weatherfish embryos exposed from fertilization to hatching, larvae revealed a higher temperature optimum (T_{Opt}), a lower vulnerability to high temperatures and a higher vulnerability to low temperatures (Drozd et al., 2009). Consequently, weatherfish larvae showed an upwards shift of both, T_{Opt} and critical temperatures (T_C) as ontogeny proceeds. This observation might be best explained with regard to the natural temperature conditions weatherfish experience in their spawning season in early spring (Kottelat & Freyhof, 2007): due to the seasonal warming of water, larvae are exposed to increasing temperatures with proceeding ontogeny, resulting in a simultaneous shift in the zone of thermal tolerance (Rombough, 1997). Regarding the lower end of tested temperatures, an exposure over longer time periods (>10 days) might be critical for survival of weatherfish larvae at temperatures ≤15° C. Since weatherfish habitats with low water levels are likely to suffer from large temperature fluctuations (Meyer & Hinrichs, 2000), a drop in temperature below critical limits might occur and hence lead to severe losses in offspring. However, short term exposures to these temperatures (<5 days) did not affect survival of weatherfish larvae.
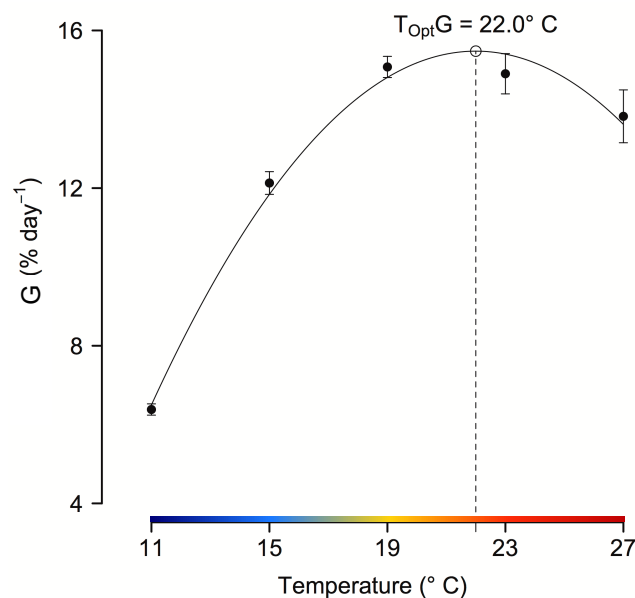

Fig. 4. Mean mass-specific growth rates (G) and associated standard deviations of weatherfish larvae after acclimation to 11, 15, 19, 23 and 27° C (% d^{-1}). White point with dotted vertical line indicates the optimum temperature for growth ($T_{Opt}G$) calculated from a second-order polynomial regression (p < 0.001, r^2 = 0.9801; y = -20.44 + 3.27x – 0.07x^2). All treatments were replicated four times (expect 11° C where *n* = 3). Figure adapted from *Appendix II*.

Mean mass-specific growth rates (G) of weatherfish larvae calculated after 25 days of temperature acclimation were higher in the upper temperature treatments (19, 23 and 27° C) compared to lower temperature treatments (11 and 15 °C) (Fig. 4). These differences can be traced back to increasing reaction rates for growth that ectothermic organisms like fishes show with increasing water temperature (Angiletta et al., 2002; Jobling, 1997; Wang & Overgaard, 2007). Furthermore, the decreasing G observed at 27° C might be linked to a mismatch between the increased metabolic costs induced by higher temperatures and the energy uptake necessary to maximize growth (Pörtner & Knust, 2007; Jonassen, 2000; Neuheimer et al., 2011). Furthermore, the calculated optimum temperature for growth ($T_{Opt}G$) of weatherfish larvae (22.0° C) is similar to the optimum reported for larvae of the closely related spined loach (*Cobitis taenia*; 20 to 24° C) (Bohlen, 2003).
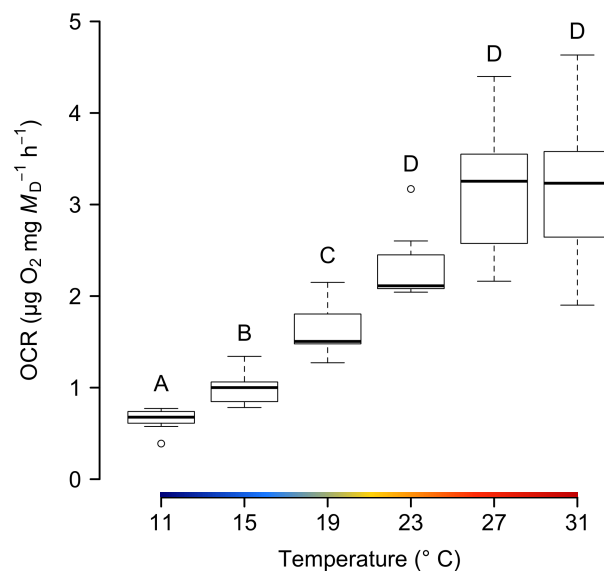

Fig. 5. Box-and-whisker plots of oxygen consumption rates (OCR; $\mu g\ O_2$ mg M_D^{-1} h^{-1}) of weatherfish larvae exposed to 11, 15, 19, 23, 27 and 31° C. Different capital letters indicate significant differences ($\alpha = 0.05$) between treatments. All treatments were replicated three times with 3 larvae per replicate. Figure adapted from *Appendix II*.

Regarding OCR of weatherfish larvae during acute exposure (Fig. 5), a metabolic temperature compensation could be measured as the oxygen demand of the larvae increased with increasing exposure temperatures from 11 to 27° C (Rombough, 1988). However, temperatures >27° C did not induce higher OCR because $\dot{M}O_2$ of the larvae reached a maximum (Hardewig et al., 2004; Breau et al., 2011). These results support the plausibility of growth impairments observed at 27° C during 25 days of acclimation (Fig. 4) and hence, temperatures ≥ 27° C can be stated as critical for weatherfish larvae from a metabolic point of view (Wang & Overgaard, 2007). Furthermore, as all larvae died within the acclimation period at 35° C, the upper critical

temperature threshold of 3 dph weatherfish larvae for an acute exposure is likely to lie between 31 and 35° C. Prior to these investigation, 25° C were declared as the maximum temperature tolerance of weatherfish, irrespective of age and exposure duration (Leuven et al., 2011; FishBase, 2017b).

BOX 3.2: CONCLUSIONS – AUTECOLOGY

Thermal requirements of larval weatherfish were presented for the first time and thereby contributed to the knowledge about the species autecology. The obtained results show that weatherfish larvae are susceptible to low water temperatures that are in a range that is likely to occur in their habitats. On the other hand, temperatures that exceeded the previously reported upper critical limit of the species were tolerated by weatherfish larvae. Furthermore, results from the experiments were used to determine the optimum temperature for growth of larvae. An application of these findings for stocking measures have the potential to contribute to the prospects of such conservation programs.
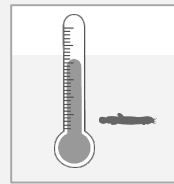
CHEMICAL STRESS AS A POTENTIAL THREAT

4.1 FEASIBILITY OF WEATHERFISH FOR TOXICITY TESTING

In order to investigate the applicability of a toxicity test method for weatherfish that complies with requirements demanded for alternative animal tests, the FET as proposed by the Organization for Economic Co-Operation and Development (OECD; test guideline No. 236 (OECD-236)) was transferred to weatherfish embryos (OECD, 2013). Criteria to be examined in this context were: feasibility of embryo production, determination of teratogenic effects, sensitivity to laboratory handling, compliance with reported validity criteria, repeatability of results and sensitivity to contaminants. For this purpose, 3,4-dichloroaniline (DCA) was chosen as a reference substance because it was extensively studied in FETs with different species and therefore allowed interspecific comparisons regarding sensitivity. In total, one range-finding experiment and three main tests were carried out by exposing weatherfish embryos from two different broodstock populations to DCA for 72 h. Furthermore, the tolerance of weatherfish embryos against limited DO concentrations were investigated, as this factor was assumed to be critical in FETs, especially when using environmental samples (Küster & Altenburger, 2008). Here, weatherfish embryos were exposed to 14 different DO concentrations for 48 h.
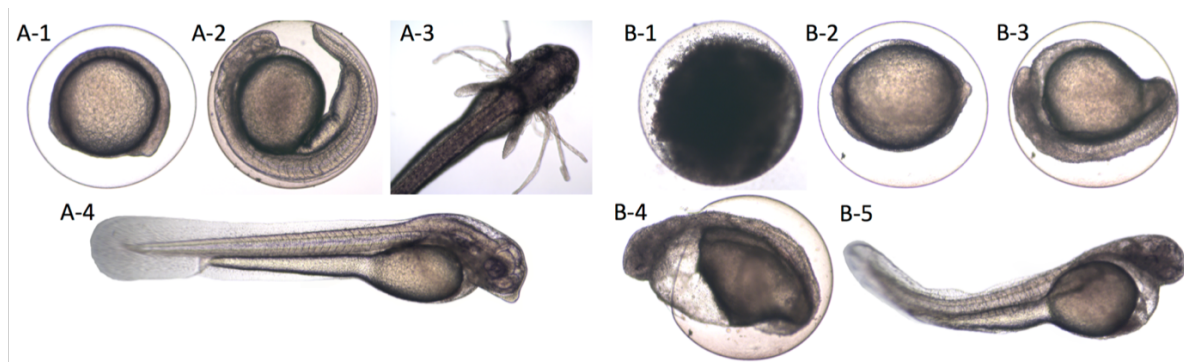

Fig. 6. Pictures of normally developed weatherfish embryos (indicated with A) from control setups (artificial water) after 24 (A-1), 48 (A-2) and 72 hpf (A-4), respectively. Picture A-3 shows outer filamentous gills of embryo 96 hpf. Pictures showing examples for apically observable effects in (Indicated with B), including: coagulation (B-1), edema and underdevelopment after 24 hpf (B-2), edema and malformation after 48 hpf (B-3), edema, delayed hatching and malformation after 72 hpf (B-4), edema and malformation after 72 hpf (B-5). Figure adapted from *Appendix III*.

As already mentioned in previous chapters, the production of weatherfish embryos can be considered as effective and stable since fertilization rates (≥70%) and number of eggs per female (6540±2778) were constantly high. In the context of FET, these figures offer benefits compared to acute toxicity tests conducted with juvenile or adult fish as high numbers of replicates might be less expensive (Wedekind et al., 2007). Furthermore, practical restrictions that were reported for embryo production (e.g. complex procedure and limited amount of eggs) when using the widespread test species fathead minnow and Japanese medaka (Braunbeck & Lammer, 2006). Thus, the practical prerequisites provided by weatherfish are not a matter of course. A disadvantage of the procedure for weatherfish embryo production at the current state might be its seasonality (March to July) since test material (i.e. embryos) is not available throughout the whole year. However, Kostomarova (1991) reported year-round embryo production for weatherfish in Russia and Suzuki (1983) for the closely related pond loach (*Misgurnus anguillicaudatus*) in Asia. Thus, existing methods might be successfully adopted. The determination of apically observable teratogenic effects was possible due to the transparent embryonic membrane of weatherfish (Kostomarova, 1991) (Fig. 6). Moreover, a classification of effects into sublethal (edema, malformation, non-hatching) and lethal (coagulation, lack of heartbeat) was provided for weatherfish embryos after 72 h that is nearly identical to effects defined for zebrafish in OECD-236 (OECD, 2013). The stable survival rates that were achieved in all negative controls (≥90%) indicate both, low sensitivity of weatherfish embryos against laboratory handling as well as minor risk to contravene validity criteria.

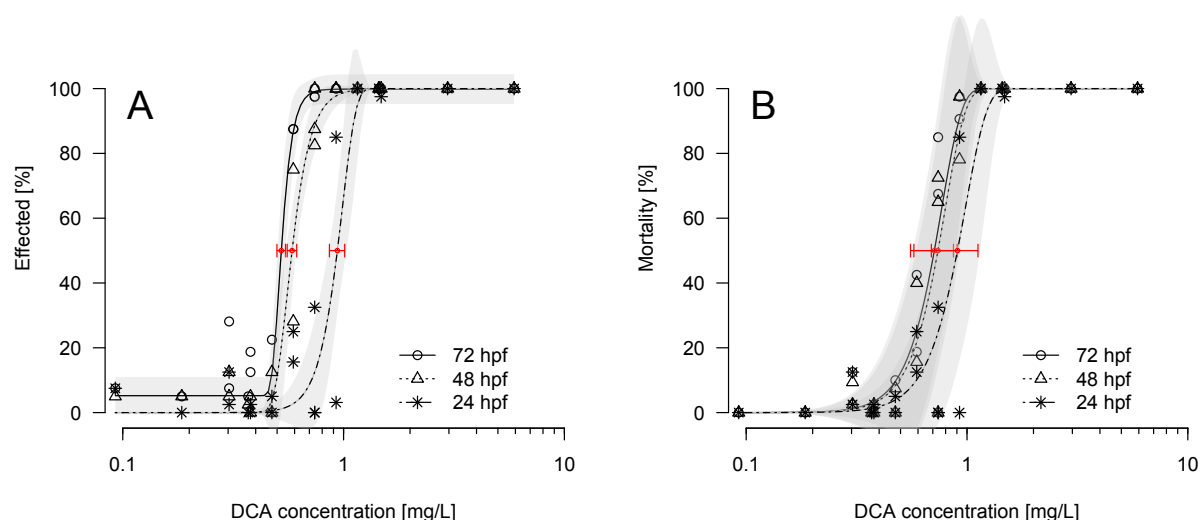
Fig. 7. Dose-response curves with associated 95% confidence intervals (grey) for three acute fish embryo tests (FET) conducted with weatherfish embryos exposed for 24, 48 and 72 h to 3,4-dichloroaniline (DCA): sublethal (A) and lethal (B) effects. Red dots indicate EC_{50} and LC_{50} values with the corresponding 95% confidence interval, respectively. Figure taken from *Appendix III*.

Lethal (LC_X) and effect concentrations (EC_X), including confidence intervals (CI) calculated on the basis of all three main tests conducted with DCA (72 h) were 0.52 (0.50-0.55) mg/l (EC_{50}) and 0.71 (0.55-0.87) mg/l (LC_{50}), respectively. Even if DCA is known to induce dose-response curves with steep slopes (Lange et al., 1995) (Fig. 7), only slight deviations were observed between all tests conducted with weatherfish embryos (mean EC_{50} ± standard deviation (SD): 0.58±0.04 mg/l; mean LC_{50}±SD: 0.71±0.11 mg/l), indicating good reproducibility of results. An inter-specific comparison of EC_{50} and LC_{50} values based on literature data (Braunbeck et al., 2005; Braunbeck & Lammer, 2006) revealed that weatherfish embryos are more sensitive to DCA than embryos of zebrafish (factor of three), fathead minnow (factor of 11) and Japanese medaka (factor of 40). As all latter mentioned species are warm-adapted, the recommended test temperatures (25-26° C) exceed the temperature optimum documented for weatherfish embryos (15-24° C) (Drozd et al., 2009). Therefore, weatherfish embryos were tested at lower temperatures (20±1° C), which might decrease or increase toxicity of certain substances (Li et al., 2014). However, Schäfers & Nagel (1993) showed that toxicity of DCA was not influenced by temperature when testing early life stages of perch (*Perca fluviatilis*).

Table 3. Teratogenic effects (grouped as lethal, heavy, medium, slight) in weatherfish embryos exposed to different oxygen concentrations. Given are oxygen concentrations and the associated 95% confidence intervals at which a certain proportion (10, 20, 50, or 90%) was affected. Footnotes indicate a list of effects that were taken into account (see table captions). Table taken from *Appendix III*.

	Proportion affected			
	10%	20%	50%	90%
Intensity of effects	Oxygen concentration (mg/l)			
Lethal[†]	0.85 (0.70-1.00)	0.76 (0.66-0.85)	0.64 (0.59-0.68)	0.53 (0.47-0.59)
Heavy[‡]	1.04 (0.76-1.33)	0.88 (0.72-1.04)	0.68 (0.61-0.75)	0.52 (0.42-0.61)
Medium[§]	1.52 (1.16-1.89)	1.31 (1.09-1.53)	1.04 (0.96-1.13)	0.82 (0.73-0.90)
Slight[¶]	2.18 (1.25-3.11)	1.71 (1.18-2.23)	1.18 (1.00-1.37)	0.80 (0.65-0.95)

[†] coagulation, lack of heartbeat
[‡] coagulation, lack of heartbeat, severe deformation
[§] coagulation, lack of heartbeat, severe deformation, deformation, edema
[¶] coagulation, lack of heartbeat, severe deformation, deformation, slight deformation, edema, delayed development

Based on the results on oxygen requirements of weatherfish embryos, even slight effects caused by DO scarcity can be excluded at concentrations ≥ 2.2 mg/l (i.e. EC_{10} for slight effects) (Table 3). On the other hand, DO concentrations ≤ 0.53 mg/l induced 90% mortality of weatherfish embryos (i.e. LC_{90}). Comparing these results to the DO tolerance of temperate fish species from north-western Europe (lowest observed effect concentrations (LOEC): 3-9 mg/l; LC_{100} values: 1.5-4.8 mg/l) (Elshout et al., 2013), weatherfish embryos might be most tolerant against low DO levels. With regard to the applicability of weatherfish for FETs, it can be concluded that DO should not limit the feasibility since concentrations >2 mg/l were reported as unproblematic for zebrafish embryos (Strecker et al., 2011) and this value can also be adopted for weatherfish embryos.

BOX 4.1: CONCLUSIONS – FEASIBILITY FOR TOXICITY TESTING

An existing test protocol for investigations of adverse effects on fish embryos was successfully transferred to weatherfish. The obtained results demonstrated a high robustness of weatherfish embryos for experimental studies and a high tolerance against DO depletion. Furthermore, the sensitivity of weatherfish embryos towards a tested reference substance was highest compared to other species and three times higher than reported for the commonly used zebrafish. As an endangered species that occurs in agriculturally impacted landscapes, the ecological relevance for European surface waters might additionally qualify the weatherfish as a prospective species in toxicity testing. In the context of weatherfish conservation, the findings represent a methodical foundation to assess the risks posed by chemical stress and therefore a conceptual and methodological basis for further investigations.

4.2 EVALUATION OF MULTIPLE SEDIMENT BIOASSAYS WITH WEATHERFISH

As described above, a toxicity test method for the determination of effects derived from contaminants dissolved in water was successfully transferred to weatherfish. However, as the benthic weatherfish is likely to be exposed to sediment-associated contaminants, it is important to additionally provide methods that can be applied for sediment toxicity testing. In order to evaluate various sediment bioassays, weatherfish embryos were exposed to sediments from four sites (hereafter referred to with the acronyms ST, ES, AL, HH) with assumedly differing contamination levels by applying SCAs (96 h) with whole freeze-dried sediments and FETs (96 h) with aqueous solutions gained by accelerated solvent extraction (ASE). The latter included acetone extracts (ex-ACE) and extracts produced by PHWE (ex-PHWE). In addition, zebrafish embryos were tested in FETs (96 h) with ex-ACE and ex-PHWE from the two most contaminated sediments (i.e. AL and HH) in order to compare the results to a frequently used test organism (Fig. 8). The contamination levels of all tested extracts were analyzed regarding polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs). Furthermore, total lipid content and fatty acid composition of unfertilized weatherfish eggs were quantified.
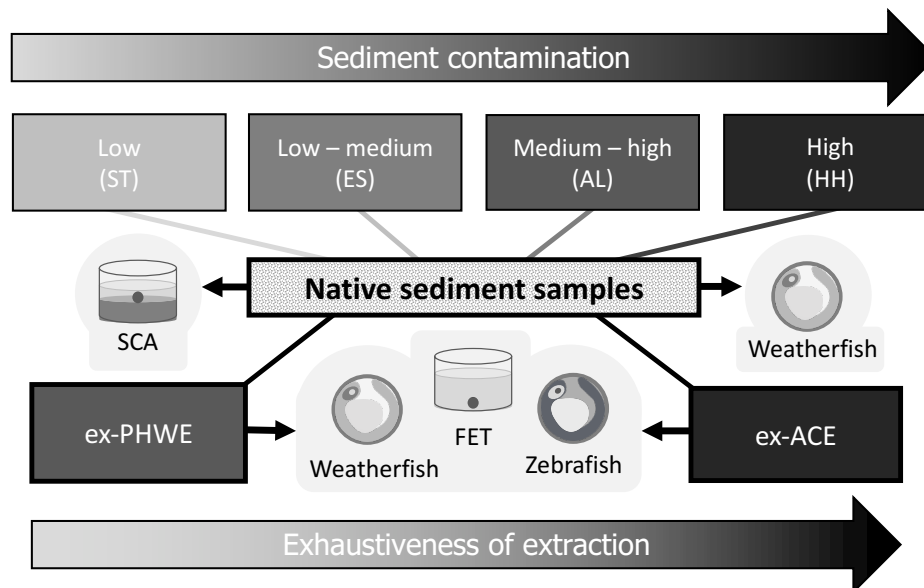
Fig. 8. Structure of the experimental approach: Four native sediment samples (ST, ES, AL, HH) of varying contamination degree were tested in sediment contact assays (SCA) with weatherfish embryos. Furthermore, extracts gained by pressurized hot water extraction (ex-PHWE; low exhaustiveness) and acetone extraction (ex-ACE; high exhaustiveness) were tested in acute fish embryo toxicity tests (FET) with weatherfish and zebrafish embryos. Figure taken from *Appendix IV*.

Regarding the results derived from FETs with weatherfish embryos, mortality rates were ≤5% in all negative (i.e. artificial water alone) and method controls (i.e. artificial water including 1% of the additional solvent dimethyl sulfoxide (DMSO) and extracts from quartz sand extraction). Therefore, it can be concluded that weatherfish embryos generally met the requirements for FETs with sediment extracts and that an influence from DMSO (1%) on survival could be excluded. Furthermore, LC_{50} values could be calculated for all sediments tested as ex-ACE and for three of four sediments tested as ex-PHWE (i.e. ES, AL, HH) (Fig. 9).
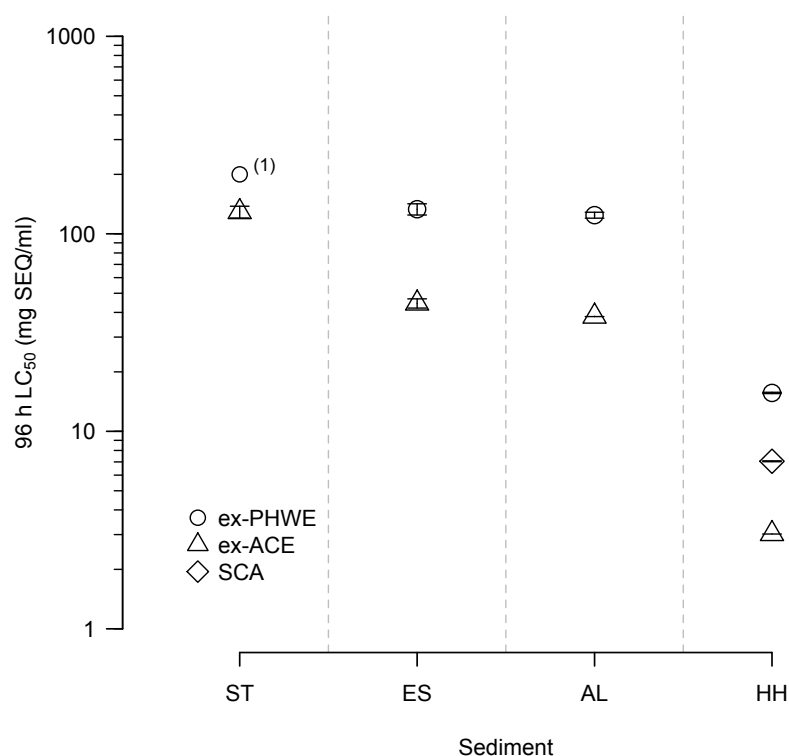
Fig. 9. 96 h LC_{50} values and the corresponding standard errors (SE) of weatherfish embryos, given for sediments from ST, ES, AL, and HH. All sediments were tested as pressurized hot water extracts (ex-PHWE; ○), as acetone extracts (ex-ACE; △) and in a sediment contact assay (SCA; ◇). However, in the case of SCA, calculation of LC_{50} value was only feasible for HH. Symbol labeled with (1) indicates that no LC_{50} value could be calculated but given value corresponds to the highest concentration tested (200 mg SEQ/ml) at which <50% of weatherfish embryos died. Figure taken from *Appendix IV*.

When comparing the LC_{50} values derived from FETs with weatherfish embryos (Fig. 9), both extract types (i.e. ex-ACE and ex-PHWE) revealed the same toxicity gradient of tested sediments (i.e. ST<ES<AL<HH). Previously published studies from Schiwy et al. (2015) and Hafner et al. (2015) also revealed this toxicity gradient for sediments from ES, AL and HH, when tested inter alia in SCAs or as Soxhlet-generated acetone extracts in FETs (both with zebrafish embryos). Hence, the observed compliance indicates a good reliability of sediment toxicity evaluations based on FETs with weatherfish embryos exposed to sediment extracts.

The higher toxicity of ex-ACE compared to ex-PHWE (Fig. 9) supports the assumption that organic solvent extracts represent worst-case scenarios (Kosmehl et al., 2007; Seiler et al., 2008). More specifically, the ability of organic solvents to recover sequestered compounds from sediments that are unavailable under natural conditions (Kelsey et al., 1997; Mayer & Reichenberger, 2006) might overestimate sediment toxicity when applied in bioassays. Therefore, acetone extracts risk to bias an ERA (Brack et al., 2009). On the other hand, the lower toxicity of ex-PHWE supports the initially hypothesized intermediate character of the underlying extraction method (Hawthorne et al., 2000). Considering that PHWE was already successfully applied to simulate bioavailability of contaminants (Tao et al., 2006; Konda et al., 2002), there is increasing evidence that it might also be a promising method for an application in ERA of sediment toxicity with fish embryos.
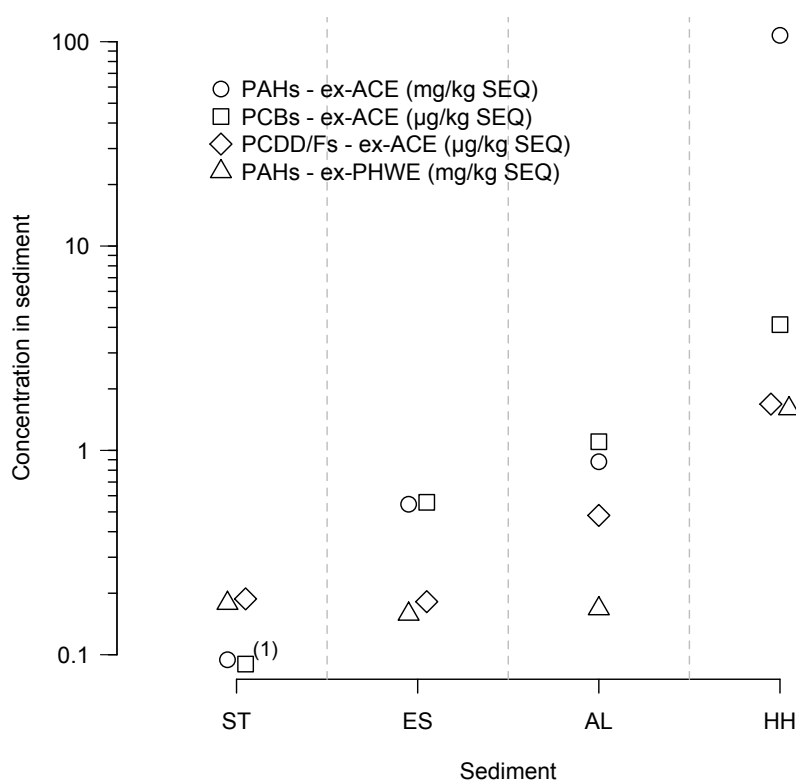
Fig. 10. Sum concentrations of PAHs (○/△), PCBs (□) and PCDD/Fs (◇), measured in acetone extracts (ex-ACE) and pressurized hot water extracts (ex-PHWE) of sediments from ST, ES, AL, and HH. Symbol labeled with (1) indicates that all measured substances were below the limit of quantification (PCDD/Fs in ex-ACE from ST). Sum concentrations of PCBs and PCDD/Fs in ex-PHWE are not shown because all measured substances were below the limit of quantification. Figure taken from *Appendix VI*.

SCAs conducted with weatherfish embryos revealed that the main requirements for an application of this bioassay could also be fulfilled as mortality rates were 0% in negative controls conducted with and without quartz sand. However, effect concentrations could only be calculated for the most toxic sediment from HH as high concentrations (>600 mg SEQ/ml) of the other sediments (i.e. ST, ES, AL) led to limited free water volume and DO scarcity in the test system. Problems resulting from this might be that the quantified toxicity effects cannot be allocated to the decisive cause. Comparable limitations when applying SCAs were also reported for zebrafish embryos (Schiwy et al., 2015; Strecker, 2013). However, the intermediate toxicity observed in the SCA conducted with sediment from HH (Fig. 9) supports the assumption that contaminant uptake via direct contact plays a major role (Hollert et al., 2003).

The results from chemical analyses of ex-ACE revealed mainly the same contamination gradient that was determined based on FETs with weatherfish embryos (ST<ES<AL<HH) (Fig. 9; Fig. 10). A comparison of measured contamination with effect concentrations was restricted for ex-PHWE because most chemicals (PCBs and PCDD/Fs) were below the limit of quantification in sediments from ST, ES and AL.
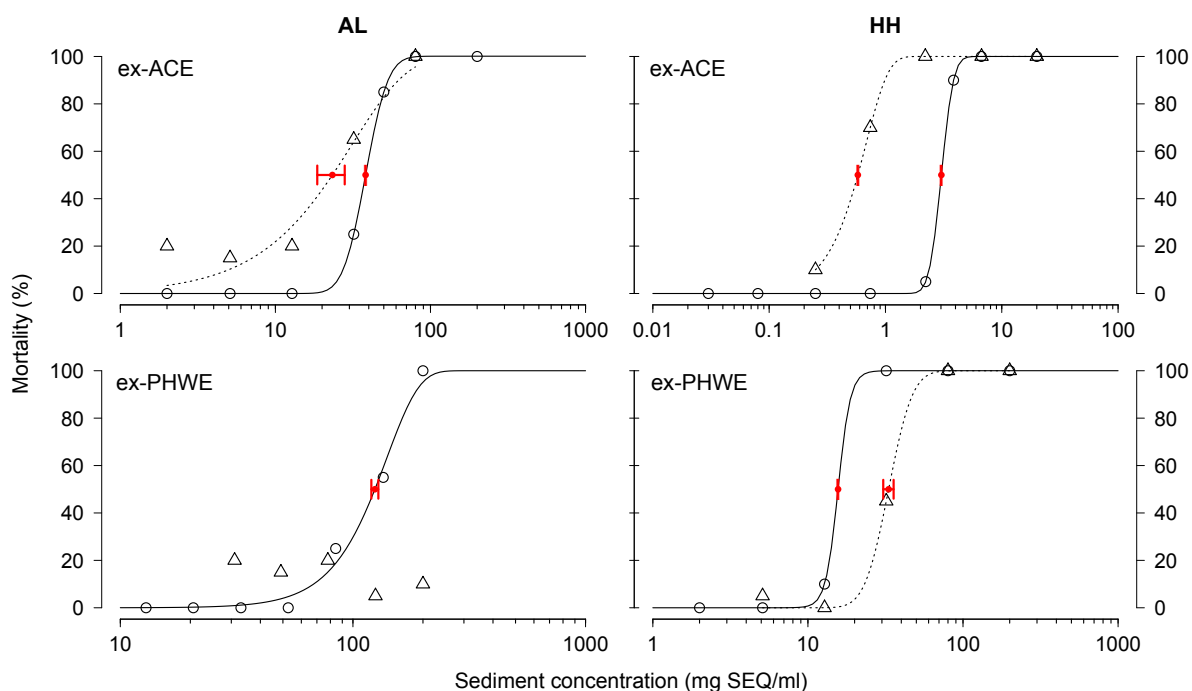
Fig. 11. Concentration-response curves (mortality) for weatherfish (○; solid lines) and zebrafish (△; dashed lines) embryos exposed for 96 h to acetone extracts (ex-ACE) and pressurized hot water extracts (ex-PHWE) of sediments from AL (left) and HH (right). Red dots with error bars indicate LC_{50} values with the corresponding standard errors (SE). No LC_{50} value could be calculated for zebrafish embryos exposed to ex-PHWE from AL as even the highest test concentration (200 mg SEQ/ml) induced ≤50% mortality. Figure taken from *Appendix IV*.

The LC$_{50}$ values derived from FETs conducted with weatherfish and zebrafish embryos revealed that differences in sensitivity were exposure scenario specific: zebrafish embryos showed higher sensitivity to ex-ACE, whereas weatherfish embryos exhibited higher sensitivity to ex-PHWE (Fig. 11). With regard to the differences in polarity of the used solvents (water vs. acetone), it can be assumed that ex-PHWE might contain more compounds of higher polarity (e.g. chlorinated phenols) and ionic compounds (e.g. heavy metals), whereas ex-ACE might predominately contain compounds with low-polarity (Hawthorne et al., 1994). This assumption could partly be confirmed by the results from chemical analyses, as the sums of low-polarity compounds, including PAHs, PCBs and PCDD/Fs, were higher in ex-ACE than in ex-PHWE (Fig. 10). However, as sediments can be burdened by complex mixtures of contaminants, an identification of cause-effect relationships traced to single compounds or compound classes is highly challenging (Schiwy et al., 2015).

BOX 4.2: CONCLUSIONS – SEDIMENT BIOASSAYS

The presented investigations successfully applied weatherfish embryos for a variety of bioassays that might be used for an ERA of sediment toxicity. As the gradient of effect concentrations derived from FETs with sediment extracts complied with analytically measured sediment contaminations, as well as with results reported in previous studies, a good reliability of data obtained by weatherfish testing can be assumed. However, an application of weatherfish embryos for SCAs was limited to sediment with high contamination levels. In comparison to zebrafish, weatherfish embryos displayed significantly higher sensitivity towards extracts gained by PHWE, a promising alternative extraction method for more realistic bioavailability simulation in sediment toxicity bioassays. With regard to conservational issues, the risk posed by sediment contamination might be a critical factor for weatherfish that should be considered for an assessment of potential stocking waters.

CONCLUSIONS AND OUTLOOK

The weatherfish is a species that stayed below the radar of fish conservation efforts for a long time even though many unique features emphasized it as an exceptional representative of the European ichthyofauna. In order to counteract the critical situation of weatherfish, the present thesis achieved contributions to its conservation on different levels.

The presented reintroduction and stock enhancement program for weatherfish represents a conservation approach with focus on direct intervention on a regional scale. However, the success of such measures depends on thorough planning while respecting a variety of factors, always under consideration of the species ecology. The present thesis provided information about weatherfish breeding, emphasized significant criteria for the selection of reintroduction sites and recommended improvements for future activities. Furthermore, the provided course of action might be successfully transferred to further federal states and countries, or even partly to other species, and thereby contribute to stable populations on larger scales. The presented results on thermal requirements of weatherfish larvae are directly applicable with regard to stocking measures, as they broaden the knowledge about its autecology. More specifically, provided information about T_{Opt} and T_C of the particularly sensitive weatherfish larvae helped to refine rearing conditions and to improve the identification of suitable waters for stocking. Furthermore, the provided data can be utilized in the development of temperature related distribution models or interspecific comparisons of temperature tolerance and thereby contribute to the conservation of other species than weatherfish.

The presented investigations on potential threats posed by chemical stress provided a methodical basis for toxicity testing with weatherfish embryos. Moreover, since first results indicated a high sensitivity of weatherfish to the tested reference substance, chemical stress might constitute a considerable risk for the species. However, an exposure of weatherfish embryos to further substances with varying mode of actions can contribute to deeper understanding of the severity of this risk. As weatherfish embryos were also successfully applied for different sediment bioassays, first investigations on the hazard potential of sediment-associated contaminants for weatherfish could be presented. Here, the demonstrated sensitivity of weatherfish embryos to water extractable and consequently easily bioavailable substances indicated that sediment contamination might be among the reasons for population

declines, especially because of the species benthic lifestyle and the resulting proximity to the hazard. With regard to an ERA of sediment toxicity in Europe, the application of weatherfish embryos for sediment bioassays could allow investigations with a temperate species of high ecological relevance, while respecting animal welfare concerns. Consequently, a reduction of animal testing and improvements for the protection of native (i.e. European) fishes might be achieved, potentially leading to benefits for various species.

Overall, the present thesis demonstrated that different efforts conducted for the conservation of weatherfish can be bundled in a targeted manner in order to achieve long-lasting improvements. Thorough knowledge about weatherfish autecology (e.g. thermal requirements; tolerance against DO scarcity) can not only increase the efficiency of weatherfish rearing or the prospects of stocking measures, but also provides essential basics for experimental approaches (e.g. toxicity testing), as it helps to adjust test conditions to species requirements. Furthermore, the presented investigations on threats posed by chemical stress can be integrated into reintroduction programs for weatherfish in order to evaluate the suitability of potential stocking waters. For this purpose, on-site sediment samples could be tested in bioassays with weatherfish embryos in order to evaluate the hazard potential for the species. Consequently, investigations on all research objectives pursued within the present thesis, not only contributed to the conservation of weatherfish individually, but rather collaboratively and further synergies might still be discovered on this basis.

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DECLARATION

I hereby declare that I independently conducted the work presented in this PhD thesis with the title "Conservation of the European weatherfish *Misgurnus fossilis* – Stocking measures, autecology and potential threats". All used assistances and involved contributors are mentioned either as co-authors or in the acknowledgements of the respective publication. This thesis has never been submitted elsewhere for an examination, as a thesis or for evaluation in a similar context to any department of this university or any scientific institution. I am aware that a violation of the aforementioned conditions can have legal consequences.

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Place, Date   Signature

(Benjamin Schreiber)

CONTRIBUTIONS TO APPENDIX I-IV

APPENDIX I

Benjamin Schreiber, Egbert Korte and Ralf Schulz conceived and designed the study. Weatherfish breeding and rearing, as well as monitoring and stocking measures were conducted by Benjamin Schreiber and Egbert Korte. Thomas Schmidt conducted the geographical representation of the data. The first draft of the manuscript was written by Benjamin Schreiber and all other authors contributed to the final version.

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APPENDIX II

Benjamin Schreiber, Matthias Hundt and Ralf Schulz conceived and designed the study. The experiments were conducted by Benjamin Schreiber and Julian Monka. Benjamin Schreiber conducted the statistical analyzation of the data and wrote the first draft of the manuscript. All other authors contributed to the final version of the manuscript.

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APPENDIX III

Benjamin Schreiber, Henner Hollert and Ralf Schulz conceived and designed the study. The experiments were conducted by Benjamin Schreiber, Marius Petrenz and Julian Monka. Benjamin Schreiber analyzed the data statistically and wrote the first draft of the manuscript and all other authors contributed to the final version of the manuscript.

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APPENDIX IV

Benjamin Schreiber, Jonas Fischer, Henner Hollert and Ralf Schulz contributed to the design of the study. Benjamin Schreiber and Jonas Fischer conducted the experiments. Benjamin Schreiber analyzed the data statistically and wrote the first draft of the manuscript and all other authors contributed to the final version of the manuscript.

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APPENDICES

REINTRODUCTION AND STOCK ENHANCEMENT OF EUROPEAN WEATHERFISH (*Misgurnus fossilis* L.) IN RHINELAND-PALATINATE AND HESSE, GERMANY

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The European weatherfish (*Misgurnus fossilis* L.) belongs to the family Cobitidae and occurs in densely vegetated waters with muddy substratum, including oxbows, flood plains or ditch systems (Kottelat & Freyhof, 2007; Lelek, 1987). It is a demersal species native to Europe that can be found north of the alps, from the Volga basin to France (Lelek, 1987). Presumably, as a result of habitat degradation, habitat management (i.e. dredging) and water pollution, weatherfish populations declined or even became extinct in many regions across its original range (Hartvich, Lusk, & Rutkayová, 2010). Today, the weatherfish is listed under Annex II of the Habitats Directive (92/43/EEC) and classified as vulnerable or nearly extinct on Red Lists of numerous countries, including Germany (E.U., 1992; Hartvich, Lusk, & Rutkayová, 2010). The Red Lists of the German federal states Rhineland-Palatinate and Hesse classify weatherfish as highly endangered (Dümpelmann & Korte, 2013; Röter-Flechtner & Simon, 2015)

In order to counteract the ongoing decline of weatherfish populations, a regional joint conservation program in Rhineland-Palatinate and Hesse was initiated in 2014. The program area lies at the south-western boundary of the species distribution range and is supposed to play a significant role for weatherfish conservation on a national level as the Rhine floodplains provide rare primary habitats for this species (Copp, 1989; Dister, Gomer, Obrdlik, Petermann & Schneider, 1990). Main objectives of the program were determined as (i) the support of existing weatherfish populations by stock enhancement and (ii) reintroduction of weatherfish to waters with suitable conditions where the specific reasons for its eradication can be excluded. For both objectives, hatchery-reared weatherfish fry derived from local broodstock should be utilized (Lelek, 1987).

As a first step, a large-scale monitoring within the study area was carried out to identify relic weatherfish populations for broodstock sampling. Furthermore, habitat assessments identified potential waters for reintroduction measures that meet the species requirements. In this context, special attention was given to good connectivity and year-round water bearing, none to moderate maintenance practices (e.g. mowing of the banks), presence of spawning habitats (i.e. connected flood plains) as well as absence of nocturnal predators (in particular the European eel (*Anguilla anguilla* L.)).

Weatherfish spawners were caught with baited fish traps at the beginning of the spawning season (March to April) and kept separated by populations origin at ≤11 °C, until further treatment. In order to induce stripping, males and females were isolated from each other and water temperature was gradually increased to 18 °C, before a gonadotropin-releasing hormone (GnRH) containing preparation (Ovopel, Unic-trade, Hungary) was intramuscularly injected in two consecutive dosages. Stripped eggs of one female were fertilized with sperm of

two males and subsequently transferred to hatching troughs. Incubation of embryos (18 °C) and rearing of larvae (21 °C) was carried out in recirculating aquaculture systems. Weatherfish fry were fed with decapsulated *Artemia salina* nauplii and reared until they reached a size of approximately 2 to 4 cm, which took 4 to 8 weeks. This rearing duration was chosen as it represents a feasible compromise between the aim of overcoming the most sensitive life span on the one hand, and avoiding an irreversible adaptation to artificial conditions on the other hand. After a recovery period of several weeks, stripped spawners were released to their origin without observable fitness restrictions.

Stocking was carried out in several batches per year and site by evenly distributing weatherfish fry in shallow and densely vegetated water stretches. Stocking batches were divided to apply the principle of risk diversification because weatherfish habitats often suffer from unpredictable fluctuations in water level (Meyer & Hinrichs, 2000), which might negatively influence the survival rate of a stocking batch. One half of the cultured fry was released in the same waters where the broodstock originated (i.e. endogenous enhancement), the other half was used for reintroductions to habitats that were previously assessed as suitable (Table 1). The only exception to this procedure happened at waters of the Schwarzbach catchment, where stock enhancement was carried with fry that came from the Horloff catchment (i.e. exogenous enhancement).

The stocking success was monitored bi-annually beginning in late summer of the first year of stocking (2014). The last monitoring that was considered in the dataset presented here took place in late summer of 2016. As stocked individuals could not be distinguished from previously occurring weatherfish, only waters selected for reintroduction could be used for a reliable assessment of stocking success.
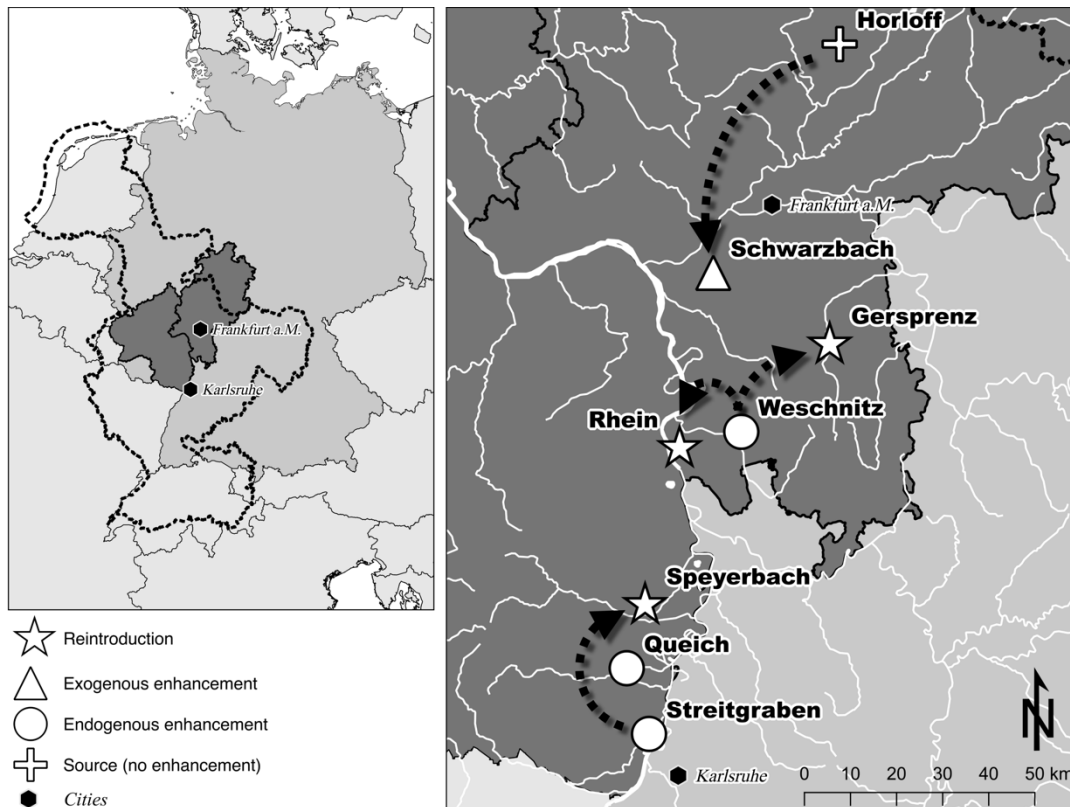
Fig. 1. Map of the study area. Overview map on the left shows the location of the federal states of Rhineland-Palatinate and Hesse (dark grey) in Germany (grey) and Europe (light grey; GADM, 2012). The dotted black line indicates the international Rhine river basin district (European Environment Agency, 2011). Detailed map on the right shows the sites used for the different stocking measures (reintroduction, exogenous enhancement, endogenous enhancement) and the broodstock source used where no enhancement took place. Black arrows indicate the broodstock origin used for reintroductions. White lines show main rivers (GADM, 2012).

In the initial monitoring, weatherfish populations suitable for broodstock sampling could be detected in four water systems (i.e. Streitgraben, Weschnitz, Queich, Horloff) and further three systems were assessed as suitable for weatherfish reintroduction (i.e. Speyerbach, Gersprenz, Rhein) (Fig. 1; Table 1). During the study period (2014-2016), a total amount of approximately 275,000 eggs was gained from 38 female spawners (mean total length (L_T) ± standard deviation (SD) = 202±27 mm; mean wet mass (M_W) ± SD = 47±15 g). From these eggs, approximately 168,500 weatherfish reached the juvenile stage (resulting mortality rate: 39%) and were released to the designated waters (Table 1). In the Gersprenz catchment, seven individuals (size class: 7-8 cm) were recaptured in 2015 and one individual (10 cm) in 2016. In the Rhein catchment, 37 individuals of two size classes (10-14 cm (34 individuals); 16-18 cm (3 individuals)) were recaptured in 2016.

Table 1. Number of stocked weatherfish subdivided into stock enhancement and reintroduction measures, including year of stocking, stocked catchment and catchment of broodstock origin. For a geographical orientation, the reader is referred to Fig. 1.

| Year | Stock enhancement | | Reintroduction | | |
	Stocked catchment	No. of stocked individuals	Stocked catchment	No. of stocked individuals	Catchment of broodstock origin
2014	Streitgraben[†]	24,000	Speyerbach	11,000	Streitgraben
2014	Weschnitz[†]	12,000	Gersprenz	13,000	Weschnitz
Total 2014	-	36,000	-	24,000	-
2015	Streitgraben[†]	13,000	Speyerbach	7,500	Streitgraben
2015	Queich[†]	10,000	Gersprenz	8,000	Weschnitz
2015	-	-	Rhein	18,000	Weschnitz
Total 2015	-	23,000	-	33,500	-
2016	Streitgraben[†]	6,000	Speyerbach	14,000	Streitgraben
2016	Schwarzbach[‡]	20,000	Gersprenz	5,000	Horloff
2016	-	-	Rhein	7,000	Horloff
Total 2016	-	26,000	-	26,000	-
Total	-	85,000	-	83,500	-

[†] Stocking site is equivalent to the site of broodstock origin (i.e. endogenous enhancement).

[‡] Broodstock originates from the Horloff catchment (i.e. exogenous enhancement).

The low number of weatherfish populations identified during the initial monitoring illustrates the species critical situation within the study area and consequently underlines the need for conservation measures. However, the successful determination of weatherfish refuges ensured the opportunity of broodstock sampling from populations that might be genetically adapted to the local conditions – a factor considered crucial for reintroduction success (Cochran-Biederman, Wyman, French, & Loppnow, 2014; Weeks et al., 2011). Numerous ditch systems located in the study area generally met the species requirements, but lacked suitable spawning habitats like floodplains because ditches typically prevent water from bursting its banks (Dollinger, Dagès, Bailly, Lagacherie, & Voltz, 2015). This observation might explain the frequently reported absence of juvenile weatherfish in comparable systems and leads to the assumption that successful reproduction in ditches might be limited to years with high water

levels in spring. As the long-term absence of appropriate spawning habitats can result in a lack of reproduction success, stock enhancement might be particularly effective in these systems.

The methods applied for artificial propagation and rearing of weatherfish can be stated as feasible for the objectives pursued in the present program as sufficient stocking material could be provided every year ($\geq$ 52,000 juveniles per year). A good feasibility of weatherfish production was previously reported in several studies (Demény et al., 2009; Kouril et al., 1996; Schauer, Ratschan, Wanzenböck, Gumpinger, & Zauner, 2013). However, comprehensive investigations on the thermal requirements of weatherfish larvae carried out along with the program helped to increase their survival and growth rates during the rearing period (Schreiber et al., 2017). This illustrates how scientific guidance of different steps in comparable conservation programs may improve the prospects and hence is explicitly recommended.

The total number of individuals recaptured at two reintroduction sites (45) can be considered as relatively high because traditional capturing methods (e.g. traps or electric-fishing) were frequently reported as inefficient when applied for weatherfish (Meyer & Hinrichs, 2000; Sigsgaard, Carl, Møller & Thomsen, 2015). A solution to these problems might be the implementation of environmental DNA (eDNA) monitoring (Sigsgaard et al., 2015). An expansion of catching efforts and eDNA sampling is planned for the near future, especially for the third so far unsuccessfully monitored reintroduction site (Speyerbach). However, the clear size classification of recaptured individuals indicates perennial reintroduction success in terms of survival of stocked weatherfish. Therefore, it can be assumed that the conducted habitat assessment indicated suitable waters for reintroduction and that the stocking strategy is generally appropriate (for reintroduction and stock enhancement). Since weatherfish reach maturity not earlier than after two to three years (Kottelat & Freyhof, 2007), a confirmation of successful reproduction, and thus of self-sustainable reintroduced populations (IUCN, 1998), is still pending. However, as the presence of appropriate spawning habitats was chosen as a factor of primary importance, reproduction success can be stated to be a realistic prospect. In order to assess the success of stock enhancement, the establishment of a chemical marking protocol for weatherfish fry is conceivable (e.g. with oxytetracycline). However, marking success and mortality during marking are hard to predict as the outcome of both is driven by various factors (Hundt et al., 2015). Furthermore, a parental assignment based on microsatellite markers can be a promising alternative, but the polyploidy of weatherfish might limit the feasibility of this method (Dozd, Flajšhans, & Ráb, 2010; Zhao et al., 2015).

As a conclusion, regarding the documented success of reintroduction and stock enhancement measures conducted with hatchery-reared weatherfish fry, the present approach

might be effective for further federal states and countries with critical populations. However, for sustainable improvements, stocking has to be maintained in future and individual steps can be refined.

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THERMAL REQUIREMENTS FOR GROWTH, SURVIVAL AND AEROBIC PERFORMANCE OF WEATHERFISH LARVAE *Misgurnus fossilis*

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ABSTRACT

Thermal requirements of larval weatherfish *Misgurnus fossilis* were investigated in terms of growth, survival and aerobic performance. Growth and survival of *M. fossilis* larvae acclimated to five temperatures (11, 15, 19, 23 and 27° C) were measured over 25 days. In the upper temperature treatments (19, 23 and 27° C), survival of larvae was stable throughout the entire rearing period (>75%), whereas 11 and 15° C resulted in severe declines in survival (to <10%). Growth of larvae (expressed as dry mass and total length) was highest at 19° C and 23° C but significantly decreased at 27° C. Routine metabolic rate of three days post-hatch (dph) larvae was estimated as oxygen consumption rate ($\dot{M}O_2$) during acute exposure (30 min to 1 h) to seven temperatures (11, 15, 19, 23, 27, 31 and 35° C). Larval oxygen uptake increased with each consecutive temperature step from 11 to 27° C, until a plateau was reached at temperatures >27° C. However, all larvae of the 35° C regime died within the $\dot{M}O_2$ measurement period. *M. fossilis* larvae show greater than expected tolerance of high temperatures. On the other hand, low temperatures that are within the range of likely habitat conditions are critical because they might lead to high mortality rates when larvae are exposed over time periods >10 days. These findings help to improve rearing conditions and to identify suitable waters for stocking, and thus support the management of reintroduction activities for endangered *M. fossilis*.

INTRODUCTION

The European weatherfish *Misgurnus fossilis* L. 1758 has a wide distribution throughout Europe - originally ranging from northwest France to western Russia (Lelek, 1987; Kottelat & Freyhof, 2007). Primary habitats are oxbows, backwaters and periodically flooded meadows, all containing muddy sediments and aquatic vegetation (Pekárik *et al.*, 2008; Hartvich *et al.*, 2010). In modern agricultural landscapes, *M. fossilis* populations also inhabit drainage channels with comparable conditions (Meyer & Hinrichs, 2000; Sigsgaard *et al.*, 2015). Despite its adaptation to oxygen deficient habitats – *M. fossilis* has the ability for cutaneous respiration and oxygen uptake via its gut - it has exhibited a distinctive decline in many regions (Hartvich *et al.*, 2010). As a result, the species is listed as vulnerable or nearly extinct across its original range, including Germany, Hungary, Czech Republic, Slovenia, Austria, Switzerland, Slovakia and Denmark (Zulka & Wallner, 2006; Haupt *et al.*, 2009; Sigsgaard *et al.*, 2015). The European Union listed *M. fossilis* under Annex II of the Council Directive 92/43/EEC, representing species of community interest that need Special Areas of Conservation (SAC)

(Council of the European Union, 1992). The main reasons for the decline are assumed to be habitat loss, ditch maintenance practices and degradation of water quality by pollution (Lelek, 1987; Kouril et al., 1996; Meyer & Hinrichs, 2000). Promising conservation measures include stock enhancement and reintroduction activities, conducted with fry reared within captivity (Lelek, 1987; Demény et al., 2009). The artificial propagation of M. fossilis has been successfully demonstrated and rearing of its larvae can be carried out under controlled conditions in aquaculture systems (Geldhauser, 1992; Kouril et al., 1996; Demény et al., 2009). However, a production of vigorous stock material, as well as implementation of stocking measures presupposes an accurate consideration of ecological requirements. This is particularly important for early life stages, since they often represent the most sensitive period in the life cycle of fishes and are therefore considered a bottleneck for maintenance of stable populations (Houde, 2002). In this context, water temperature is an important abiotic factor because it influences functions at various levels in ectothermic organisms, including foraging ability, behavior, growth and survival (Beitinger et al., 2000; Angilletta et al., 2002; Killen et al., 2013). Furthermore, thermal tolerance of fishes is typically narrowest in eggs and early larval stages (Brett, 1956; Rombough, 1997; Pörtner & Farrell, 2008).

Due to the lack of economic value and the cryptic behaviour of M. fossilis (Sigsgaard et al., 2015) few data are available about thermal requirements of this species. Available data include effects of temperature on developing embryos (Drozd et al., 2009) and measured temperature ranges in occupied waters (FishBase Consortium, 2016). Temperature effects on the larval stage, including an integration of physiological responses, have not been investigated.

In order to assess thermal requirements, fish larvae are acclimated to environmentally realistic temperatures with an exposure duration adapted to seasonal temperature fluctuations (Selong et al., 2001; Zale, 1984). Besides variables like growth and survival that are routinely monitored during these experiments, the detection of physiological responses to thermal stress (e.g. oxygen consumption rate ($\dot{M}O_2$)) can provide additional insights into optimal (T_{Opt}) as well as critical (T_C) temperatures for fishes (Clark et al., 2013; Li et al., 2014; Pörtner & Peck, 2010). In this study, the above mentioned approaches were combined to determine the temperature optimum and physiological constraints at critical temperatures of M. fossilis larvae. For this purpose, artificially propagated M. fossilis larvae were studied using two methods under various temperature regimes: (i) a thermal acclimation, including five temperatures and a rearing period of 25 days (measuring growth and survival); and (ii) $\dot{M}O_2$ measurements during acute exposure (30 min to 1 h) to seven temperatures.

MATERIALS AND METHODS

ORIGIN AND MAINTENANCE OF FISH

All experiments were carried out using *M. fossilis* larvae from artificial propagation of wild spawners. Adult *M. fossilis* were collected in March 2014 from stable wild populations in Hesse, Germany (Kreuzlachgraben; 49° 40' 25.5" N; 8° 34' 56.1" E) and transported to the Eusserthal research facility of the University of Koblenz-Landau, Germany. During a 30 day acclimation period (11° C), adult *M. fossilis* were kept outdoors in 800 l tanks provided with sediment and submersed macrophytes (*Elodea* spec.). Propagation of the spawners was induced by a gradual temperature increase from 11 to 18° C, followed by a treatment with a gonadotropine-releasing hormone (GnRH) analogue and metoclopramide-containing hormonal preparation (Ovopel, Unic-trade; www.ovopel.hu). The total dose of the hormonal preparation (1 pellet kg^{-1} body mass) was applied by two intramuscular injections with a time interval of 12 h. All treated fish received the same dose, independent of sex. Gametes from 3 females (L_T = 21.2±1.8 cm; M_W = 54.4±11.1 g) and 8 males (L_T = 18.6±0.6 cm; M_W = 27.6±2.0 g) were obtained approximately 24 h after the second injection. Stripped eggs of all females were poured together and fertilized using the dry method (Drozd *et al.*, 2009). Incubation of the eggs was carried out in a hatching trough connected to a recirculating aquaculture system (RAS) at a water temperature of 18.5±1.2° C. Hatching of *M. fossilis* larvae occurred 62 – 70 h after fertilization. Two days post-hatch (dph) old larvae were transferred to aquaria (100 l; approximately 50 larvae l^{-1}) that were equipped with gentle aeration. Constant temperature of aquaria was maintained by placing them in water baths (18.5±1.2° C). Exogenous feeding of larvae started 3 dph, and fish were fed with decapsulated *Artemia salina* nauplii (Sanders, Great Salt Lake Artemia, www.gsla.us). Light cycles throughout maintenance and experiments followed a European spring photoperiod (10 h light: 14 h dark).

SURVIVAL AND GROWTH DURING THERMAL ACCLIMATION

M. fossilis larvae were acclimated to five thermal treatments (11±0.31, 15±0.14, 19±0.27, 23±0.3 and 27±0.3° C (±SD)), each with a rearing period of 25 days. Test temperatures were chosen on the basis of the optimal range (15 to 24° C) and the upper lethal temperature (27° C) reported for *M. fossilis* embryos (Drozd *et al.*, 2009). All treatments were replicated four times with 5 l aquaria and 250 larvae (3 dph) per replicate (50 larvae l^{-1}). Prior to test start, experimental aquaria with randomly selected larvae were kept at a temperature of 18.5 ±1.2° C. Target temperature regimes were achieved by placing the experimental aquaria into

temperature controlled water baths that were adjusted to the respective target temperature of the treatment, allowing gentle acclimation of fish larvae ($1°$ C h^{-1}).

The test was carried out under semi-static conditions: throughout the feeding regime (from 900 h to 1900 h; *ad libitum* with *Artemia salina* nauplii ten times a day), a flow-through system of five head tanks provided each experimental aquarium with fresh water at a renewal rate of 60% h^{-1}. During night hours (from 1900 h to 900 h) all aquaria were kept as static systems without water renewal. All aquaria were cleaned twice a day by siphoning off food residues, feces and dead larvae. Dissolved oxygen (70-105%), pH (7.0-7.5), total ammonia (<0.02 mg/l), nitrite (<0.015 mg/l), nitrate (<5 mg/l) and water hardness (3 °dH) were measured daily in one replicate per treatment (Multi 340i SET - WTW; www.wtw.com; MACHEREY-NAGEL visocolor® ECO; www.mn-net.com), changing the replicate for the measurement every day. Temperature was monitored daily in each replicate (Multi 340i SET – WTW; www.wtw.com). To analyse growth rates, eight larvae from each replicate were subsampled on day 5, 10, 15, 20 and 25 of the test. The sampled larvae were euthanized with eugenol, total length (L_T) was measured to an accuracy of 0.1 mm with an image processing software (ImageJ; www.imagej.net) and dried for 24 hours at $60°$ C before determining the dry mass (M_D) to the nearest 0.01 mg (Mettler-Toledo; www.mt.com). Survival rates were calculated on the basis of dead larvae counted during cleaning of aquaria and remaining live larvae counted at the end of the test. One replicate of the $11°$ C treatment had to be excluded from statistical analyses due to a technical problem.

ROUTINE METABOLIC RATE DURING ACUTE THERMAL EXPOSURE

The routine metabolic rate of unfed *M. fossilis* larvae was estimated by measuring $\dot{M}O_2$ in static respirometers (according to Hundt *et al.*, 2015). Prior to each measurement, 10 larvae (3 dph) were randomly selected from the rearing tank ($18.5\pm1.2°$ C) and acclimated for 30 minutes to the respective temperature regime ($11\pm0.5°$ C, $15\pm0.5°$ C, $19\pm0.5°$ C, $23\pm0.5°$ C, $27\pm0.5°$ C, $31\pm0.5°$ C, $35\pm0.5°$ C ($\pm$SD)). After acclimation, three larvae were transferred into separate 4 ml HPLC glass vials (Sigma-Aldrich, www.sigmaaldrich.com) filled with fully aerated water at the respective test temperature. One further vial was treated identically as temperature-specific blank (no larva was introduced), to correct for bacterial oxygen consumption. Temperature during measurements was kept constant with a temperature controlled water bath ($\pm0.5°$ C). Oxygen micro-optodes (needle-type, fiber-optic microsensor, flat broken tip, 140 µm, Presens precision sensing, www.presens.de) connected to a microsensor oxygen meter with four channels (Presens precision sensing) were used to measure oxygen saturations in the vials

(measurement at 15 seconds intervals). The $\dot{M}O_2$ measurements were repeated three times at each temperature, with three larvae per replicate. As larvae were inactive under resting conditions, the vials were gently turned every five minutes to avoid oxygen gradients within the respirometer (Rodgers *et al.*, 2016). Measurements were performed until the initial oxygen saturation in each vial containing a larva was reduced by ten percent or ended after 60 minutes regardless of the degree of reduction. After $\dot{M}O_2$ measurements, M_D of larvae were determined following the method described for the thermal acclimation experiment.

DATA ANALYSES

Survival rates for the thermal acclimation were calculated by performing a survival analysis via cox proportional hazard test, incorporating random effects in the replicated temperature treatments by using mixed effects cox modelling. Modelled survival rates of the different treatments were compared using Tukey's HSD test for multiple comparisons. Differences in L_T and M_D after the rearing period (day 25) were compared using linear mixed effects models (LME), followed by Tukey's HSD test. Mass-specific growth rates (G) were calculated for the period from day 0 to day 25 based on the measured M_D (according to Jobling, 1983). A second-order polynomial regression was used to calculate the optimal temperature for growth ($T_{Opt}G$), i.e. the maxima of the regression curve. $\dot{M}O_2$ was calculated in µg O_2 mg M_D^{-1} h^{-1} and compared following the method described by Herberich *et al.* (2010); a max-t test for multiple comparisons of means using the heteroscedastic consistent covariance estimation in presence of unequal variances was used followed by Tukey's HSD test. The 35° C treatment was excluded from the analyses since all larvae died during measurements. Mean Q_{10} values were calculated on the basis of $\dot{M}O_2$ results as a measure of the increase in metabolic rate with increasing temperature (Crawley, 2013). All statistical analyses and graphics were performed with the open source software package R Version 3.0.2 (R Development Core Team, 2013) and appropriate packages (coxme, multcomp, nlme, sandwich, survival). Significance levels of all tests were set at $\alpha = 0.05$.

RESULTS

SURVIVAL AND GROWTH DURING THERMAL ACCLIMATION

There were significant differences in survival of *M. fossilis* larvae among temperature regimes over 25 days (Fig. 1). Larvae exposed to higher temperatures (19, 23 and 27° C) showed survival rates over 75% over the whole rearing period (85.8%; 93.7%; 75.2%), whereas the

lower temperatures (11 and 15° C) resulted in survival rates lower than 10% (6.5%; 9.7%). The highest survival rates were of larvae at 23° C (93.7%). Survival was stable (>90%) in all treatments, within the first four days. A sharp decline in survival of larvae exposed to 15° C occurred from day 5 to day 13 (from 86.4% to 36.7%) and most larvae exposed to 11° C died within the period from day 8 to day 25 (from 98.0% to 6.5%). Larvae exposed to 11° C rested continuously on the aquarium floor, with no foraging behaviour. Additionally, the highest levels of food residues were observed at 11° C. Survival in the 27° C treatment was stable for approximately 16 days until a slight decline occurred (from 94.2% to 75.2%).
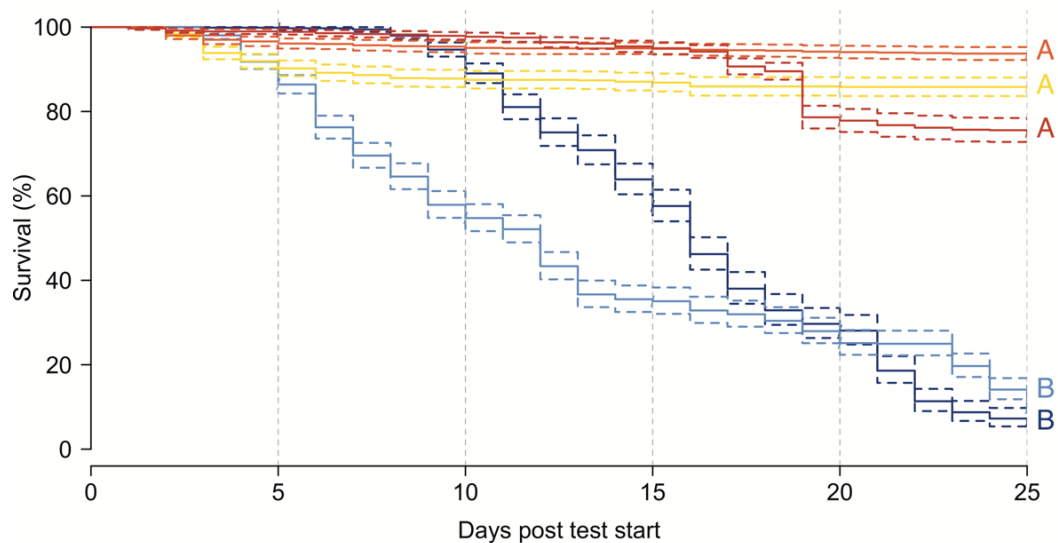

Fig. 1. Survival rates (± 95% confidence intervals) of *Misgurnus fossilis* larvae during 25 days lasting thermal acclimation to 11 (●), 15 (●), 19 (●), 23 (●) and 27° C (●). Dotted grey vertical lines indicate dates when subsamples for growth analyses were taken. Different capital letters indicate significant differences between treatments (α = 0.05). All treatments were replicated four times (except 11° C with *n* = 3).

Both, L_T and M_D of *M. fossilis* larvae exposed over 25 days differed significantly among temperature regimes (Fig. 2). From 11 to 19° C L_T and M_D increased with each consecutive temperature step. Larvae exposed to 23° C neither further increased in mean L_T (20.91 mm) nor mean M_D (7.67 mg) compared with larvae at 19° C (L_T = 21.29 mm; M_D = 7,97 mg). A significant decrease in L_T and M_D was detected for larvae exposed to 27° C (L_T = 17.30 mm; M_D = 5.88 mg). Consequently, the highest mean values of L_T and M_D were in the larvae at 19 and 23° C. $T_{Opt}G$ based on G calculated for the whole thermal acclimation period (i.e. day 0 to day 25) was 22.0°C (Fig. 3).
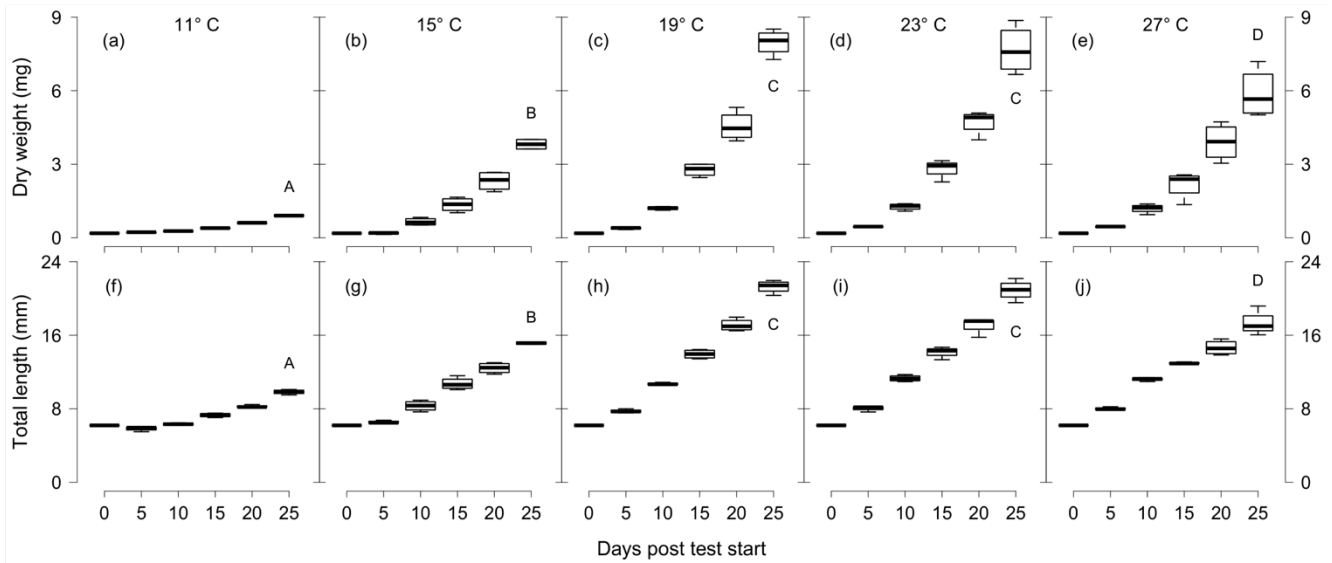
Fig. 2. Box-and-whisker plots of dry mass (M_D; a - e) and total length (L_T; f - j) of *Misgurnus fossilis* larvae prior to test start (day 0) and 5, 10, 15, 20 and 25 days after thermal acclimation to 11, 15, 19, 23 and 27° C, respectively. Different capital letters indicate significant differences ($\alpha = 0.05$) between treatments after 25 days of exposure. All treatments were replicated four times (except 11° C with $n = 3$). However, two replicates of the 15° C treatment did not contain 8 larvae on day 25, therefore only two replicates could be considered at this point in time.
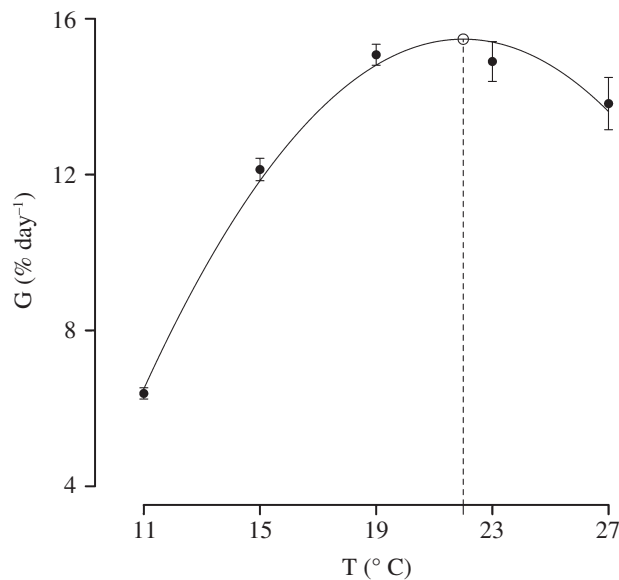
Fig. 3. Mean mass-specific growth rates (G) and associated standard deviations of *Misgurnus fossilis* larvae after a 25 days lasting thermal acclimation to 11, 15, 19, 23 and 27° C (% d^{-1}). White point with dotted vertical line indicates the optimum temperature for growth ($T_{Opt}G$) calculated from a second-order polynomial regression ($p < 0.001$, $r^2 = 0.9801$; $y = -20.44 + 3.27x - 0.07x^2$). All treatments were replicated four times (expect 11° C where $n = 3$). However, two replicates of the 15° C treatment did not contain 8 larvae on day 25, therefore only two replicates could be considered at this point in time.

None of the *M. fossilis* larvae showed any behavioural abnormalities during the exposure period to the respirometer. However, all larvae at 35° C died during the trial run (including acclimation time (±SD): 62.3±2 min). There was an increase in mean $\dot{M}O_2$ with each consecutive temperature from 11 to 27° C, representing the same temperature range tested during thermal acclimation. Between 23 and 27° C the measured difference in $\dot{M}O_2$ was not statistically significant but with a tendency toward increasing ($P = 0.087$) (Fig. 4). The $\dot{M}O_2$ was highest in larvae exposed to 27° C (3.17±0.72 µg O_2 mg M_D^{-1} h^{-1}) and 31° C (3.25±0.84 µg O_2 mg M_D^{-1} h^{-1}) with no differences between both treatments ($P = 0.99$). Deviations in $\dot{M}O_2$ between individuals increased with increasing exposure temperatures, particularly apparent at 27 and 31° C (Fig. 4).
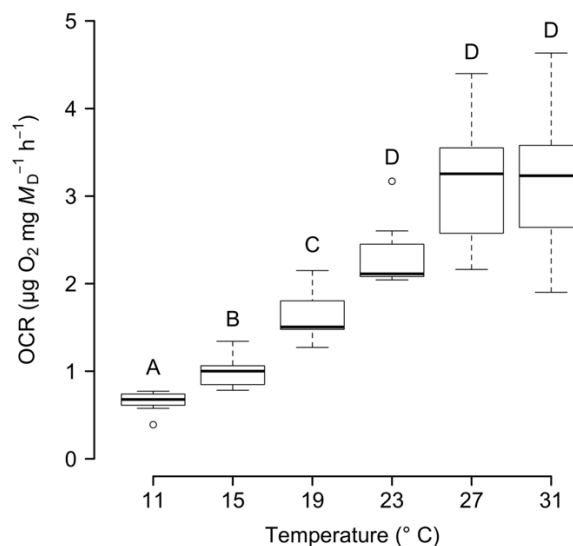
Fig. 4. Box-and-whisker plots of oxygen consumption rates (OCR; µg O_2 mg M_D^{-1} h^{-1}) of *Misgurnus fossilis* larvae exposed to 11, 15, 19, 23, 27 and 31° C. Different capital letters indicate significant differences ($\alpha = 0.05$) between treatments. All treatments were replicated three times with 3 larvae per replicate.

The mean Q_{10} values for $\dot{M}O_2$ ranged between 2.19 and 3.43 at temperatures between 11 to 27° C, respectively, with the highest value between 15 and 19° C ($Q_{10} = 3.43$). A Q_{10} value of 1.07 was obtained for exposure to temperatures between 27 and 31° C (Table 1).

Table 1. Q_{10} values for *Misgurnus fossilis* larvae calculated on the basis of oxygen consumption rate ($\dot{M}O_2$) at temperatures of 11-15, 15-19, 19-23, 23-27 and 27-31° C.

11-15° C	15-19° C	19-23° C	23-27° C	27-31° C
2.82	3.43	2.45	2.19	1.07

DISCUSSION

Highest survival rates of *M. fossilis* larvae during thermal acclimation were observed at 23° C (Fig. 1). This temperature lies within, but at the upper end of, the optimum range of 15-24° C, reported by Drozd *et al.* (2009), where *M. fossilis* embryos were exposed from fertilisation until hatching. Additionally, Drozd *et al.* (2009) observed 100% mortality of embryos exposed to temperatures higher than or equal to 27° C, whereas in the present study >75% of the larvae survived at 27° C. This difference can be attributed to the higher sensitivity of embryos compared to larvae, as shown in many marine and freshwater fish species (Rombough, 1997). At the lower end of temperatures tested during thermal acclimation, temperatures ≤15° C have to be considered as critical for the long-term survival of larvae (survival rate lower than 10%), whereas survival of embryos was not adversely affected by 15° C (Drozd *et al.*, 2009). With regard to the thermal situation during spawning season of *M. fossilis* in early spring, larvae are likely to experience a natural increase in temperature through seasonal warming with on-going development. Therefore, larvae presumedly are adapted to a temperature increase, resulting in an upwards shift of T_{Opt} and T_C as ontogeny proceeds. This shift is associated with a higher vulnerability to temperature decreases. Similar changes in the zone of thermal tolerance with on-going development were reported for many temperate fish species, always as a reflection of the natural conditions these fishes experience (Rombough, 1997).

The maximum temperature tolerance of *M. fossilis* previously was thought to be 25° C, irrespective of differences between developmental stages and exposure duration (Leuven *et al.*, 2011; FishBase Consortium, 2016). Based on the current observations, , the maximum temperature tolerance of 3 dph *M. fossilis* larvae for an acute exposure is most likely to be within the range of 31 to 35° C.

Regarding acute exposure to the lower end of tolerable temperatures, it can be concluded that 11 and 15° C are tolerable by *M. fossilis* larvae for short time periods. In contrast to the thermal acclimation approach used in the present study, alternative methods that

predominately focus on the thermal tolerance of fish towards acute exposure (i.e. critical thermal maximum and minimum; upper and lower lethal temperature) risk to underestimate the lethal effects of chronic exposure to temperature extremes (Selong *et al.*, 2001). This risk becomes particularly apparent when considering the time dependency for survival as observed at 11 and 15° C, since significant mortality of larvae did not occur prior to five days. Spawning of *M. fossilis* is induced by temperatures around 19° C, which are frequently reached in its habitats by March or April (Kottelat & Freyhof, 2007). At this time of the year, seasonal temperature fluctuations can lead to a drop in temperature that persists for several weeks, especially since habitats are often characterized by low water levels and weak temperature buffering (Meyer & Hinrichs, 2000). The implication of this might be a critical loss in the offspring, negatively affecting population performance.

The calculated $T_{Opt}G$ (22.0° C) for *M. fossilis* larvae lies slightly below T_{Opt} stated for survival (23° C). Since growth is known to be a more sensitive indicator for thermal stress than survival (Jobling, 1997), it is reasonable that 27° C affected growth but did not lead to a significant decrease in survival. A reasonable explanation for the higher growth rates observed at 19, 23 and 27° C compared with 11 and 15° C is the increase in reaction rates for growth of fishes associated with increasing environmental temperatures (Angilletta *et al.*, 2002; Jobling, 1997; Wang & Overgaard, 2007). The significant decrease in growth of larvae exposed to 27° C may represent a mismatch between the increased metabolic costs induced by higher temperatures and the energy uptake necessary to maximize growth (Pörtner & Knust, 2007). This mismatch is corroborated by previous lab and field studies conducted with other fish species and varying life stages (Jonassen, 2000; Neuheimer *et al.*, 2011; He *et al.*, 2014; Li *et al.*, 2014). $T_{Opt}G$ of *M. fossilis* larvae (22.0° C) is similar to the optimum presented for larvae of the spined loach *Cobitis taenia* L. 1758 (20 to 24° C), a closely related species (Bohlen, 2003). Other limnophilic European species that can occasionally be found in *M. fossilis* habitats are tench *Tinca tinca* L. 1758 and crucian carp *Carassius carassius* L. 1758 (Schauer *et al.*, 2013). These are the two most thermo-tolerant native European fish species (Leuven *et al.*, 2011). Larval *T. tinca* show the highest growth rate at temperatures up to 31° C, when fed live or mixed diets (Wolnicki & Korwin-Kossakowski, 1993). However, a direct comparison with *M. fossilis* larvae is difficult since spawning of *T. tinca* mostly occurs when water temperature reaches 22 to 24° C (Kottelat & Freyhof, 2007). Thus, *T. tinca* larvae are adapted to considerably higher temperatures. No literature is available about the thermal requirements of larval *C. carassius*. Considering M_D and L_T of *M. fossilis* larvae obtained during the thermal acclimation, further improvements in terms of food composition during first-feeding seem

possible in order to produce a more vigorous stock material. Demény *et al.* (2009) achieved *M. fossilis* larvae lengths of almost 30 mm after 15 days of cultivation, when fish were fed a mixture of *Artemia* and *Tubifex*, i.e. twice the mean L_T recorded after 15 days at 23° C in the present study (14.16 mm). However, this difference might also be due to the higher rearing density (50 larvae L^{-1}) used during thermal acclimation as growth performance is inversely related to rearing density in many cultivated fish species (Barcellos *et al.*, 1999).

The increased $\dot{M}O_2$ induced by higher temperatures (Fig. 4) represents a metabolic temperature compensation for the increased oxygen demand of larvae (Rombough, 1988). The Q_{10} values calculated for temperatures between 11 and 27° C ranged from 2.19 to 3.43 (Table 1), and therefore lie within the range of Q_{10} values that characterize freshwater fish larvae (Rombough, 1988). However, the $\dot{M}O_2$ of *M. fossilis* larvae reaches a plateau at temperatures above 27° C, representing the point at which routine metabolic rate of larvae is at a maximum. When this threshold is exceeded, additional energy demands (e.g. for swimming or feeding) require anaerobic metabolic pathways (Hardewig *et al.*, 2004; Breau *et al.*, 2011). Thus, temperatures above 27° C are critical for *M. fossilis* larvae from an $\dot{M}O_2$ point of view, which is also in agreement with the growth impairment observed during thermal acclimation (Wang & Overgaard, 2007). Moreover, the Q_{10} value calculated between 27 and 31° C dropped to 1.07 (Table 1), underlying the thermal stress *M. fossilis* larvae experienced (Rombough, 1988).

In summary, this paper is the first investigation of thermal requirements of *M. fossilis* larvae and therefore provides essential information that should be considered in further investigations, such as modelling approaches or interspecific comparisons. Furthermore, the results can be applied for the conservation of an endangered and poorly studied fish species, as thorough knowledge of thermal requirements is fundamental for this purpose. Previous studies that aimed at producing *M. fossilis* for stocking purposes used 16 to 20° C not only for incubation of eggs but also for rearing of larvae (Kouril *et al.*, 1996; Schauer *et al.*, 2013). A higher rearing temperature of about 22° C ($T_{Opt}G$) could possibly improve survival and growth results for larvae. In conclusion, the particularly high survival rates of larvae observed during thermal acclimation (more than 95% when exposed to 23° C) support the practical feasibility of *M. fossilis* production under artificial conditions (Geldhauser, 1992; Kouril *et al.*, 1996; Demény *et al.*, 2009; Schauer *et al.*, 2013).

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WEATHERFISH (*Misgurnus fossilis*) AS A NEW SPECIES FOR TOXICITY TESTING?

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ABSTRACT

Selection of appropriate test species is a critical issue when assessing effects of environmental contamination on fish because the ecological relevance of commonly used test species might be restricted due to their exotic origin. In the present study, a European freshwater fish with frequent occurrence in agricultural areas is suggested as a potential alternative: the European weatherfish (*Misgurnus fossilis*). The suitability for acute embryo toxicity tests (FET) was investigated with regard to practical implementation, sensitivity to contaminants and tolerance against environmental conditions of concern. For this purpose, weatherfish embryos were exposed (72 h) to the reference substance 3,4-dichloroaniline (DCA) in three independent tests. Furthermore, the effects of dissolved oxygen (DO) deficiency on weatherfish embryos were studied to evaluate their suitability e.g. for sediment bioassays. Obtained results revealed that the sensitivity of weatherfish embryos towards DCA (72 h-EC_{50}=0.52 mg/l; 72 h-LC_{50}=0.71 mg/l) was highest compared to other species and three times higher than reported for the commonly used zebrafish (*Danio rerio*). Even though knowledge of DO requirements during the embryonic period of European fish species is scarce, weatherfish can be stated as one of the most tolerant native species (120 h-LC_{90} for DO=0.53 mg/l). Its high ecological relevance for Europe, the particular sensitivity towards DCA and high tolerance against DO depletion highlights the potential of weatherfish as additional species for toxicity testing.

INTRODUCTION

Due to their economic and ecological value, fish receive attention for protection against aquatic pollution and therefore play a major role as test organisms in ecotoxicological bioassays (EU, 2000; EU, 2006; van der Oost et al., 2003; Powers, 1989). This applies not only for tests focusing on hazard classification of chemicals but also for the ecological risk assessment (ERA). For the latter, a selection of appropriate test species is particularly critical, as the scientific community recommends a wide range of considerations to be addressed (Chapman, 2002; Wedekind et al., 2007):

- Ecological relevance and importance for the region to be investigated;
- General suitability for experimental investigations;
- Sensitivity to contamination; and,
- Tolerance against environmental conditions of concern.

Despite the long history of ERA research and the implementation of management measures in recent European Union legislation (EU, 2008a; EU, 2008b), fish species selected for bioassays rarely meet all criteria mentioned above. In particular, the restricted relevance of standard test organisms (e.g., *Danio rerio*, *Pimephales promelas*) is repeatedly scrutinized, as they are predominately used irrespective of site-specific characteristics. Major concerns in this debate are inconsistencies concerning species origin (exotic vs. temperate) and lifestyle (pelagic vs. benthic) (Chapman, 2002; Chapman et al., 2002; Hallare et al., 2011; Wedekind et al., 2007). Without depriving the numerous advantages commonly used fish species undoubtedly offer (e.g., easy to culture, genetically stable, functional genetic endpoints, demonstrated test repeatability, well quantified descriptions of ecotoxicological test endpoints) (Hollert and Keiter, 2015; Laale, 1977), an integration of ecology and aquatic toxicology as claimed by Chapman (2002) is only possible by "improvements in the manner in which we select species for testing" (Chapman, 2002).

A species that could bridge the gap of restricted relevance might be the European weatherfish (*Misgurnus fossilis*; Linnaeus 1758). The weatherfish originally inhabited wide areas across Europe but is recently suffering a populations decline in many regions (Meyer and Hinrichs, 2000; Sigsgaard et al., 2015). Consequently, it is categorized as endangered in many European countries and listed under Annex II of the Council Directive 92/43/EEC (species of Community interest) (EU, 1992). Typical weatherfish habitats, such as oxbows or backwaters, as well as ditch system or temporarily filled channels and ponds, are characterized by low currents, heavy vegetation and muddy substratum (Meyer and Hinrichs, 2000).The weatherfish burrows into the sediment by day, desiccation or to avoid predation – making it a strongly

bottom-related species and therefore its presence is highly dependent on sediment quality. Furthermore, the weatherfish is physiologically and morphologically adapted to hypoxic conditions including dependence on cutaneous respiration, oxygen-uptake via the gut and development of external filamentous gills a few days after hatching (Kottelat & Freyhof, 2007). Among other reasons, environmental pollution in agriculturally used landscapes is assumed to be responsible for its decline (Drozd et al., 2009).

In the early 1970's, Russian researchers revealed the suitability of weatherfish embryos for early developmental studies, due to the possibility to obtain large numbers of embryos by hormonal stimulation, relatively fast embryonic development with a hatching 50-70 hours post fertilization (hpf) and transparency of the embryonic membrane (Kostomarova, 1991; Sleptzova et al., 2000). However, to our knowledge only one study used the weatherfish in an ecotoxicological context, dealing with the effects of cyanobacteria on chromosomal aberrations (Palíková et al., 2007).

Irrespective of which fish species is selected for toxicity tests, the question of ethical concerns regarding bioassays with vertebrates inevitably arises. A shift towards alternative test methods according to the 3Rs principle (replacement, reduction and refinement of animal experiments) (Russel & Burch, 1959) is emphatically requested from public authorities and the scientific community (Scholz et al., 2013). A promising method that could perform that function is the acute fish embryo toxicity test (FET) because EU animal welfare legislation protects fish as experimental organism from the onset of exogenous feeding, which is not applicable to embryonic life stages (EU, 2010; Strähle et al., 2012). The FET is already standardized for whole effluent testing in Germany (DIN, 2001; ISO, 2007) and internationally proposed as an additional test guideline since 2013 (OECD Test Guideline 236 (OECD TG 236)) (Braunbeck et al., 2015; OECD, 2013). Furthermore, it was established for important standard fish organisms, including zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*) (Braunbeck et al., 2005). The excellent correlation between acute fish toxicity tests conducted with juveniles or adults as reported by Lammer et al. (2009) and Belanger et al. (2013) even supports a substitution of acute fish toxicity tests with FET.

On the basis of the above-mentioned aspects, the weatherfish can be stated as a native European freshwater species with high ecological relevance for agriculturally impacted waters, therefore a potential test species in Europe. However, questions remain about the practical feasibility of FET with weatherfish embryos, their sensitivity towards contaminants and their tolerance against environmental conditions of concern. To take account of these uncertainties,

the present study aimed to transfer the FET protocol as proposed for zebrafish embryos in OECD TG 236 to weatherfish embryos, with regard to: embryo production, determination of apically observable effects (including a categorization into lethal and sublethal), sensitivity to laboratory handling, compliance with validity criteria (i.e., fertilization rate, survival in controls), sensitivity to contaminants and repeatability of results. For this purpose, the reference substance 3,4-dichloroaniline (DCA) was tested in three independent tests in two consecutive years (2014 and 2015), using two different wild parental populations. Additionally, the dissolved oxygen (DO) requirements of weatherfish embryos were investigated in a separate experiment, since this factor might be particularly critical in future applications of sediment bioassays with a reasonable likelihood of hypoxic conditions in the test system (Küster and Altenburger, 2008; Strecker et al., 2011).

MATERIALS AND METHODS

FISH MAINTENANCE AND EMBRYO PRODUCTION

Wild weatherfish spawners from two different populations in Germany (DE-I, DE-II) were caught in March 2014 and 2015, using fish traps (Table 1). Spawners from DE-I and DE-II were treated according to Schreiber et al. (2016). In brief, after a stepwise temperature acclimation to 18°C, a gonadotropine-releasing hormone analogue and metoclopramide containing hormonal preparation (Ovopel, Unic-trade, Hungary) was injected intramuscularly in two consecutive dosages (total dose = 1 pellet kg^{-1} body mass) with a time interval of 12 h. A subsample of approximately 50 eggs was weighed to the nearest 0.01 mg (Mettler-Toledo, Switzerland) from each female to determine the amount of eggs per female. Fertilization rates were checked under a binocular microscope (Olympus SZX9, Japan) by counting the proportion of fertilized eggs in a subsample of approximately 100 eggs. After stripping, all stripped spawners were released to their original localities without any observable health restrictions.

Table 1. Overview of weatherfish populations used in the present study, including their origin, the number of stripped females (with mean total length (L_T) and wet weight (W_W)) and the mean number of eggs gained per female (± standard deviations).

Population	Origin	No. of females	No. of males	No. of eggs/female
DE-I	Streitgraben, Germany (49° 5'42.98"N; 8°19'3.64"E)	9 (L_T = 169±11 mm; W_W = 29±8 g)	18 (L_T = 176±14 mm; W_W = 22±4 g)	4938±2074
DE-II	Kreuzlachgraben, Germany (49° 40' 25.5" N; 8° 34' 56.1" E)	5 (L_T = 211±13 mm; W_W = 51±9 g)	12 (L_T = 185±7 mm; W_W = 27±3 g)	8945±1991
Total		14	30	6540±2778

ACUTE FISH EMBRYO TEST (FET) WITH 3,4-DICHLOROANILINE (DCA)

Embryo selection and test performance were carried out according to OECD TG 236 with slight adjustments for an implementation of weatherfish embryos (OECD, 2013).

Fertilized eggs were thoroughly rinsed in fresh water to remove potential dirt and blood particles. Afterwards, eggs were transferred to artificial water that was prepared according to ISO 7346-3 (ISO, 1996), pursuant to the same water composition recommended for toxicity tests with *Danio rerio*. Prior to selection of eggs, 24-well plates (Orange Scientific, Belgium) were prepared with DCA (98% purity, Sigma-Aldrich, Germany) stock solutions, so that target concentrations could be achieved by adding 0.5 ml artificial water including one egg per well (total volume per well: 2.5 ml). Normally developed eggs were selected from the beginning of the 8-cell stage (2.5±0.4 hpf) using a binocular microscope (Olympus SZX9, Japan) and pipetted to the wells. Hence, exposition of eggs to the test chemical started approximately 2-3 hpf. All plates were covered, randomly arranged and incubated for 72 h at 20±1° C and a photoperiod of 12 h light: 12 h dark. Every 24 hpf, eggs were checked under a microscope (Olympus CX31, Japan) for apically observable abnormalities in embryonic development of weatherfish (Fig. 2). Due to uncertainties concerning the severity (lethal vs. sublethal) and temporal definition of some observed effects (i.e., lack of somite formation, lack of blood circulation, tail malformations, underdevelopment), only coagulation (lethal), lack of heartbeat

(lethal), pericardial edema (sublethal), malformations (sublethal) and delayed hatching (sublethal) were eventually taken into account for data analysis (Table 2). Pictures of eggs were taken with a microscope (Olympus CX31, Japan) equipped with a camera (AxioCam ERc 5s, Carl Zeiss, Germany).

As a preparatory test, a range-finding experiment with DCA was performed, using six concentrations (0.03, 0.15, 0.74, 3.70, 18.50, 92.50 mg/l) and a negative control, each with 24 replicates. Embryos for the range-finding experiment were obtained from population DE-I.

Three definitive tests (Test-I, Test-II and Test-III) were carried out with two well plates per test concentration and additional internal controls for each well plate (4 (Test-I and Test-II) and 8 wells (Test-III), respectively). Test-I included seven concentrations (0.09, 0.19, 0.37, 0.74, 1.48, 2.96, 5.92 mg/l), conducted with fertilized eggs from population DE-I. In Test-II and Test-III, the concentrations used were identical (0.30, 0.38, 0.47, 0.59, 0.74, 0.93, 1.16, 1.45 mg/l) but tests were performed in two consecutive years (2014 and 2015), using population DE-II. A test was considered as valid when overall fertilization rate in the batch of stripped eggs was ≥70%, mortality in the internal control was ≤25% (i.e. ≤1 dead embryo per internal control) and survival in the control plate was ≥90%.

Quantification of DCA was performed for exposure verification. The highest (1.47 mg/l), the middle (0.74 mg/l) and the lowest (0.30 mg/l) concentration from a dilution series were measured in triplicate from samples taken subsequent to solution preparation (T-0) and after 72 h (T-72). Furthermore, a sample of the stock solution (63 mg/l) was measured (T-0). Measurements were carried out by high performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) (supporting information Table S3). The analytical standard of DCA (purity >99%) was obtained from Sigma-Aldrich (Germany).

DISSOLVED OXYGEN (DO) REQUIREMENTS OF EMBRYOS

Teratogenic effects of low DO concentrations were investigated based on the method by Strecker et al. (2011). Weatherfish embryos (DE-I, DE-II) were subjected to 14 different DO concentrations (0.41, 0.54, 0.62, 0.70, 0.77, 0.79, 0.85, 0.94, 1.05, 1.15, 1.49, 1.62, 1.83, 2.54 mg/l) for 48 h; each concentration was replicated three times (± 20% deviation tolerated). Additional to that, one replicate with DO saturated water (≥ 6 mg/l) served as a control for each test.

At 24 h prior to test start, 1000 ml bottles (Schott, Germany) were filled completely with artificial water (ISO, 1996) and bubbled with nitrogen to lower the DO concentration. Afterwards, bottles were closed with a screw cap in a way that no air was between cap and

water surface, in order to avoid recent DO re-enrichment or depletion. Six embryos were chosen as described for FET and put into each bottle. DO concentrations were measured using the physico-optical oxygen sensor FireStingO$_2$ (PyroScience, Germany) immediately before closing the test bottles (initial concentration). After 48 h, bottles were opened and DO concentrations were measured again (final concentration). Subsequently, eggs were transferred into 6-well plates (1 egg/well) that contained artificial water saturated with DO and incubated for further 72 h. Eggs were checked for apically observable effects every 24 h in the same way described for FET experiments. DO concentrations were defined as mean of initial and final concentration and intensity of effects after 120 h were divided into four categories: lethal, heavy, medium and slight (Table 4). A test was considered as valid when overall fertilization rate in the batch of stripped eggs was ≥70% and mortality in the control was ≤ 17% (i.e. ≤1 dead embryo per control).

DATA ANALYSES

The mean numbers of eggs gained per female and the associated standard deviations (SD) were calculated for both populations both separately and collectively (Table 1). Statistical analyses and graphs for FET and DO requirements were performed with the open source software package R Version 3.0.2 (R Development Core Team, 2013) extended with the 'drc'-package (Ritz, 2013). In brief, effect concentrations (for FET with DCA and DO requirements) were calculated by fitting dose-response models and by selecting the best-fitted model based on Akaike's information criterion and visual judgement. For FET results with DCA, 50% effect concentrations (EC_{50}, LC_{50}), including the associated 95% confidence intervals (CI), were calculated for 24, 48 and 72 hpf, respectively (Table 3). Furthermore, mean effect concentrations (72 h-EC_{50} and 72 h-LC_{50}) and the associated SD were calculated for the three definitive tests. The plotted dose-response curves for sublethal and lethal effects of DCA also include 95% confidence intervals (Fig. 3). Analysis of DO requirements data were carried out by calculating EC_{90}, EC_{50}, EC_{20} and EC_{10} values (±CI) after 120 h for each effect category mentioned above (lethal, heavy, medium and slight).

RESULTS

In total, more than 89,000 weatherfish eggs were obtained from 14 females (Table 1). The minimum amount of stripped eggs per female was 1,489 (L_T = 163 mm; W_W = 27.5 g), and as a maximum, 12,193 eggs were obtained per female (L_T = 200 mm; W_W = 52.6 g; DE-II). Fertilization rates exceeded 70% in all cases. The mean time interval (±SD) between the first hormonal injection and fertilization of eggs was 36.8±1.7 h. The mean time interval (±SD) between fertilization and achievement of the 8-cell-stadium was 2.5±0.4 h.
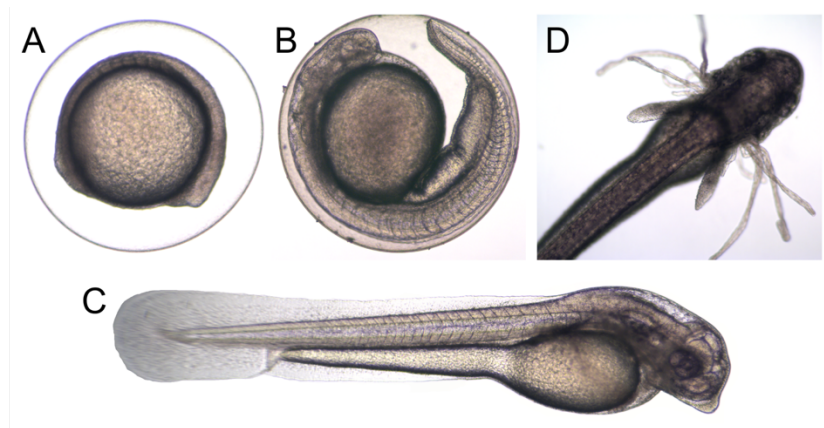
Fig. 1. Pictures of normally developed weatherfish embryos from control setups (artificial water) after 24 (A), 48 (B) and 72 hpf (C), respectively. Picture D shows outer filamentous gills of embryo 96 hpf.
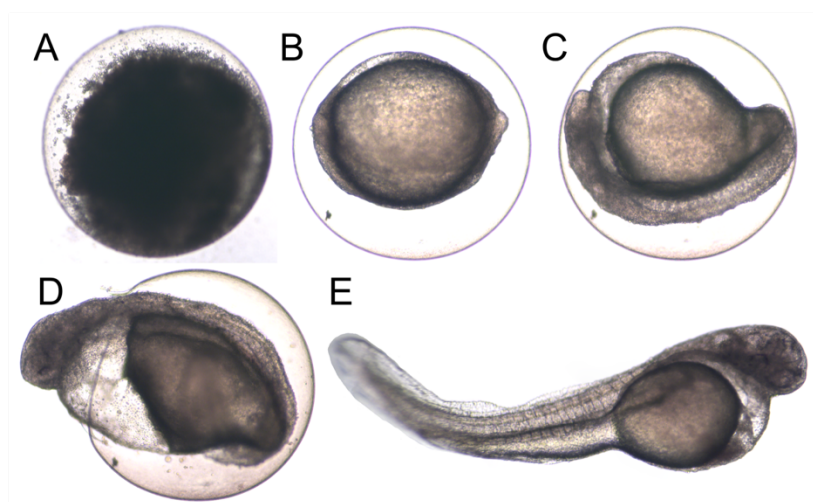
Fig. 2. Pictures showing examples for apically observable effects in weatherfish embryos, including: coagulation (A), edema and underdevelopment after 24 hpf (B), edema and malformation after 48 hpf (C), edema, delayed hatching and malformation after 72 hpf (D), edema and malformation after 72 hpf (E). Pictures indicated with B, C and D show the same embryo at 24, 48 and 72 hpf, respectively.

The developmental stages of weatherfish from fertilization to active feeding were described in detail by Kostomarova (1991). Examples for normal development (Fig. 1) as well as effects that may occur (Fig. 2) are provided here. At 24 hpf, normally developed weatherfish embryos are elongated around the yolk and show multiple pairs of somites, as well as slight eye rudiments (Fig. 1A). At 48 hpf, the tail is detached from the yolk; embryos show movements and heartbeat, blood circulation and pericardium are visible (Fig. 1B). At 72 hpf, embryos are hatched; the head is already raised over the yolk with only slight curvature and first rudiments of outer gills are visible (Fig. 1C). Development of outer gills further proceeds (Fig. 1D) and reaches its maximum approximately 120 hpf (Kostomarova, 1991). The effects finally considered for an evaluation in the FET and the definition as lethal or sublethal are presented in Table 2.

Table 2. Apically observable effects in weatherfish embryos defined as lethal or sublethal in dependency of time, following OECD-236 with slight deviations (OECD, 2013).

Intensity of effect	Effect		
	24 hpf	48 hpf	72 hpf
Lethal	Coagulation	Coagulation	Coagulation
		Lack of heartbeat	Lack of heartbeat
Sublethal	Edema	Edema	Edema
	Malformation	Malformation	Malformation
			Non-hatching

Survival rates in the negative controls were $\geq 90\%$ ($95\pm 3\%$) for all the three definitive tests. Three of 46 well plates tested in the three definitive tests had to be excluded from data analyses because of violation of validity criteria (mortality in the internal control $\geq 25\%$) and were not further considered. DCA showed a clear dose-response relationship for weatherfish embryos and toxicity increased with exposure time from 24 to 48 hpf but increased only slightly from 48 to 72 hpf (Fig. 3). Mean effect concentrations ($\pm$SD) for the three definitive tests (72 hpf) were 0.58 ± 0.04 mg/l (EC_{50}) and 0.71 ± 0.11 mg/l (LC_{50}), respectively. A calculation of effect concentrations considering the results of all three definitive tests (72 hpf; CI) revealed 0.52 (0.50-0.55) mg/l (EC_{50}) and 0.71 (0.55-0.87) mg/l (LC_{50}), respectively.
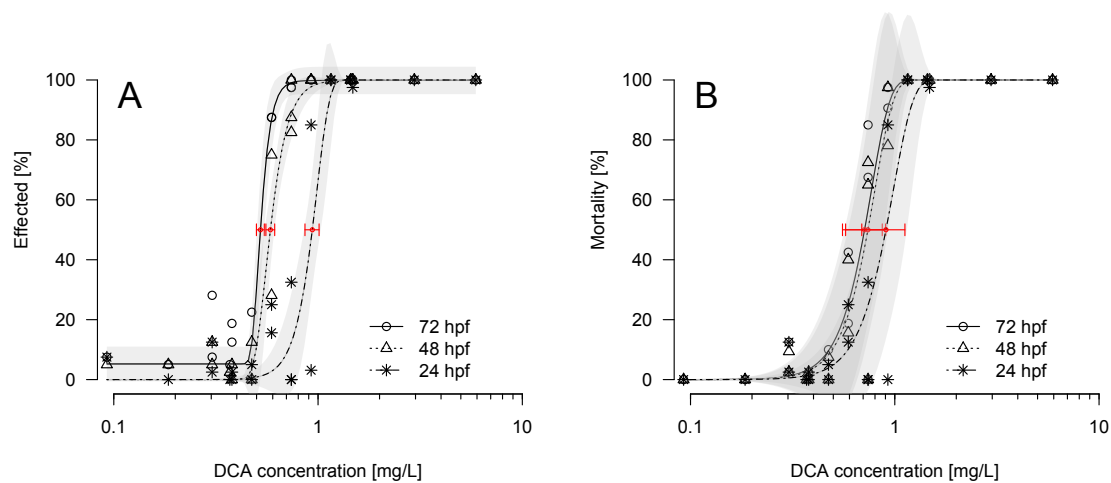
Fig. 3. Dose-response curves with associated 95% confidence intervals (grey) for three acute fish embryo tests (FET) conducted with weatherfish embryos exposed for 24, 48 and 72 h to 3,4-Dichloroaniline (DCA): sublethal (A) and lethal (B) effects. Red dots indicate EC_{50} and LC_{50} values with the corresponding 95% confidence interval, respectively (Table 3).

Measured concentrations of DCA were generally lower than the target nominal concentrations and decreased over time (T-0>T-72). However, deviations were in a range of ≤10% for all samples taken at T-0 and in a range of ≤20% for samples taken at T-72. Only the lowest concentration measured (0.23 mg/l) deviated by 23% from the nominal concentration (0.30 mg/l) at T-72 (supporting information Table S2).

Table 3. Interspecific comparison of EC_{50} and LC_{50} values derived from acute fish embryo tests (FET) conducted with 3,4-Dichloroaniline (DCA).

Species	Time (h)	EC_{50} (mg/l)	LC_{50} (mg/l)	Temperature (°C)	Reference
Misgurnus fossilis	24	0.94 (0.86-1.01)[a]	0.91 (0.69-1.12)[a]	20±1	(1)
	48	0.58 (0.56-0.61)[a]	0.74 (0.57-0.90)[a]	20±1	(1)
	72	0.52 (0.50-0.55)[a]	0.71 (0.55-0.87)[a]	20±1	(1)
Danio rerio	48	1.8±0.4[b]	2.4±0.7[b]	26±1	(2)
Oryzias latipes	30	24.1±11.88[b]	–	26±1	(3)
	78	21.8±2.97[b]	–	26±1	(3)
Pimephales promelas	28	17.1±0.62[b]	–	25±1	(3)
	56	8.08±2.57[b]	–	25±1	(3)
	72	5.93±4.13[b]	–	25±1	(3)
	96	4.35±2.10[b]	–	25±1	(3)
Gobiocypris rarus	72	2.97 (2.62-3.37)[a]	4.15 (3.67-4.71)[a]	25±1	(4)

[a] lower and upper 95% confidence intervals; [b] standard deviations from independent tests; (1) Present study; (2) Braunbeck & Lammer, 2006; (3) Braunbeck et al., 2005 (4) Zhu et al., 2013

Teratogenic effects were observed for weatherfish embryos exposed to low DO concentrations and showed an inverse dose-response relationship (increasing effects with decreasing DO concentration). Some of the sublethal effects observed were reversible after embryos were transferred to DO-saturated water (after 48 h), hence effect concentrations decreased with test duration. Furthermore, effect concentrations calculated revealed that slight effects did occur for at most 10% when exposed to an DO concentration of 2.18 mg/l. Values of ≤0.53 mg/l led to mortality of 90% (Table 4).

Table 4. Teratogenic effects (grouped as lethal, heavy, medium, slight) in weatherfish embryos exposed to different oxygen concentrations. Given are oxygen concentrations and the associated 95% confidence intervals at which a certain proportion (10, 20, 50, or 90%) was affected.

	Proportion affected			
	10%	20%	50%	90%
Intensity of effects	Oxygen concentration (mg/l)			
Lethal	0.85 (0.70-1.00)	0.76 (0.66-0.85)	0.64 (0.59-0.68)	0.53 (0.47-0.59)
Heavy	1.04 (0.76-1.33)	0.88 (0.72-1.04)	0.68 (0.61-0.75)	0.52 (0.42-0.61)
Medium	1.52 (1.16-1.89)	1.31 (1.09-1.53)	1.04 (0.96-1.13)	0.82 (0.73-0.90)
Slight	2.18 (1.25-3.11)	1.71 (1.18-2.23)	1.18 (1.00-1.37)	0.80 (0.65-0.95)

Lethal: coagulation, lack of heartbeat; Heavy: coagulation, lack of heartbeat, severe deformation; Medium: coagulation, lack of heartbeat, severe deformation, deformation, edema; Slight: coagulation, lack of heartbeat, severe deformation, deformation, slight deformation, edema, delayed development

DISCUSSION

GENERAL SUITABILITY FOR EXPERIMENTAL INVESTIGATIONS

A successful production of weatherfish embryos by hormonal stimulation could be shown in numerous studies that applied various hormonal preparations (Drozd et al., 2009; Geldhauser, 1992; Kostomarova, 1991; Kouril et al., 1996; Schreiber et al., 2016). The method used in the present study can be stated as effective and feasible for developmental investigations. Weatherfish embryos could be obtained with relatively little effort and high precision in terms of predicting the time of stripping (36.8±1.7 h), which ensures a realization of schedules during experimental procedures. Furthermore, all spawners survived the handling process without visible health restrictions and could be returned to the wild after an acclimation period of a few days. The large number of embryos obtained per female (6540±2778; Table 1) and the high fertilization rates achieved (≥70% in all cases) allowed an application of test designs with high numbers of replicates. Compared to acute fish toxicity tests conducted with juveniles or adults, these numbers provide advantages in terms of economy, statistical power and multi-factorial test designs (Wedekind et al., 2007). When using fathead minnow for FET, the relatively complex production of embryos (sticky eggs are attached to tiles) might pose a restriction with regard to methodological feasibility of large-scale applications (Braunbeck and Lammer, 2006). Same restrictions might be true for Japanese medaka as only 20-40 eggs can be removed from the anal fin of females and spiny hooks might reduce visibility of teratogenic effects (Braunbeck and Lammer, 2006). The seasonality of weatherfish production as carried out in the present study (March to July) limits the availability of test material throughout the year. However, continuous egg production has been documented for weatherfish in the Russian literature (Kostomarova, 1991). Moreover, a method to induce multiple spawning of females within one year has been reported for the pond loach in Asia (*Misgurnus anguillicaudatus*) (Suzuki, 1983). As the weatherfish is also known to be a multiple spawning fish (Drozd et al., 2009) methods might be successfully transferred. Since wild weatherfish populations might further decline, a large production of test material (i.e. weatherfish embryos) comprises the development of laboratory stocks for testing purposes. Such an approach requires a fundamental concept for sustainable weatherfish production, including detailed studies on the populations genetics. However, the conservation of weatherfish might benefit from an application for ERA since the public perception of the largely cryptic species rises when giving more emphasis to it.

The transparency of the embryonic membrane allows a detection of various characteristics during weatherfish ontogeny, such as differentiation of somites, development of optical and auditory organs, presence of heartbeat and blood circulation (Kostomarova, 1991).

A definition of the timing of teratogenic effects possibly leading to death of weatherfish embryos could not yet be given for (i) lack of somite formation and (ii) non-detachment of the tail (i.e., effects defined as lethal for FET with zebrafish after 24 h) (OECD, 2013). Consequently, only coagulation and lack of heartbeat were defined as lethal for weatherfish embryos in the present study, in order to achieve more conservative results (Table 2). However, it remains questionable if weatherfish embryos with observed severe spinal malformations (Fig. 2D), that were defined as sublethally affected in the present study, were really capable of surviving. Long-term observations of developing weatherfish, including larval and juvenile stages are necessary to provide more specific definitions for the lethality of these effects. Considering negative and internal controls of all conducted tests, stable survival rates could be achieved that were in compliance with validity criteria defined in OECD TG 236 (survival of ≥90% in the control, mortality in the internal control ≤25%) (OECD, 2013). Additionally, high synchronicity of embryo development was observed between unaffected embryos and is also reported by Drozd (2011): start of hatching at 18 and 21° C occurred 2.97±0.10 and 1.95±0.11 days post fertilization, respectively. Both facts emphasize that weatherfish embryos are robust against laboratory procedures like handling stress or temporary fluctuations of conditions. When discussing toxicity tests with weatherfish embryos in the context of alternative test methods, the onset of exogenous feeding is a critical aspect since it defines the maximum exposure time possible, according to criteria defined for animal experiments (EU, 2010, Strähle et al., 2012). Drozd (2011) reported that activation of the digestive system of weatherfish eleutheroembryos does not occur prior to 144 hpf when incubated at 21° C, followed by a first exogenous food intake at 156 hpf. Similar results are reported for the closely related *M. anguillicaudatus* that shows active feeding behavior at 132 hpf when cultured at 20 °C (Fujimoto et al., 2006) and opening of mouth and anus at three days post hatching (19–21° C) (Zhang et al., 2014). Consequently, FET duration of 120 h, as claimed for zebrafish (Strähle et al., 2012), is ethically justifiable also for the weatherfish. Moreover, FET with weatherfish might even be extended to 144 h without requiring additional permissions in terms of animal experiments when tested at the recommended temperatures.

SENSITIVITY TO CONTAMINATION

DCA is a comprehensively studied degradation product of pesticides like diuron, linuron or propanil, with proven toxicity for a wide range of aquatic organisms (Crossland, 1990; Zhu et al. 2013). Furthermore, DCA was chosen as the substance for positive control in OECD TG 236, implying that it is extensively used in FET. An interspecific comparison including

literature data (Braunbeck et al., 2005; Braunbeck & Lammer, 2006; Zhu et al., 2013) revealed that acute effect concentrations for DCA ranged from 0.52 to 24.1 mg/l (EC_{50}) and 0.71 to 4.15 mg/l (LC_{50}), respectively. The highest sensitivity was observed for weatherfish embryos, yielding a factor of three lower 48 h-EC_{50} and LC_{50} values compared to *Danio rerio*. Least sensitive towards DCA were embryos of *Oryzias latipes*, followed by *Pimephales promelas* and *Gobiocypris rarus* (Table 3).

According to its physiological temperature optimum (15-24° C) (Drozd et al., 2009), weatherfish embryos should be tested at lower temperatures (Table 3), yet it has been inferred from early life stage tests with perch (*Perca fluviatilis*) carried out at 20.2±0.3° C that temperature does not affect the toxicity of DCA (Schäfers & Nagel, 1993). A steep slope of the dose-response curve (Fig. 3) has also been reported for zebrafish exposed to DCA (Lange et al., 1995). As a consequence, results might vary disproportionally between tests. However, mean effect concentrations obtained in the present study show only slight deviations between tests (±SD) even with embryos from different wild catches (EC_{50}: 0.58±0.04 mg/l; LC_{50}: 0.71±0.11 mg/l), suggesting a good reproducibility of FET results. Moreover, DCA can be recommended for positive controls in further investigations with a test concentration of 1.0 mg/l. However, in order to assess the sensitivity of weatherfish embryos in a broader sense, additional substances with varying mode of actions or lipophilicity should be tested.

TOLERANCE AGAINST ENVIRONMENTAL CONDITIONS OF CONCERN

Experiments conducted to assess the DO requirements of weatherfish embryos confirm the tolerance expected based on the species autecology (Kottelat & Freyhof, 2007). Elshout et al. (2013) compared the DO tolerance of fish embryos of four temperate species. Based on their results, LOEC and LC_{100} values varied between 3-9 mg/l and 1.5-4.8 mg/l, respectively. Both, EC_{10} (2.18 mg/l for slight and 1.52 mg/l for medium effects) and LC_{90} values (0.53 mg/l) determined for weatherfish embryos revealed that weatherfish might be one of the native species in Europe most tolerant to low DO levels. Strecker et al. (2011) concluded that concentrations ≥2 mg/l do not induce teratogenic effects in non-native zebrafish embryos. Therefore, an application of weatherfish embryos for FET is not restricted by DO, as this value can also be adopted for weatherfish embryos and even slightly lower DO concentrations (≥1.7 mg/l) should not limit its feasibility. Another factor of concern for testing is the tolerance of embryos towards temperature. Drozd et al. (2009) showed that weatherfish embryos can tolerate temperatures during incubation from 9–24° C, with an optimum in the range of 15–24° C. Moreover, Schreiber et al. (2016) showed that weatherfish larvae can survive temperatures

from >15 to 27° C for a period of 25 days, emphasizing the broad thermal tolerance of this species.

CONCLUSIONS

This study demonstrates a high potential of weatherfish embryos to meet many criteria for use as a standard species in toxicity testing. As a rare species that can be found in agriculturally impacted areas, the weatherfish possesses high ecological relevance for European surface waters with particular exposure to hazardous contaminants. The general suitability of weatherfish embryos for experimental investigations is illustrated by compliance with all validity criteria of the FET protocol proposed in OECD TG 236. Furthermore, a protocol for weatherfish embryo production and determination of apically observable teratogenic effects is provided. A table with recommended test conditions for FET with weatherfish embryos can be found in the supporting information (Table S1). Transparency of the embryonic membrane, the brief developmental period from fertilization to hatching and fulfillment of criteria qualifying the FET as alternative test method, underline the practical feasibility. However, restrictions may exist in terms of availability of embryos throughout the year. The sensitivity of weatherfish embryos to DCA was clearly higher than those for zebrafish, Japanese medaka and fathead minnow. Finally, weatherfish embryos are tolerant against DO depletion and show a broad thermal tolerance. Weatherfish (*M. fossilis*) thus appears a suitable additional species for toxicity testing. As an outlook, further investigations might apply weatherfish embryos for sediment toxicity assessments, as both its benthic lifestyle and tolerance against low DO levels implies a close relation to potentially contaminated sediment. For this purpose, the FET protocol can be extended to an application with sediment extracts, pore water or eluates.

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Table S1. Recommended test conditions for fish embryo toxicity assays (FET) conducted with weatherfish embryos in aqueous solutions.

Test conditions	Recommendations
Test medium	reconstituted water according to ISO 7346-3[a]
Temperature	20±1° C[b]
Dissolved oxygen	>2 mg/l[c]
Test duration	72 hpf (extension to 120 hpf possible)[d]
Test chambers	24-well plates (2.5 ml solution per well; 1 embryo per well)[e]
Negative control	20 wells with reconstituted water (additional solvent control if necessary)[e]
Positive control	20 wells with 3,4-Dichloroaniline (1 mg/l)[c, e]
Internal control	4 wells with reconstituted water on each plate[e]
Validity criteria	overall fertilization rate ≥70%; survival in the negative control ≥90%; survival in internal control ≥75%; survival in the positive control ≤70%[e]

[a] ISO, 1996; [b] Drozd et al., 2009; [c] Present study; [d] Drozd, 2011; [e] OECD, 2013

Table S2. Nominal and mean measured concentrations (triplicate; ± standard deviations) from samples taken subsequent to (T-0) and 72 hours after (T-72) preparation of solutions. Measurements included the stock solution (63.0 mg/l) and the highest, the middle and the lowest test concentration.

DCA concentrations		
Nominal (mg/l)	Measured T-0 (mg/l)	Measured T-72 (mg/l)
63.0	62.50 (±0.35)	–
1.45	1.39 (±0.00)	1.23 (±0.00)
0.74	0.70 (±0.01)	0.61 (±0.00)
0.30	0.27 (±0.00)	0.23 (±0.00)

Table S3. Settings for the HPLC-UV system for the chemical analysis of 3,4-Dichloroaniline (DCA).

Pump	PU-2080 (JASCO, Germany)
Mobile phase	CH_3CN/H_2O
Time program	00.00 – 10.00 min: 05/95 to 90/10
	10.00 – 12.00 min: 90/10
	12.00 – 13.00 min: 90/10 to 05/95
	13.00 – 16.00 min: 05/95
Buffer	0.1% CH_2O_2
Flow rate	1 ml/min
Autosampler	AS-2050 (JASCO, Germany)
Injection volume	100 µl
Column	Synergi Hydro-RP (Phenomenex, USA)
Length	50 mm
Internal diameter	4.6 mm
Particle size	2.5 µm
Column oven	JetStream 2 Plus (KNAUER, Germany)
Temperature	20° C
UV detector	UV-2075 (JASCO, Germany)
Wavelength	254 nm

TOWARDS MORE ECOLOGICAL RELEVANCE IN SEDIMENT TOXICITY TESTING WITH FISH: EVALUATION OF MULTIPLE BIOASSAYS WITH EMBRYOS OF THE BENTHIC WEATHERFISH (*Misgurnus fossilis*)

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ABSTRACT

The effects of sediment contamination on fish are of high significance for the protection of ecosystems, human health and economy. However, standardized sediment bioassays with benthic fish species, that mimic bioavailability of potentially toxic compounds and comply with the requirements of alternative test methods, are still scarce. In order to address this issue, embryos of the benthic European weatherfish (*Misgurnus fossilis*) were exposed to native sediment samples (via sediment contact assays (SCA)) and sediment extracts (via acute fish embryo toxicity tests (FET)) varying in contamination level. The extracts were gained by accelerated solvent extraction with (i) acetone (ex-ACE) and (ii) pressurized hot water (ex-PHWE) and subsequently analyzed for polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs). Furthermore, the extracts were exposed to embryos of the predominately used zebrafish (*Danio rerio*), as a reference species. Results indicated a high robustness of weatherfish embryos towards varying test conditions and sufficient sensitivity towards relevant sediment-bound compounds. Furthermore, a compliance of effect concentrations derived from weatherfish embryos exposed to sediment extracts (96 h-LC_{50}) with both measured gradient of sediment contamination and previously published results was observed. In comparison to zebrafish, weatherfish embryos showed higher sensitivity to the bioavailability-mimicking extracts from PHWE but lower sensitivity to extracts gained with acetone. SCAs conducted with weatherfish embryos revealed practical difficulties that prevented an implementation with three of four sediments tested. In summary, an application of weatherfish embryos, using bioassays with sediment extracts from PHWE might increase the ecological relevance of sediment toxicity testing: it allows investigations using benthic and temperate fish species considering both bioavailable contaminants and animal welfare concerns.

INTRODUCTION

Sediments play a major ecological role as they provide essential ecosystem functions and habitats to numerous organisms in aquatic systems (Apitz, 2012; de Castro-Català et al., 2016). On the other hand, their ability to accumulate especially hydrophobic chemicals makes them an important sink and potential source for hazardous contaminants (Ahlf et al., 2002; Power and Chapman, 1992). The alarming deterioration of sediment quality has raised a lot of attention in ecotoxicological research since the early 1970s (Burton, 1991) and led to several legislative regulations (EU, 2008a, 2008b). Furthermore, an integration of bioassays in environmental risk assessment (ERA) of sediment toxicity is highly requested by numerous studies in order to comprehensively investigate possible adverse effects on ecosystems (Burton and Scott, 1992; Chapman et al., 2002).

Besides organisms of lower trophic levels, like bacteria, algae, plants or invertebrates (Feiler et al., 2013; Hafner et al., 2015), special emphasis concerning the protection of ecosystems against sediment contamination is given to fish (Hallare et al., 2011). This is due to their wide distribution, including freshwater, marine and estuarine systems (van der Oost et al., 2003), their fundamental (e.g. nutrient cycling) and demand-derived ecosystem services (e.g. economic and recreational value) (Holmlund and Hammer, 1999) and their close linkage to human health (Kannan and Falandysz, 1997). However, (i) the restricted suitability of exotic and pelagic (vs. native and benthic) fish species, (ii) animal welfare concerns related to the usage of vertebrates and (iii) the selection of appropriate exposure scenarios are emphasized as major critical issues for widespread application of fish in sediment toxicity testing (Burton, 2013; Chapman et al., 2002, Hallare et al., 2011).

Benthic species are considered more vulnerable to sediment contamination because of their proximity to the potential hazard and the resulting multiple exposure pathways (e.g. aqueous and dietary exposure) (Costa et al., 2011; Egeler et al., 2001). However, toxicity testing with indigenous benthic fish species is rather an exception for site-specific investigations of European freshwater sediments (Viganò et al., 2015). This lack is particularly apparent in the case of sediments from European edge-of-field surface waters like drainage channels and ponds, that are under high agricultural influence (Stoate et al., 2009).

The endangered European weatherfish (*Misgurnus fossilis*) inhabits systems described above and is known to be highly dependent on sediment quality because of its benthic lifestyle (Kottelat and Freyhof, 2007; Meyer and Hinrichs, 2000). Environmental pollution in agriculturally used landscapes is assumed to be among the reasons for the species decline observed in many regions across its original range (Hartvich et al., 2010). A test protocol for

conducting the acute fish embryo toxicity test (FET) with weatherfish embryos, that complies with the requirements for alternative test methods - fish embryos are not considered as experimental organisms prior to the start of exogenous feeding (EU, 2010; Strähle et al., 2012) - has already been established (Schreiber et al., 2017a). Furthermore, first results indicated high sensitivity of weatherfish embryos towards a reference substance (3,4-dichloroaniline (DCA)) and high tolerance against low dissolved oxygen concentrations (Schreiber et al., 2017a). Consequently, the weatherfish might be a promising indigenous species for an ERA of sediment toxicity in Europe based on the alternative FET, especially when focusing on sediments from agriculturally impacted waters.

In the context of sediment toxicity testing, the FET can be applied as long as aqueous solutions of particle bound chemicals are prepared. This can be achieved by a variety of extraction methods and subsequent exposure to the gained extract (Hallare et al., 2011; Schulze et al., 2012). Organic solvent extraction (e.g. with acetone) can be used to investigate the overall hazard potential of a sediment (i.e. worst-case scenario) as also hardly bioavailable fractions are extracted (Kosmehl et al., 2007). In contrast, elutriates are extracted from sediments with water to study the toxicity of easily dissolved and rapidly desorbing (i.e. bioavailable) sediment fractions (Haring et al., 2012). Alternatively, pressurized hot water extraction (PHWE) might be used as an intermediate extraction method because it combines the naturally occurring solvent water - also used for elutriate extraction - with supernatural extraction conditions (i.e. high temperature and pressure) - also part of the organic solvent extraction (Hawthorne et al., 2000). PHWE allows the extraction of environmentally relevant contaminants like PAHs or pesticides over a wide range of polarities from various environmental matrices (Hawthorne et al., 1994, 2000; Teo et al., 2010; Yang et al., 1997). However, to our knowledge PHWE was not yet applied for sediment toxicity bioassays with fish. As a further development of the FET, Hollert et al. (2003) presented a sediment contact assay (SCA) where zebrafish embryos are incubated in direct contact to freeze-dried sediment samples and later also with native sediments (Feiler et al., 2013). This exposure scenario is assumed to be most realistic for sediment toxicity testing with fish embryos (Hallare et al., 2011) and might be successfully transferred to weatherfish (Schreiber et al., 2017a).

In order to provide a comprehensive approach to the above listed challenges in the field of sediment toxicity testing with fish, this study investigated the suitability of weatherfish embryos for an ERA of sediment toxicity, including a comparison of different exposure scenarios that comply with the requirements for alternative test methods (i.e. FET and SCA). Moreover, the potential of PHWE was investigated as a novel extraction method in the context

of sediment toxicity bioassays with fish embryos. For this purpose, four sediments of varying contamination degree were extracted using organic solvent extraction with acetone (ex-ACE) and PHWE (ex-PHWE). Weatherfish embryos were acutely (96 h) exposed to the extracts, using FETs. Furthermore, SCAs (96 h) were conducted to study the effects of freeze-dried sediment samples. In order to compare the results to an established test organism, zebrafish embryos were exposed to ex-ACE and ex-PHWE from the two most contaminated sediments tested here, also using FETs (96 h) (Fig. 1). Additionally, chemical analyses of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs) in ex-ACE and ex-PHWE, as well as quantification of total lipid content and fatty acid composition of weatherfish eggs were carried out.

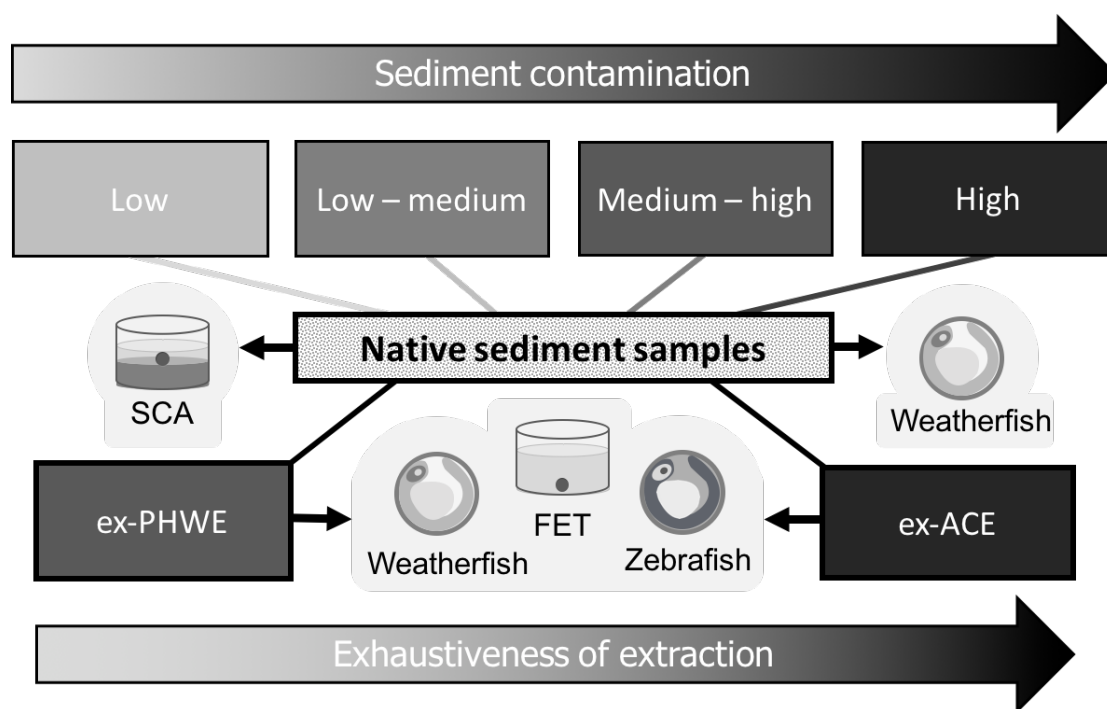**Fig. 1**. Structure of the present study. Four native sediment samples of varying contamination degree were tested in sediment contact assays (SCA) with weatherfish embryos. Furthermore, extracts gained by pressurized hot water extraction (ex-PHWE; low exhaustiveness) and acetone extraction (ex-ACE; high exhaustiveness) were tested in acute fish embryo toxicity tests (FET) with weatherfish and zebrafish embryos.

MATERIAL AND METHODS

SEDIMENT SAMPLING AND PROCESSING

The used sample scheme aimed at choosing sediments along a contamination gradient, including a sediment from an agricultural ditch with stable weatherfish occurrence (ST; presumed low contamination), two previously studied sediments from the Rhine (ES and AL; medium contamination), and a previously studied sediment from a large port (HH; high contamination) (Table 1). Sediment sampling of ST was carried out with a scoop (maximum sediment depth = 10 cm), samples were roughly sieved to remove coarse vegetation components and freeze-dried (Alpha 1-2 LDplus, Christ, Osterode, Germany). ES, AL, and HH were sampled with a Van-Veen gripper, samples were immediately shock-frozen with liquid nitrogen and subsequently freeze-dried (Alpha 2-4 LDplus, Christ, Osterode, Germany) (Schiwy et al., 2015). Finally, all freeze-dried sediments were sieved to a grain size of 740 μm and stored in dark at -20°C until further processing.

Table 1. Overview of sediments investigated in the present study.

Acronym	Site	Waterbody description	Assumed level of contamination
ST	Streitgraben, Jockrim	Agricultural ditch with weatherfish occurrence	Low
ES	Rhine, Ehrenbreitstein	Backwater	Low - medium
AL	Rhine, Altrip	Main stream	Medium - high
HH	Vering Canal, Hamburg	Port water	High

References of previous studies conducted with ES, AL, and HH: Feiler et al., 2009; Hafner et al., 2015; Höss et al., 2010; Schiwy et al., 2015; Bräunig et al., 2015.

Sediment extracts were produced by accelerated solvent extraction (ASE 350, Thermo Scientific Dionex, USA) using (i) acetone and (ii) water (Milli-Q purified; Millipore, USA) as solvents. Each extraction was carried out with six stainless steel cells (34 ml; Agilent Technologies, Germany) that were filled with 8 g of freeze-dried sediment. Top and bottom of the cells were covered with cleaned and glowed analytical sea sand (Dinkelberg Analytics, Germany) and sealed with cellulose (for acetone extraction; Restek, USA) and glass microfiber

filters (for water extraction; Ahlstrom, Finland), respectively. All extractions were performed at 100°C with three cycles of 5 min heat-up and 5 min static extraction. Cells were rinsed with the respective solvent (40% of cell volume) and pressurized nitrogen (1 MPa; 60 seconds) was used to purge the extracts from the cells. Method controls were produced identically but with cells filled exclusively with analytical sea sand and sealed with the respective filters.

Both extract types, acetone extracts (ex-ACE) and water extracts (ex-PHWE), were subsequently evaporated under a nitrogen stream for 4 h (ex-ACE) and 48 h (ex-PHWE), respectively. During evaporation, vessels were periodically vortexed to minimize extract residuals on the glass surface. After evaporation to dryness, all ex-ACE, except from HH, were re-dissolved in 2.4 ml of dimethyl sulfoxide (DMSO) (99.98%; Fisher Scientific, USA) to achieve a storage concentration of 20 g of dry weight sediment equivalent (SEQ) per ml DMSO. Due to the high amount of sediment bound compounds gained from HH, it was re-dissolved in 4.2 ml DMSO, resulting in a storage concentration of 11.4 g SEQ/ml DMSO. ex-PHWE were re-dissolved in 4.8 ml of a 1:1 DMSO:water solution, resulting in a storage concentration of 10 g SEQ/ml solution (equivalent to 20 g SEQ/ml DMSO). All extracts were stored in dark at -20°C until usage for toxicity testing.

FISH MAINTENANCE AND EMBRYO PRODUCTION

Adult weatherfish caught from three different wild populations in April 2015 were treated according to Schreiber et al. (2017a). To induce stripping, water temperature was gradually increased from 11 to 18°C and spawners were treated with a hormonal preparation (Ovopel, Unic-trade, Hungary). Fertilization percentage of eggs were checked under a binocular microscope (Olympus SZX9, Japan). For each test, only males and females that belong to the same population were used.

Zebrafish embryos were provided by the Department of Zoology, University of Heidelberg, Germany. Adult zebrafish maintenance and embryo production followed the conditions and procedures reported by Braunbeck et al. (2005).

FISH EMBRYO EXPOSURE TO SEDIMENT EXTRACTS

Exposure of fish embryos to sediment extracts (i.e. ex-ACE and ex-PHWE) was carried out according to OECD test guideline 236 (OECD, 2013) with minor adjustments for weatherfish embryos, following the protocol provided by Schreiber et al. (2017a). In brief, after fertilization, embryos were transferred to fully aerated artificial water (ISO, 1996) that also served as basis for all test solutions and controls. Each concentration was tested in a 24-well plate (Orange

Scientific, Belgium) with 20 replicates and 4 internal negative controls (artificial water). Three additional plates with the same design served as (i) negative control (artificial water), (ii) positive control (1.05 mg/l of 3,4-dichloroaniline (98% purity, Sigma-Aldrich, Germany; DCA) for weatherfish and 4.0 mg/l DCA for zebrafish embryos), and (iii) method control (ex-ACE and ex-PHWE method controls). The method control investigated the influence of the extraction method and the additional solvent, in our case DMSO (1%). Well plates were prepared with 1 ml of test solution per well and embryos were added with 1 ml of artificial water. DMSO concentration was adjusted to 1% in all test solutions for reasons of comparability. All test concentrations are given in Table S1 in the supporting information.

Exposure duration was 96 h, photoperiod was set to 12 h light:12 h dark, and embryos were examined for apically observable lethal effects under a microscope (Olympus, CX31, Japan) every 24 h (Table 2). The incubation temperatures differed between test species because of different temperature optima and were 26±1°C for zebrafish (OECD, 2013) and 20±1°C for weatherfish embryos (Drozd et al., 2009; Schreiber et al., 2017b). Observations of test concentrations and controls were considered as valid when mortality in the internal control was ≤25%. Furthermore, a whole test was accepted when overall fertilization percentage in the batch of stripped eggs was ≥70%, all method control plates were valid (see above), survival in the negative and method control were ≥90%, and survival was ≤70% in the positive control.

Table 2. Apically observable effects in weatherfish and zebrafish embryos defined as lethal after 96 h in acute fish embryos toxicity tests (FET) and sediment contact assays (SCA).

Weatherfish embryos[a]	Zebrafish embryos[b]
Coagulation	Coagulation
Lack of heartbeat	Lack of heartbeat
	Non-detachment of the tail
	Lack of somite formation

[a] according to Schreiber et al. (2017a)
[b] according to OECD 236 (2013)

SEDIMENT CONTACT ASSAY WITH WEATHERFISH EMBRYOS

The sediment contact assay (SCA) was conducted following Hollert et al. (2003). Target concentrations were achieved by mixing freeze-dried sediments with washed quartz sand (F36, Bassermann minerals GmbH, Germany). Subsequently, the mixture was mortared in order to increase homogeneity, transferred into 4 wells of a 6-well plate (3 g per well) and 4 ml of aerated artificial water (ISO, 1996) were added. Additionally, 4 control plates were prepared for each test, including (i) a negative water control (8 wells with 4 ml artificial water), (ii) a negative quartz sand control (4 wells with 3 g quartz sand + 4 ml artificial water), (iii) a positive water control (4 wells with 4 ml DCA solution (final concentration of 1.05 mg/l)), and (iv) a positive quartz sand control (4 wells with 3 g quartz sand + 4 ml DCA solution (final concentration of 1.05 mg/l)). In order to achieve oxygen saturation in the water column prior to test start, the prepared plates were closed and put on an orbital shaker (KL-2, Edmund Bühler GmbH, Germany) at 50 rpm for 24 h (Strecker et al., 2011). Tests were started by gently adding 5 embryos together with 1 ml artificial water to each well. All test concentrations are shown in Table S1 in the supporting information.

Weatherfish embryos were incubated at 20±1°C and examined for apically observable effects after 48 and 96 h. Over the whole test duration, closed well plates were kept on the orbital shaker at 50 rpm. Dissolved oxygen was measured in three wells of the highest test concentration at 96 h for sediments from ST, AL and ES, using the physico-optical oxygen sensor FireStingO$_2$ (PyroScience, Germany) (mean ± standard deviation (SD): ST=5.3±0.6 mg/l; AL=3.9±0.9 mg/l; ES=5.9±0.3 mg/l). Evaluation of apically observable effects was identical to FETs with sediment extracts (Table 2). Validation criteria in SCA were identical to the criteria mentioned for FETs with sediment extracts.

CHEMICAL ANALYSES OF PAHs, PCBs AND PCDD/Fs IN SEDIMENT EXTRACTS

Test solutions of all sediments, containing the highest test concentration of ex-ACE and ex-PHWE (Table S1) and 1% of DMSO were analyzed for PAHs (Naphthalene; Acenaphthylene; Fluorene; Phenanthrene; Anthracene; Fluoranthene; Pyrene; Benzo[a]anthracene; Chrysene; Benzo[b+j]fluoranthene; Benzo[a]pyrene; Indeno-1,2,3-[c,d]pyrene; Benzo[g,h,i]perylene; Dibenz[a,h]anthracene), PCBs (77; 81; 126; 169; 105; 114; 118; 123; 156; 157; 167; 189) and PCDD/Fs (2,3,7,8-TCDD; 1,2,3,7,8-PeCDD; 1,2,3,4,7,8-HxCDD; 1,2,3,6,7,8-HxCDD; 1,2,3,7,8,9-HxCDD; 1,2,3,4,6,7,8-HpCDD; OCDD; 2,3,7,8-TCDF; 1,2,3,7,8-PeCDF; 2,3,4,7,8-PeCDF; 1,2,3,4,7,8-HxCDF; 1,2,3,6,7,8-HxCDF; 1,2,3,7,8,9-HxCDF; 2,3,4,6,7,8-HxCDF; 1,2,3,4,6,7,8-HpCDF; 1,2,3,4,7,8,9-HpCDF; OCDF). The chemical analyses were

performed by the Bayreuth Institute of Environmental Research (Ökometric GmbH, Bayreuth, Germany) and followed standard method protocols (ISO, 2000, 2004, 2007).

TOTAL LIPID CONTENT AND FATTY ACID COMPOSITION OF WEATHERFISH EGGS

For the determination of total lipid content and fatty acid composition, triplicates (mean wet weight $\pm$ SD = 1.02$\pm$0.01 g) of unfertilized weatherfish eggs from two batches were used. The following described procedures were performed at the Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice, Czech Republic. Methods applied for lipid extraction followed the protocol of Hara and Radin (1978) with slight modifications. In brief, egg samples were homogenized in 10 ml of hexane-isopropanol (3:2) and 6 ml of Na_2SO_4 (6.67%) were added to the obtained homogenates. After centrifugation, the upper lipid phase was transferred into pre-weighted tubes and subsequently evaporated under nitrogen. Final determination of lipid content was carried out gravimetrically. Methylation of 2 mg of lipids was induced with boron trifluoride-methanol complex solution and NaOH as described by Appelqvist (1968). Resulting fatty acid methyl esters (FAME) were checked on TLC plate and analyzed using a gas chromatograph (Trace Ultra FID; Thermo Scientific, USA) equipped with a BPX 70 column (SGE, USA). Subsequently, comparison of FAME retention times of sample and standard GLC-68D (Nu-Chek Prep, USA) were used to identify fatty acid compositions.

DATA ANALYSES

All data analyses and graphs were carried out with the open source software package R Version 3.0.2 (R Development Core Team, 2013) extended with the 'drc'-package (Ritz, 2013). To calculate lethal concentrations after 96 h (LC_{50}), including the corresponding standard errors (SE), concentration-response models were fitted and the best fitted model was chosen based on Akaike's information criterion and visual judgement. All concentrations are given in mg of sediment equivalent per ml (mg SEQ/ml), regardless of the applied test method (FET or SCA). In order to compare the calculated LC_{50} values, ratio tests were conducted following the method reported by Wheeler et al. (2006). The mortality rates and concentration-response curves of all tests, are given in the supporting information (Fig. S1; Fig. S2).

RESULTS

SEDIMENT TOXICITY TO WEATHERFISH EMBRYOS

In all FETs conducted with weatherfish embryos, survival rates were ≥95% in the negative and the method controls. Moreover, in the same tests, survival rates in the positive controls (1.05 mg/l DCA) were ≤70%. LC_{50} values could be calculated for ex-ACE from all sediments (±SE) and showed the highest value for ST (129.08±8.86 mg SEQ/ml), followed by ES (44.46±2.39 mg SEQ/ml), AL (38.16±2.38 mg SEQ/ml) and HH (3.03±1.37 mg SEQ/ml), all differing significantly from each other (Fig. 2). Concerning ex-PHWE, no LC_{50} value could be calculated for ST, as even the highest test concentration (200 mg SEQ/ml) induced only 15% mortality of weatherfish embryos. However, LC_{50} values calculated for ex-PHWE from ES (133.20±8.70 mg SEQ/ml), AL (124.30±4.31 mg SEQ/ml), and HH (15.64±0.12 mg SEQ/ml) showed a decrease of LC_{50} values in the same order as observed for ex-ACE (Fig. 2), even though ES and AL did not differ significantly.

Considering SCAs conducted with weatherfish embryos, survival rates after 96 h were 100% in all negative (with and without quartz sand) and 0% in all positive controls (1.05 mg/l DCA; with and without quartz sand). However, LC_{50} values could only be calculated for HH (7.05±0.04 mg SEQ/ml) because methodical difficulties - that are specified and discussed below - impaired the feasibility of SCAs with sediments from ST, ES and AL (Fig. 2).

When comparing the LC_{50} values of both extraction methods (i.e. ex-ACE vs. ex-PHWE) derived for sediments from ES, AL, and HH, all values differ significantly and ex-ACE always lies below ex-PHWE by a mean factor (±SD) of 3.81±0.97 (ES=3.00; AL=3.26; HH=5.17). The LC_{50} value (±SE) calculated for SCA conducted with HH (7.05±0.04 mg SEQ/ml) is significantly higher than the LC_{50} derived for ex-ACE (3.03±1.37 mg SEQ/ml) but significantly lower than the LC_{50} derived for ex-PHWE (15.64±0.12 mg SEQ/ml) (Fig. 2).
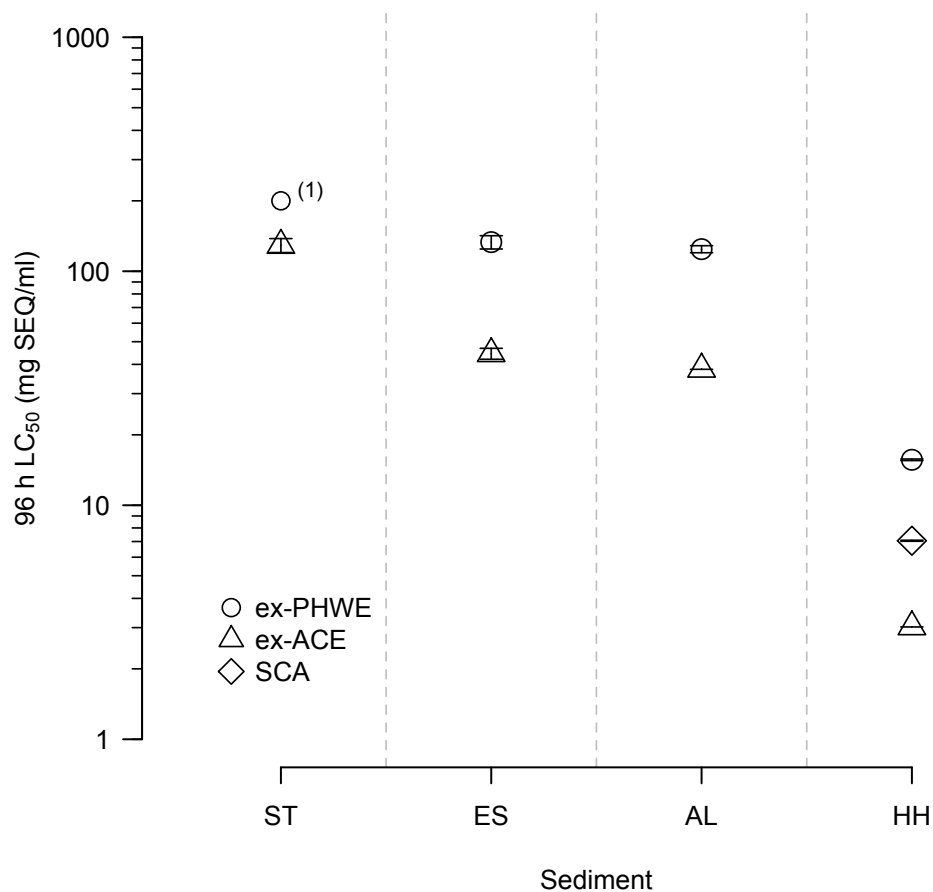
Fig. 2. 96 h LC_{50} values and the corresponding standard errors of weatherfish embryos, given for sediments from Streitgraben (ST), Ehrenbreitstein (ES), Altrip (AL), and Vering Canal (HH). All sediments were tested as pressurized hot water extracts (ex-PHWE; ○), as acetone extracts (ex-ACE; △) and in a sediment contact assay (SCA; ◇). However, in the case of SCA, calculation of LC_{50} value was only feasible for HH. Symbol labeled with (1) indicates that no LC_{50} value could be calculated but given value corresponds to the highest concentration tested (200 mg SEQ/ml) at which <50% of weatherfish embryos died.

COMPARISON BETWEEN WEATHERFISH VS. ZEBRAFISH EMBRYOS EXPOSED TO EX-ACE AND EX-PHWE

When comparing the calculated LC_{50} values (±SE) (Fig. 3), ex-ACE from AL shows a lower, even though not significantly differing, value for zebrafish (23.31±4.69 mg SEQ/ml) compared to weatherfish embryos (38.16±0.02 mg SEQ/ml; ratio: 1.64). The same applies for ex-ACE from HH but with a significant difference between both values (LC_{50} zebrafish ±SE: 0.58±0.00 mg SEQ/ml; LC_{50} weatherfish ±SE: 3.03±1.37 mg SEQ/ml; ratio: 5.22).

A comparison of LC_{50} values was not possible for ex-PHWE from AL, as only 10% of zebrafish embryos died in the highest test concentration (200 mg SEQ/ml) (Fig. 2). However, the same concentration induced a mortality rate of 100% in weatherfish embryos (LC_{50} ±SE:

124.30±4.31 mg SEQ/ml), indicating that sensitivity of weatherfish was higher in this treatment. ex-PHWE from HH revealed a significant lower LC_{50} value for weatherfish (15.64±0.12 mg SEQ/ml) compared to zebrafish embryos (33.14±2.55 mg SEQ/ml; ratio: 2.12).
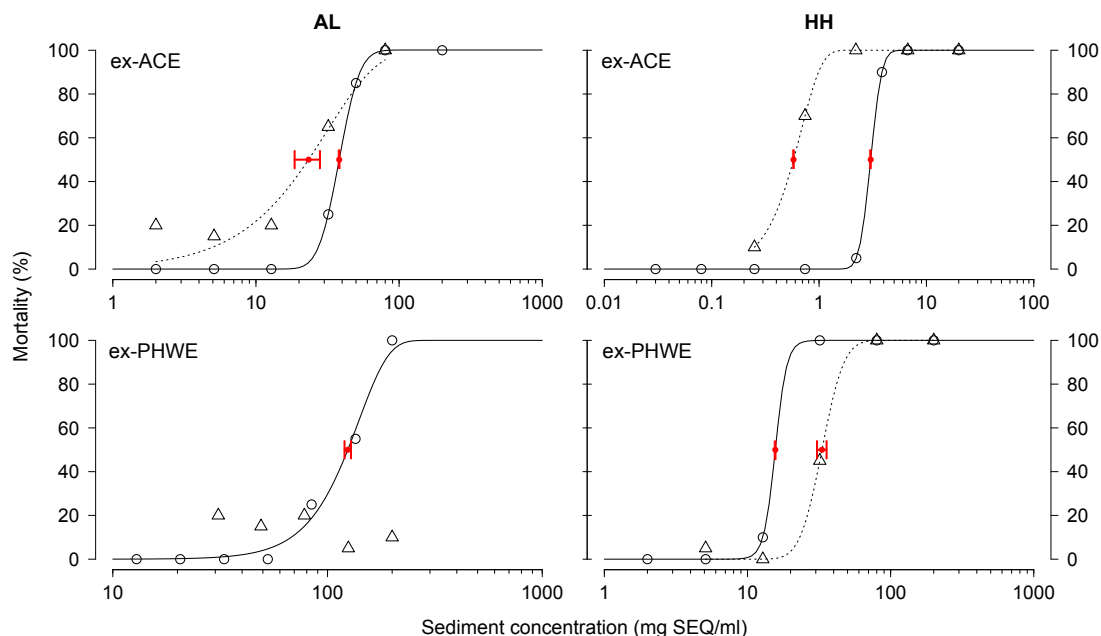

Fig. 3. Concentration-response curves (mortality) for weatherfish ($\bigcirc$; solid lines) and zebrafish ($\triangle$; dashed lines) embryos exposed for 96 h to acetone extracts (ex-ACE) and pressurized hot water extracts (ex-PHWE) of sediments from Altrip (AL; left) and Vering Canal (HH; right). Red dots with error bars indicate LC_{50} values with the corresponding standard errors. No LC_{50} value could be calculated for zebrafish embryos exposed to ex-PHWE from AL as even the highest test concentration (200 mg SEQ/ml) induced ≤50% mortality.

CHEMICAL ANALYSES OF PAHS, PCBS AND PCDD/FS IN SEDIMENT EXTRACTS

Comparing the sum concentrations of PAHs in ex-ACE from all sediments, the highest concentration was measured for HH (107.42 mg/kg SEQ), followed by AL (0.88 mg/kg SEQ), ES (0.55 mg/kg SEQ) and ST (0.10 mg/kg SEQ). Hence, the sum concentration of PAHs measured for ST was three orders of magnitude lower than for HH. The same pattern was present for the sum concentrations of PCBs measured in ex-ACE (i.e. HH (4.13 µg/kg SEQ) > AL (1.10 µg/kg SEQ) > ES (0.56 µg/kg SEQ) > ST). However, the concentrations of all measured PCBs in ex-ACE from ST were below the limit of quantification (Table S4). The sum concentrations of PCDD/Fs measured in ex-ACE were highest for HH (1.68 µg/kg SEQ), followed by AL (0.48 µg/kg SEQ), ST (0.19 µg/kg SEQ) and ES (0.18 µg/kg SEQ) (Fig. 4). However, the latter two sediments differed only by 0.01 µg/kg SEQ.

Regarding the results from chemical analyses of ex-PHWE, all PCBs and PCDD/Fs were below the limit of quantification in all sediments (Table S4; Table S3). Furthermore, the sum concentrations of PAHs measured in ex-PHWE were highest for HH (1.60 mg/kg SEQ), followed by ST (0.18 mg/kg SEQ), AL (0.17 mg/kg SEQ) and ES (0.16 mg/kg SEQ) (Fig. 4). However, differences measured between the latter three sediments were not greater than 0.02 mg/kg SEQ.
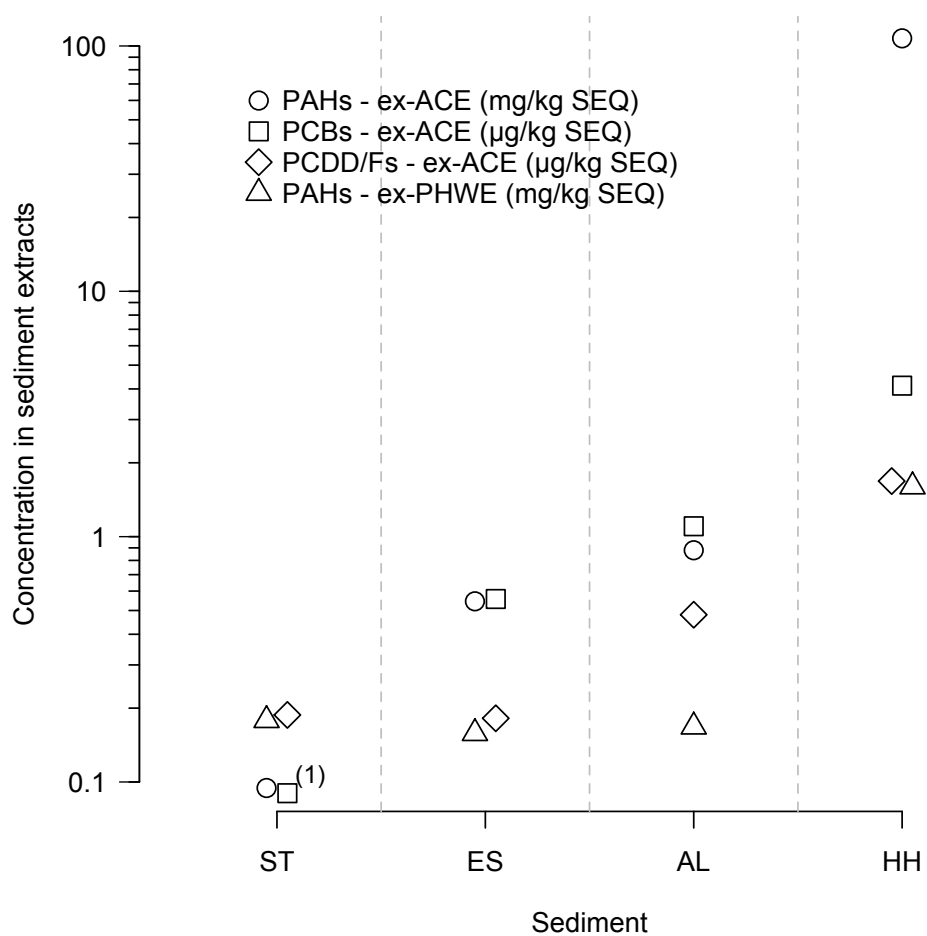
Fig. 4. Sum concentrations of PAHs (○/△), PCBs (□) and PCDD/Fs (◇), measured in acetone extracts (ex-ACE) and pressurized hot water extracts (ex-PHWE) of sediments from Streitgraben (ST), Ehrenbreitstein (ES), Altrip (AL), and Vering Canal (HH). Symbol labeled with (1) indicates that all measured substances were below the limit of quantification (PCDD/Fs in ex-ACE from ST). Sum concentrations of PCBs and PCDD/Fs in ex-PHWE are not shown because all measured substances were below the limit of quantification (Table S4; Table S3).

LIPID CONTENT AND FATTY ACID COMPOSITION OF WEATHERFISH EGGS

The total lipid content of unfertilized weatherfish eggs ranged between 54 and 66 mg/g wet weight (mean ± SD = 58±5 mg/g wet weight), which is equal to 5.4 – 6.6% of lipid content per individual egg (mean ± SD = 5.5±0.5%).

The highest relative amounts among all measured fatty acids in weatherfish eggs were determined for the saturated palmitic acid (C16:0; 21.7%) and the polyunsaturated docosahexaenoic acid (C22:6n3; 19.4%). Further fatty acids that accounted for more than 6% were found to be the polyunsaturated arachidonic acid (C20:4n6; 9.6%) and oleic acid (C18:1n9; 9.3%), the saturated stearic acid (C18:0; 7.0%) and the polyunsaturated cis-vaccenic acid (C18:1n7; 6.5%) and eicosapentaenoic acid (C20:5n3, 6.1%). Further information about the fatty acid composition of weatherfish eggs are shown in Fig. S3 in the SI.

DISCUSSION

SUITABILITY OF WEATHERFISH EMBRYOS FOR SEDIMENT TOXICITY BIOASSAYS

The high survival rates of weatherfish embryos that were obtained in all method and negative controls (i.e. ≥95%) endorse the previously stated robustness of this species for experimental investigations (Schreiber et al., 2017a). Furthermore, a direct influence on survival of weatherfish embryos can be excluded from a DMSO concentration of 1% that was used in FETs with sediment extracts and from quartz sand and orbital shaking that were used in SCAs. Hence, weatherfish embryos met the requirements of all applied bioassays.

However, the mortality rates of weatherfish embryos observed in the positive controls (1.05 mg/l DCA) of FETs (30-50%) were lower than expected based on results by Schreiber et al. (2017a), where DCA concentrations of more than 1 mg/l led to 100% mortality after 72 h. An explanation for this deviation might be the steep slope of the concentration-response curve of DCA which can lead to a high variability of mortality (Schreiber et al., 2017a). Yet, it cannot be excluded that these variations were caused by differences in sensitivity of the batches of embryos (e.g. due to maternal or genetic differences). However, positive controls of SCAs showed expected mortality rates in absence (100%) and presence of quartz sand (100%). Further interlaboratory comparisons and repeated investigations focusing on consistency of the results should be performed to increase the acceptance for weatherfish embryos as a new species for toxicity testing.

Effect concentrations (i.e. 96 h-LC$_{50}$) derived from FETs with weatherfish embryos and sediment extracts (i.e. ex-ACE and ex-PHWE; Fig. 2) reflected the same gradient in toxicity of

the tested sediments that was previously assumed based on other studies and sampling site information (ST<ES<AL<HH; Table 1). Extracts from ES, AL and HH revealed a toxicity gradient (ES<AL<HH; Figure 2) that is consistent with results reported by Schiwy et al. (2015). In the mentioned study, native sediments from ES, AL and HH were tested, inter alia, in SCAs using zebrafish embryos (96h-LC_{50}). In addition, the identical gradient (ES<AL<HH) was reported by Hafner et al. (2015), where Soxhlet-generated acetone extracts were tested in FETs with zebrafish embryos (48h-EC_{50}). Including the results from chemical analyses, measured sediment contaminations show the same gradient (ST<ES<AL<HH) in ex-ACE analyzed for PAHs, PCBs and, with some limitations (i.e. ST≈ES), PCDD/Fs (Fig. 4). The demonstrated compliance of sediment contamination with derived effect concentrations suggests a good reliability for an ERA of sediment toxicity based on FETs with weatherfish embryos exposed to ex-ACE. Further sediment testing should be performed to increase the database for weatherfish. Even though ex-PHWE showed the same toxicity gradient like ex-ACE (Fig. 2), results could not be linked to the contamination of the extracts as most chemical groups were below the limit of quantification (Table S2; Table S3; Table S4).

A comparison of toxicity between sediments could not be performed based on SCAs with weatherfish embryos, as effect concentrations could only be calculated for the heavily contaminated sediment from HH (Fig. 2). High oxygen consumption rates and low water absorption capacities of sediments from ST, AL, and ES prevented an application of high sediment concentrations (>600 mg SEQ/ml) in the SCA. More specifically, the associated limitation of dissolved oxygen and free water volume possibly affected survival of weatherfish embryos independently from sediment toxicity and large parts of the embryos were untraceable in the wells after 96 h. Hence, a clear allocation of observed toxicity effects to the determining cause was not possible here. Similar problems were reported for SCAs conducted with zebrafish embryos for some of the sediments tested here (AL, ES) as well as for other sediments (Schiwy et al., 2015; Strecker et al., 2013). Furthermore, Fleming and Giulio (2011) showed that hypoxic conditions can enhance the toxicity of PAHs in zebrafish. In contrast, the high amount of quartz sand used to dilute the heavily contaminated sediment from HH eliminated these methodical problems. Consequently, the SCA with weatherfish embryos similar to the one with zebrafish can be stated as a feasible screening method for highly contaminated sediments but certain sediment properties might limit its feasibility.

COMPARISON OF DIFFERENT EXPOSURE SCENARIOS

When comparing the different exposure scenarios (Fig. 2), the obtained results support the worst-case properties of organic solvent extracts (Kosmehl et al., 2007) as levels of toxicity and contamination (Fig. 4) of ex-ACE were highest. However, since the extraction with organic solvents can recover compounds from sediments that are sequestrated (Kelsey et al., 1997) and therefore unavailable to fishes under natural conditions (Mayer and Reichenberg, 2006), sediment bioassays with ex-ACE run the risk to bias an ERA of sediment toxicity (Brack et al., 2009). Moreover, additional solvents that are added to prevent precipitation of nonpolar compounds in the test solutions (in this case DMSO) are critical as they can change the embryos chorion permeability and hence increase toxic effects (Kais et al., 2013). Further drawbacks are considered to be that organic solvents are expensive and not environmental-friendly (Teo et al., 2010).

The relatively lower toxicity and contamination derived for ex-PHWE, as observed here, supports the hypothesis that PHWE is an intermediate extraction method. Regarding the simulation of bioavailability, PHWE is assumed to predominately recover fractions of sediments or soils that are available to environmental processes such as transfer into organisms (Hawthorne et al., 2000), making it a feasible method to include desorption behavior of contaminants. Konda et al. (2002) effectively applied hot water extraction to evaluate desorption behavior of organic pesticides in soil and Tao et al. (2006) reported that water extracts could simulate bioavailability of PAHs to plant roots. The sensitive reaction of weatherfish embryos to ex-PHWE, as observed in the present study, implicates a potential of PHWE to be used in sediment bioassays with fish. Furthermore, water is a 'green' and favorable solvent (Teo et al., 2010) that eliminates the need for additional solvents like DMSO as extracted fractions have sufficient water solubility. However, it should be mentioned that the ability of water to extract nonpolar compounds from sediments is highly dependent on extraction temperature (Teo et al., 2010; Yang et al., 1997). In this study, extraction temperature was generally set to 100° C in order to compare both extraction solvents. A refinement of extraction parameters, especially temperature, would allow to optimize PHWE in terms of bioavailability simulation. Moreover, aqueous sediment extracts can only be an approach in simulating the bioavailability of sediment-bound compounds.

On the other hand, whole sediment assays like SCAs allow an investigation of toxic effects induced by both, the bioavailable fractions of particle-bound substances and the compounds dissolved in the water column (Feiler et al., 2013; Hollert et al., 2003). In the present study, the SCA conducted with HH revealed a medium toxicity (i.e. between ex-ACE

and ex-PHWE), which supports the assumption that direct contact to the sediment plays an important role for contaminant uptake. This makes it reasonable that sediment-dwelling organisms like benthic fish species are particularly exposed to sediment associated contaminants. However, as already mentioned above, methodical restrictions observed in SCAs might prevent an application for large-scale ERA of sediment toxicity.

INTERSPECIES COMPARISON OF WEATHERFISH AND ZEBRAFISH EMBRYOS SENSITIVITY

A transfer of standard test protocols like FET (OECD, 2013) to new test species is recommended for evaluating their acceptance and applicability (Braunbeck et al., 2005) and should be widely applied by comparing lethal effects of species (Nguyen et al., 2001). In the case of weatherfish vs. zebrafish embryos, a comparison based on current literature is restricted to the reference substance DCA, where weatherfish embryos revealed higher sensitivity by a factor of 3 (72h-LC_{50} and 72h-EC_{50}) (Schreiber et al., 2017a). Including the total lipid content determined in the present study, unfertilized weatherfish eggs revealed 3 times higher values than zebrafish eggs (Hachicho et al., 2015). Hence, the difference in sensitivity to DCA might be explained by the lipophilic character of the test substance (log K_{OW} of DCA= 2.7).

The observed differences in sensitivity between weatherfish vs. zebrafish embryos towards the most contaminated sediments from AL and HH were exposure scenario specific: while ex-ACE of both sediments revealed a higher sensitivity of zebrafish vs. weatherfish embryos, ex-PHWE showed the opposite picture (Fig. 3). Considering the different polarity of acetone vs. water, it can be assumed that ex-ACE contain rather low-polarity compounds, whereas ex-PHWE mainly contain compounds of higher polarity (Hawthorne et al., 1994). This assumption could partly be confirmed by chemical analyses, as the sums of low-polarity compounds, including PAHs, PCBs and PCDD/Fs, were higher in ex-ACE than in ex-PHWE (Fig. 4). These so-called dioxin-like compounds are known to induce severe toxic effects in early-life stages of different fish species (King-Heiden et al., 2012) by the aryl hydrocarbon receptor (AhR)- mediated mechanism (Sarasquete and Segner, 2000). Even if zebrafish embryos are widely used in bioassays to assess AhR- mediated toxicity (Boehler et al., 2017; Kais et al., 2017; Otte et al., 2008), they are reported to be least sensitive to dioxin-like compounds when compared to other species (Doering et al., 2013). Assuming that dioxin-like compounds are responsible for the observed toxicity of ex-ACE, the higher sensitivity of zebrafish vs. weatherfish embryos implies that weatherfish is even less sensitive to AhR-mediated toxicity. However, as sediments can be burdened by complex mixtures of

contaminants, an identification of cause-effect relationships traced to single compounds or compound classes is highly challenging (Schiwy et al., 2015).

With regard to the higher sensitivity of weatherfish vs. zebrafish embryos towards ex-PHWE, two substances within the PAHs could be identified that revealed higher concentrations in ex-PHWE compared to ex-ACE: Naphthalene (for HH and AL) and Acenaphthylene (for AL) (Table S2). Since both substances have relatively low molecular weights (Naphthalene: 128.17 g/mol; Acenaphthylene: 152.20 g/mol) they can be efficiently extracted with water (Hawthorne et al., 1994). However, it remains questionable if these substances are responsible for the relatively sensitive reaction of weatherfish embryos, as the measured differences in their concentration level were very low (Table S2). A more important role for the increased toxic potential of ex-PHWE for weatherfish embryos might be attributed to polar (e.g. chlorinated phenols) and ionic compounds (e.g. heavy metals) extracted by water, particularly as a low sensitivity of adult zebrafish towards heavy metal contamination (96 h) was already stated by de Paiva Magalhaes et al. (2014). However, Redelstein et al. (2015) showed bioaccumulation of heavy metals (Cd, Cu, Ni, Zn) from spiked riverine sediments to zebrafish embryos that induced cellular stress, indicating that sublethal effects might also be assumed from exposure to ex-ACE. One further reason for the particular sensitivity of weatherfish embryos might be the formation of outer filamentous gills, observed in the eleutheroembryo stage (Kostomarova, 1991). These surface extending structures might increase the uptake of and thereby sensitivity to toxic compounds, especially because gills are seen as one major route for xenobiotics entering the fish (Randall et al., 1998). Additionally, the shorter incubation time of weatherfish embryos might play a role (hatching occurs 50 to 70 h post fertilization), since the missing protective function of the chorion after hatching can increase toxicity (Henn and Braunbeck, 2011). However, further studies, including the testing of single compounds, toxicokinetic investigations within the embryo or bioaccumulation assays in long-term experiments might help to understand species related differences.

With respect to an ERA of sediment toxicity, sensitivity to water extractable and consequently more easily bioavailable substances is a relevant feature of a test species. Furthermore, tropical species like the zebrafish might appear more sensitive than temperate species because higher temperatures in the test system can increase the bioavailability of certain substances (Kwok et al., 2007). Hence, deviations from basic environmental conditions prevailing on-site (i.e. temperature), which is necessary in the case of bioassays with exotic species, may constitute a bias in ERA of sediment toxicity.

CONCLUSIONS

An application of representative, ethically justifiable and realistic fish bioassays for an ERA of sediment toxicity is still a complex challenge in ecotoxicology. It starts with the selection of appropriate test species, continues with an implementation of alternative test methods and does not yet end with choosing from a variety of exposure scenarios and endpoints. The present study emphasizes the ecological relevance and suitability of the weatherfish for site-specific investigations in Europe, as it is a native and benthic species with demonstrated robustness for laboratory studies. Furthermore, the utilization of weatherfish embryos instead of juveniles or adults, represents a progress towards an implementation of the 3 Rs principal (replacement, reduction and refinement) according to Russel and Burch (1959) and therefore an advancement in fish welfare. The obtained compliance of effect concentrations derived from weatherfish bioassays with a predicted toxicity gradient, measured sediment contamination and results of previous studies that used differing species and endpoints, demonstrates a good reliability of the data. However, practical limitations that were already reported for zebrafish embryos became also apparent when applying weatherfish embryos for SCAs. Testing of extracts generated by PHWE might remedy practical problems arising from native sediment testing (i.e. SCA) and improve the simulation of bioavailability of sediment-bound compounds (vs. organic solvent extraction). In order to reinforce this assumption, further investigations of PHWE in the context of sediment bioassays with fish is needed, including comprehensive chemical characterizations of extracts from different sediments. The comparison of weatherfish vs. zebrafish embryos showed, that effects caused by sediment contamination might not only differ between species but also as a function of the applied exposure scenario. In this case, an exposure of weatherfish embryos to the more realistic ex-PHWE, applied under temperature conditions prevailing in waters of mid-latitudes, revealed higher sensitivity. Summarizing, the novel test species (i.e. weatherfish) and extraction method (i.e. PHWE) presented in this study reveals high potential for an ERA of sediment toxicity in Europe.

ACKNOWLEDGEMENTS

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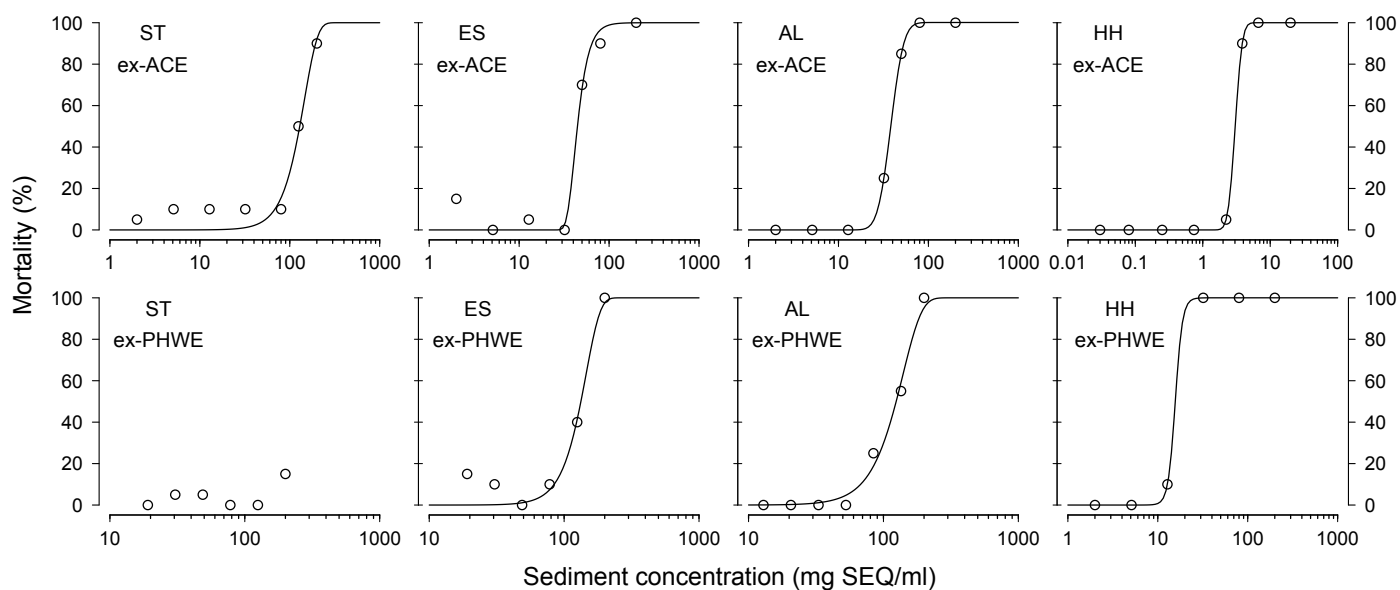
Fig. S1. Concentration-response curves for all acute fish embryo toxicity tests (FETs) conducted with weatherfish embryos (96 h) exposed to acetone extracts (ex-ACE) and pressurized hot water extracts (ex-PHWE) of sediments from Streitgraben (ST), Ehrenbreitstein (ES), Altrip (AL) and Vering Canal (HH).
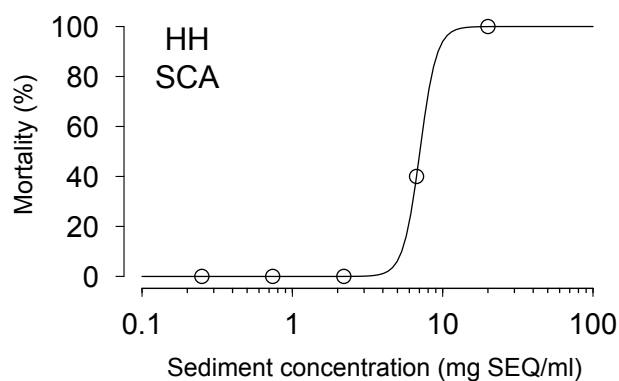
Fig. S2. Concentration-response curve for the sediment contact assay (SCA) conducted with weatherfish embryos (96 h) exposed to sediment from Vering Canal (HH).
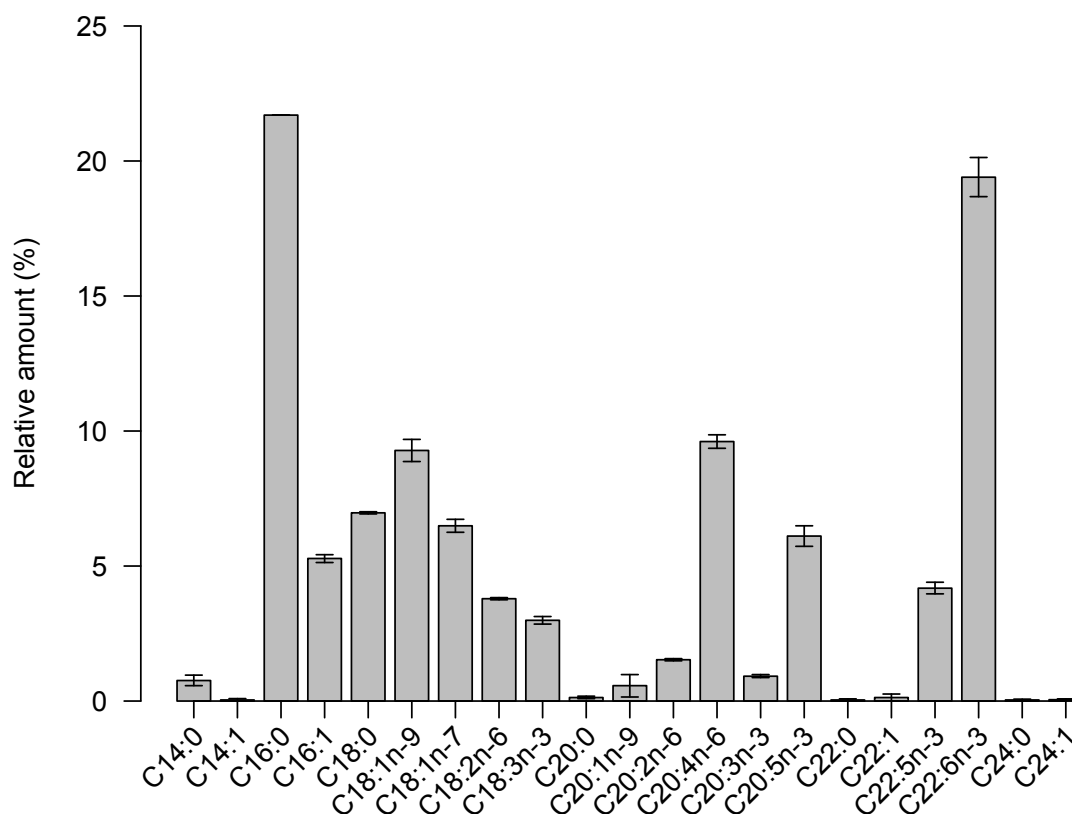
Fig. S3. Relative amounts of fatty acids in unfertilized weatherfish eggs, calculated from 2 different batches of eggs (3 samples of approximately 1 g of eggs per batch).

Table S1. Used test concentrations (mg SEQ/ml) sorted by type of exposure (ex-ACE, ex-PHWE, SCA) and sediments (ST, ES, AL, HH).

Type of exposure	Sediment	Test concentrations (mg SEQ/ml)							
ex-ACE	ST	200	125	80	32	12.8	5.1	2	
	ES	200	80	50	32	12.8	5.1	2	
	AL	200	80	50	32	12.8	5.1	2	
	HH	20	6.67	3.84	2.22	0.74	0.25	0.08	0.03
ex-PHWE	ST	200	125	78.1	48.8	30.5	19.1		
	ES	200	135	84.4	52.7	33	20.6	12.9	
	AL	200	125	78.1	48.8	30.5	19.1		
	HH	200	80	32	12.8	5.1	2		
SCA	ST	600	450						
	ES	600	450	300	150				
	AL	600	450	300	150				
	HH	20	6.7	2.2	0.74	0.25			

Table S2. Concentrations of polycyclic aromatic hydrocarbons (PAHs) in sediment extracts gained by acetone extraction (ex-ACE) and pressurized hot water extraction (ex-PHWE). Given are results for sediments from Streitgraben (ST), Ehrenbreitstein (ES), Altrip (AL) and Vering Canal (HH).

PAHs (mg/kg SEQ)	ex-ACE				ex-PHWE			
	ST	ES	AL	HH	ST	ES	AL	HH
Naphthalene	0.0945	0.104	0.0995	< LOQ	0.126	< LOQ	0.116	0.175
Acenaphthylene	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.1	0.052	< LOQ
Acenaphthene	< LOQ	< LOQ	< LOQ	0.515	< LOQ	< LOQ	< LOQ	< LOQ
Fluorene	< LOQ	< LOQ	< LOQ	2.705	< LOQ	< LOQ	< LOQ	< LOQ
Phenanthrene	< LOQ	0.1385	0.0735	18.2	0.0525	0.058	< LOQ	0.112
Anthracene	< LOQ	< LOQ	< LOQ	2.595	< LOQ	< LOQ	< LOQ	0.952
Fluoranthene	< LOQ	0.112	0.0895	25.75	< LOQ	< LOQ	< LOQ	0.14
Pyrene	< LOQ	0.083	0.0785	14.4	< LOQ	< LOQ	< LOQ	0.117
Benzo[a]anthracene	< LOQ	< LOQ	0.071	8.95	< LOQ	< LOQ	< LOQ	0.107
Chrysene	< LOQ	0.051	0.0885	11.4	< LOQ	< LOQ	< LOQ	< LOQ
Benzo[b+j]fluoranthene	< LOQ	0.0575	0.129	9.2	< LOQ	< LOQ	< LOQ	< LOQ
Benzo[a]pyrene	< LOQ	< LOQ	0.078	4.675	< LOQ	< LOQ	< LOQ	< LOQ
Indeno-1,2,3-[c,d]pyrene	< LOQ	< LOQ	0.074	4.11	< LOQ	< LOQ	< LOQ	< LOQ
Benzo[g,h,i]perylene	< LOQ	< LOQ	0.0825	3.865	< LOQ	< LOQ	< LOQ	< LOQ
Dibenz[a,h]anthracene	< LOQ	< LOQ	0.0155	1.055	< LOQ	< LOQ	< LOQ	< LOQ
Sum of PAHs	0.0945	0.545	0.88	107.42	0.1785	0.158	0.168	1.6

Table S3. Concentrations of polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs) in sediment extracts gained by acetone extraction (ex-ACE) and pressurized hot water extraction (ex-PHWE). Given are results for sediments from Streitgraben (ST), Ehrenbreitstein (ES), Altrip (AL) and Vering Canal (HH).

PCDD/Fs (µg/kg SEQ)	ex-ACE				ex-PHWE			
	ST	ES	AL	HH	ST	ES	AL	HH
2,3,7,8-TCDD	< LOQ	< LOQ	< LOQ	0.001	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,7,8-PeCDD	< LOQ	< LOQ	< LOQ	0.007	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,4,7,8-HxCDD	< LOQ	0.0002	0.00015	0.0035	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,6,7,8-HxCDD	< LOQ	0.0003	0.00095	0.0065	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,7,8,9-HxCDD	< LOQ	0.0004	0.00035	0.005	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,4,6,7,8-HpCDD	0.001	0.00845	0.0169	0.0555	< LOQ	< LOQ	< LOQ	< LOQ
OCDD	0.0042	0.149	0.3895	0.374	< LOQ	< LOQ	< LOQ	< LOQ
2,3,7,8-TCDF	< LOQ	0.0007	0.00185	0.0185	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,7,8-PeCDF	< LOQ	0.0007	0.00085	0.016	< LOQ	< LOQ	< LOQ	< LOQ
2,3,4,7,8-PeCDF	< LOQ	0.00055	0.0011	0.018	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,4,7,8-HxCDF	< LOQ	0.0009	0.00205	0.047	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,6,7,8-HxCDF	< LOQ	0.00065	0.00175	0.032	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,7,8,9-HxCDF	< LOQ	< LOQ	< LOQ	0.007	< LOQ	< LOQ	< LOQ	< LOQ
2,3,4,6,7,8-HxCDF	< LOQ	0.0003	0.0005	0.01	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,4,6,7,8-HpCDF	0.00285	0.00385	0.0073	0.1905	< LOQ	< LOQ	< LOQ	< LOQ
1,2,3,4,7,8,9-HpCDF	0.00055	0.00025	0.00075	0.048	< LOQ	< LOQ	< LOQ	< LOQ
OCDF	0.179	0.024	0.0565	0.845	< LOQ	< LOQ	< LOQ	< LOQ
Sum of PCDD/Fs	0.1876	0.1818	0.4805	1.6845	-	-	-	-

Table S4. Concentrations of polychlorinated biphenyls (PCBs)in sediment extracts gained by acetone extraction (ex-ACE) and pressurized hot water extraction (ex-PHWE). Given are results for sediments from Streitgraben (ST), Ehrenbreitstein (ES), Altrip (AL) and Vering Canal (HH).

PCBs (µg/kg SEQ) (IUPAC. No)	ex-ACE				ex-PHWE			
	ST	ES	AL	HH	ST	ES	AL	HH
77	< LOQ	0.049	0.071	0.295	< LOQ	< LOQ	< LOQ	< LOQ
81	< LOQ	< LOQ	< LOQ	0.01	< LOQ	< LOQ	< LOQ	< LOQ
126	< LOQ	0.003	0.0065	0.02	< LOQ	< LOQ	< LOQ	< LOQ
169	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
105	< LOQ	0.09	0.17	0.7	< LOQ	< LOQ	< LOQ	< LOQ
114	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
118	< LOQ	0.3	0.66	1.8	< LOQ	< LOQ	< LOQ	< LOQ
123	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
156	< LOQ	0.08	0.095	0.75	< LOQ	< LOQ	< LOQ	< LOQ
157	< LOQ	0.015	0.02	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
167	< LOQ	< LOQ	0.055	0.35	< LOQ	< LOQ	< LOQ	< LOQ
189	< LOQ	0.02	0.025	0.2	< LOQ	< LOQ	< LOQ	< LOQ
Sum of PCBs	-	0.557	1.1025	4.125	-	-	-	-

PERSONAL DATA

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EDUCATION AND CAREER

06/2004	Final secondary-school examination (Abitur) at the Wilhelm-Hittorf-Gymnasium, Münster, Germany
09/2004 – 05/2005	Civilian service at the Amt für Grünflächen und Umweltschutz, Münster, Germany
10/2006 – 06/2013	Studies in Environmental Sciences at the University of Koblenz-Landau, Landau, Germany
06/2013	Diploma thesis entitled "Studying water hardness as a factor influencing mortality of zebrafish (*Danio rerio*) during chemical marking with oxytetracycline and establishing an experimental design to investigate postmarking stress sensitivity of allis shad larvae (*Alosa alosa*) after chemical marking with alizarin red S"
Since 06/2013	PhD study at the Institute for Environmental Sciences, University of Koblenz-Landau, Landau, Germany

PUBLICATIONS

Crago, J., Tran, K., Budicin, A., **Schreiber, B.**, Lavado, R., Schlenk, D., 2015. Exploring the impacts of two separate mixtures of pesticide and surfactants on estrogenic activity in male fathead minnows and rainbow trout. Arch. Environ. Contam. Toxicol. 68, 362-370. doi: 10.1007/s00244-014-0098-3

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Löb, C., **Schreiber, B.**, Noß, C., Schmidt, T., 2011. Effektivität der Detektion von Passiven Integrierten Transpondern (PIT-Tags) zur Erfassung von Fischbewegungen in kleinen Gruppen: II. Was beeinflusst die Detektionswahrscheinlichkeit? DGL–Tagung (Freising, Germany), poster presentation.

Schreiber, B., Löb, C., Noß, C., Schmidt, T., 2011. Effektivität der Detektion von Passiven Integrierten Transpondern (PIT-Tags) zur Erfassung von Fischbewegungen in kleinen Gruppen: I. Mögliche Grenzen einer etablierten Technologie. DGL–Tagung (Freising, Germany), poster presentation.

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